FABIANA GRANJA

AVALIAÇÃO DO POTENCIAL DE USO DOS PERFIS GENOTÍPICOS DE *GSTP1, GSTO1* E *P53* COMO MARCADORES DE PREDISPOSIÇÃO AO CANCER DA TIRÓIDE E COMO PREDITORES DE RESPOSTA AO TRATAMENTO

CAMPINAS

2005

FABIANA GRANJA

AVALIAÇÃO DO POTENCIAL DE USO DOS PERFIS GENOTÍPICOS DE *GSTP1, GSTO1* E *P53* COMO MARCADORES DE PREDISPOSIÇÃO AO CANCER DA TIRÓIDE E COMO PREDITORES DE RESPOSTA AO TRATAMENTO

Tese de Doutorado apresentada à Pós-Graduação da Faculdade de Ciências Médicas da Universidade Estadual de Campinas para obtenção do título de Doutor em Clínica Médica, área de concentração em Clínica Médica.

ORIENTADORA: *PROF.ª DRA. LAURA STERIAN WARD*

CAMPINAS

2005

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

G766a

Granja, Fabiana
Avaliação do potencial de uso dos perfis genotípicos de *GSTP1, GSTO1* e *P53* como marcadores de predisposição ao câncer da tiróide e como preditores de resposta ao tratamento. / Fabiana Gontijo. Campinas, SP : [s.n.], 2005.

Orientador: Laura Sterian Ward
Tese (Doutorado) Universidade Estadual de Campinas. Faculdade de Ciências Médicas.

1. Predisposição Genética para doença. 2. Genótipo. 3. Genes p53. 4. Neoplasias da Glândula Tireóide. 5. Polimorfismo. 6. Bócio. 7. Câncer. I. Ward, Laura Sterian. II. Universidade Estadual de Campinas. Faculdade de Ciências Médicas. III. Título.

(Slp/fcm)

Banca Examinadora da Defesa de Tese de Doutorado

Orientador(a): ***Profa. Dra. Laura Sterian Ward***

Membros:

1. Prof(a). Dr(a) Rui Monteiro de Barros Maciel
2. Prof(a). Dr(a). Lea Maria Zanini Maciel
3. Prof(a). Dr(a). Alfio José Tincani
4. Prof(a). Dr(a). Lício Augusto Velloso
5. Prof(a). Dr(a). Laura Sterian Ward

Curso de Pós-Graduação em Clínica Médica, área de concentração Clínica Médica, da Faculdade de Ciências Médicas da Universidade Estadual de Campinas.

Data: 30/08/2005

DEDICATÓRIA

Aos meus pais Wilkye e Regilene e minha irmã Carolina, os quais sempre me apoiaram e me deram o suporte econômico e emocional para poder seguir em frente e superar todos os obstáculos.

Ao meu companheiro Mário Jr, que sempre me apoiou e me impulsionou e ao meu filho Mário Neto que chegou no final mais foi de grande importância para que este projeto fosse concretizado.

Primeiramente agradeço a Deus, pois para ele tudo é possível.

À Profa. Dra. Laura Sterian Ward, pela oportunidade e por investir em uma estudante de graduação e em seu desenvolvimento científico, assim como acreditar na realização deste e de outros trabalhos e pela amizade.

A Profa. Dra. Lígia V. M. Assumpção, da endocrinologia do Hospital das Clínicas da UNICAMP, por ter concedido as amostras de sangue periférico dos pacientes, assim como os dados de seguimento dos mesmos.

Aos pacientes qua participaram desta pesquisa, assim como os indivíduos controles.

As companheiras e amigas do doutorado, Janaína, Elaine, Patrícia com as quais aprendi muito, viajei muito e me diverti muito. Jana, obrigada pela companhia na faculdade, no laboratório, nas viagens, na academia, valeu pela amizade.

A amiga e estagiária Joseane, que foi uma peça fundamental no desenvolvimento deste trabalho, e com a qual aprendi muito.

As estagiárias que agora são mestrandas, Natássia e Kika, e as alunas de iniciação científica, Mariana e Inês, obrigada pela ajuda e pelos bons momentos que passamos juntas.

As estagiárias Gabriela e Fabiana Urbano, pela ajuda no trabalho, pelo aprendizado e pelos bons momentos.

Assim como todos do Laboratório de Genética Molecular do Câncer que estavam presentes em algum momento da realização deste trabalho, Hérika, Márcia, Joyce, Flávia, Mário Jr, Hélio, o meu muito obrigada

As vizinhas de laboratório, Viviane e Helen, pelos empréstimos emergenciais, dúvidas e pela amizade.

A minha amiga Ana Carolina, a qual eu conheci no período de desenvolvimento deste trabalho e a qual teve muita paciência e me deu muita força. Obrigada Amiga!

A todos que de alguma forma colaboraram para que esse trabalho fosse realizado.

O meu muito Obrigada à todos vocês pela amizade, atenção e apoio.

"A COISA MAIS INJUSTA SOBRE A VIDA É A MANEIRA COMO ELA TERMINA. EU ACHO QUE O VERDADEIRO CICLO DA VIDA ESTÁ TODO DE TRÁS PRA FRENTE. NÓS DEVERIAMOS MORRER PRIMEIRO, NOS LIVRAR LOGO DISSO. DAÍ VIVER NUM ASILO, ATÉ SER CHUTADO PRA FORA DE LÁ POR ESTAR MUITO NOVO. GANHAR UM RELÓGIO DE OURO E IR TRABALHAR. ENTAO VOCÊ TRABALHA 40 ANOS ATÉ FICAR NOVO O BASTANTE PRA PODER APROVEITAR SUA APOSENTADORIA. DEPOIS VOCÊ CURTE TUDO, BEBE BASTANTE ÁLCOOL, FAZ FESTAS E SE PREPARA PRA FACULDADE. VOCÊ VAI PRO COLÉGIO, TEM VÁRIAS NAMORADAS, VIRA CRIANCA, NÃO TEM NENHUMA RESPONSABILIDADE, SE TORNA UM BEBEZINHO DE COLO, VOLTA PRO ÚTERO DA MÃE, PASSA SEUS ÚLTIMOS NOVE MESES DE VIDA FLUTUANDO... E TERMINA TUDO COM UM ÓTIMO ORGASMO!!! NAO SERIA PERFEITO?"

CHARLES CHAPLIN

CDT	Carcinoma diferenciado de tiróide
GST	Glutationa S-Transferase
GSTT1	GSTtheta 1
GSTM1	GSTmu 1
GSTP1	GST pi 1
GSTO1	GST omega 1
CP	Carcinoma papilífero
CF	Carcinoma Folicular
TSH	Tirotrofina sérica
PCI	Pesquisa de corpo inteiro com radiodo
Tg	Tiroglobulina sérica
PCR	Reação de Polimerase em cadeia
SSCP	Single Strand Conformation Polymorphism Analysis
pb	pares de bases
ARG	Arginina
PRO	Prolina
SNPs	Single nucleotide polymorphisms
OR	Odds ratio

PÁG.

PÁG.

PÁG.

RESUMO

O desenvolvimento de métodos moleculares de rastreamento de indivíduos com risco de desenvolver câncer entre os portadores de nódulos na tiróide é uma questão urgente de Saúde Pública. O objetivo deste trabalho é analisar a utilidade do perfil genotípico de GSTP1, GSTO1 e p53 na identificação de risco para câncer de tiróide e na sua resposta terapêutica. Comparamos amostras de sangue periférico de 157 controles com 142 indivíduos com nódulos: 44 benignos (30 bócios e 14 adenomas foliculares- AF) e 98 malignos (77 carcinomas papilíferos- CP e 21 carcinomas foliculares- CF). Todos os pacientes foram tratados e seguidos de acordo com o mesmo protocolo. Os pacientes com câncer da tiróide apresentavam mais freqüentemente os polimorfismos de GSTP1 (câncer = 27.5% versus controle = 5.7% - $p<0.0001$) e os polimorfismos do códon 72 de p53 (câncer =12.2% versus controle =2% - $p<0.001$) do que na população controle. Não houve diferença entre pacientes e controles em relação ao perfil do gene GSTO. Estes genótipos variantes conferiram um aumento de risco para câncer de tiróide de mais de 7 vezes, tanto para GSTP1 (Odds ratio= 7.415; 95% CI: 2.472-22.243) como para o códon 72 de p53 (Odds ratio=7.023; 95% CI: 1.928-25.588). Os polimorfismos em GSTP1 e códon 72 p53 identificam câncer com sensibilidade de 75% e 80% e especificidade de 68% e 63%, respectivamente. Análise de regressão logística ajustada para sexo, idade e cor, mostra que estes polimorfismos de GSTP1 e p53, mas não os de GSTO1, são fatores independentes de risco para malignidade. Não houve correlação entre o perfil genotípico para qualquer gene e a resposta a terapia ou evolução dos pacientes com câncer. Sugerimos que o uso desses marcadores moleculares, combinado com as clássicas características clínico-epidemiológicas associadas à susceptibilidade ao câncer da tiróide, pode ser útil no rastreamento de malignidade entre os portadores de nódulos tiroidianos da população brasileira.

ABSTRACT

Evaluation of the potential use of GSTP1, GSTO1 and p53 genotype profiles as markers of thyroid cancer predisposition and response to treatment.

The development of screening tools designed to identify individuals at risk for thyroid odule cancer is of utmost necessity. The aim of the present research is to evaluate the utility of GSTP1, GSTO1 and p53 genotype profiles as markers of risk to thyroid cancer and its response to treatment. We compared peripheral blood samples from 157 controls to 144 patients with thyroid nodules: 44 benign (30 hyperplasias and 14 folicular adenomas - AF) and 98 malignant (77 papillary carcinomas - PC and 21 folicular carcinomas - FC). All patients were managed according to a standard protocol. Thyroid carcinoma patients presented more frequently GSTP1 (cancer = 27.5% versus controls = 5.7% - $p<0.0001$) and codon 72 of p53 (cancer =12.2% versus controls =2% - $p<0.001$) polymorphisms than the control population. There was no difference between cancer and controls regarding GSTO gene. These variant genotypes increased the risk for thyroid câncer more than 7 times for GSTP1 (Odds ratio= 7.415; 95% CI: 2.472-22.243) and codon 72 of p53 (Odds ratio=7.023; 95% CI: 1.928-25.588). GSTP1 and codon 72 p53 polymorphisms identify thyroid câncer with a sensitivity of 75% and 80% and specificity of 68% and 63%, respectively. Logistic regression analysis adjusted for gender, age and color demonstrated that GSTP1 and p53, but not GSTO1 polymorphisms are independent factors of risk for malignancy. There was no correlation between any genetic profile and the response to treatment or the outcome of cancer patients. Our data indicates that the use of these molecular markers, together with the classic clinico-epidemiologic features associated to thyroid cancer susceptibility may be useful in screening thyroid nodules malignancy in Brazilian population.

INTRODUÇÃO

Nódulos tiroidianos e câncer

Nódulos de tiróide são extremamente comuns. Estima-se que 10% da população venha a desenvolver um nódulo palpável durante a vida e vários dados indicam que este número deve ser ainda maior em nosso país, onde, até ha poucas décadas atrás, ainda havia extensas áreas carentes de aporte adequado de iodo na alimentação (WELKER & ORLOV, 2003; KNOBEL & MEDEIROS-NETO, 2004; TOMIMORI *et al,* 1995; FURLANETTO *et al*, 2000). Dois grandes estudos populacionais, o de Whickham na Inglaterra e o de Framingham nos Estados Unidos, descrevem, respectivamente, nódulos solitários palpáveis em 5,3% das mulheres e 0,8% dos homens, e 6,4% das mulheres e 1,6% dos homens (TUNBRIDGE *et al*, 1977; VANDER *et al*, 1954). No entanto, a prevalência verdadeira dos nódulos de tiróide deve ser muito maior se considerarmos dados de autópsia como os da Clínica Mayo, que mostram a presença de nódulos de tiróide em 50,5% de 821 autópsias realizadas consecutivamente em pacientes que apresentavam a tiróide clinicamente normal (MORTENSEN *et al*, 1955). Mais recentemente, o uso da ultra-sonografia como método acessível a grandes populações e de custo relativamente pequeno em nosso meio, vem aumentando sensivelmente o número de pacientes com nódulos não palpáveis já que a ultra-sonografia diagnostica nódulos em até 67% da população (CHOW *et al*, 2003; HEGEDUS *et al,* 2003; TAN & GHARIB, 1997).

A maioria dos nódulos tiroidianos é causada por doenças benignas, como nódulos colóides, cistos e neoplasias foliculares benignas, de modo que menos de 5% dos pacientes são portadores de câncer de tiróide (TAN & GHARIB, 1997; HEGEDUS *et al*, 2003). A neoplasia da tiróide não responde por mais do que 1% de todas as neoplasias malignas, correspondendo a cerca de 0.5% das neoplasias descritas em homens e 1.5% daquelas que aparecem em mulheres (NATIONAL CANCER DATA; AMERICAN CANCER SOCIETY, 2003; JEMAL *et al*, 2004). Por outro lado, a incidência do câncer da tiróide vem aumentando no mundo todo, de acordo com estatísticas recentes (NATIONAL CANCER DATA, acessado em 06/2005). Nos Estados Unidos da América, o câncer de tiróide é aquele que apresenta o maior crescimento anual de incidência em mulheres (mais de 100% de aumento na incidência de 1975 a 2002), colocando-se em segundo lugar no cômputo geral (NATIONAL CANCER DATA, acessado em 06/2005; WARD *et al*, 2004).

Embora a citologia obtida através da punção aspirativa por agulha fina (PAAF) seja um excelente método diagnóstico, de reconhecido bom custo-efetividade também em nosso meio, fazer a seleção de malignidade por PAAF torna-se impraticável a nível populacional e seu custo proibitivo se estivermos frente a percentagens assustadoras como as representadas pela prevalência do nódulo (WARD *et al,* 1993; WELKER & ORLOV, 2003; CASTRO & GHARIB, 2000).

Outro ponto crítico a ser considerado é a implicação do diagnóstico de malignidade na conduta a ser tomada. Apesar de sua baixa incidência e prognóstico favorável, o câncer de tiróide é uma doença mortal em cerca de 10% dos tumores bem diferenciados, 50% dos tumores pouco diferenciados e medulares e em 100% dos tumores anaplásicos (BHATTACHARYA, 2003).

Com a popularização do uso da ultra-sonografia e de outros métodos de imagem no Brasil, assim como em todo o mundo, aumentou de maneira considerável o número de diagnósticos de nódulos não palpáveis da tiróide, levando à identificação de um número crescente de microcarcinomas (CHOW *et al,* 2003). Estes tumores, definidos pela OMS como carcinomas com até 1 cm de diâmetro, vem sendo descritos em 1% a 35,6% das autópsias e em 5,5% a 10,5% das tiróides operadas por outras causas que não a suspeita de malignidade (FRANSSILA & HARACH, 1986; HARACH *et al*, 1985; LANG *et al*, 1988; MITSELOU *et al*, 2002; MARTINEZ-TELLO *et al* 1993; NOGUCHI *et al* 1996; HEFER *et al,* 1995). Um grande estudo nacional em 145.043 autópsias realizadas durante um período de 6 décadas mostrou elevada freqüência de lesões tiroidianas (8.38%), das quais a grande maioria foi benigna: 91,62% (BISI *et al*, 1998). No entanto, se, à semelhança de outros países, nossa incidência de câncer da tiróide varia entre 0,5 e 0,9% dos homens e 1,9 a 3,0% das mulheres, uma grande parte dos microcarcinomas detectados por ultra-sonografia, em autópsias ou em peças cirúrgicas, provavelmente nunca evolui para câncer clínico (CASELLA & FUSCO**,** 2004). Seguindo pacientes com diagnóstico de microcarcinomas sem intervir cirurgicamente, Ito e colaboradores mostraram que cerca de 70% destas lesões permanece do mesmo tamanho ou até diminui de tamanho em um período de cerca de 4 anos (ITO *et al*, 2004). Por outro lado, não faltam relatos de casos de microcarcinomas papilíferos de comportamento agressivo, que evoluem não só com

metástases regionais linfáticas, mas também sangüíneas para pulmões, ossos e cérebro (HEFER *et al,* 1995**;** LIVOLSI**,** 1996; KUSUNOKI & MURATA, 2004). Assim, frente a um carcinoma tiroidiano, seja ele detectado clinicamente ou apenas um achado de ultra-sonografia ou de cirurgia realizada por patologias benignas da glândula, é fundamental para o clínico possuir parâmetros que lhe permitam decidir por maior investigação ou conduta mais agressiva.

Parâmetros de risco para câncer da tiróide

Naturalmente, para estabelecer parâmetros de risco, necessitamos conhecer os fatores que causam ou que estão implicados no maior risco de desenvolvimento de tumores tiroidianos. O que sabemos a respeito?

O único fator clínico, claramente associado à presença de nódulos malignos e benignos tumorais na tiróide, é a exposição à radiação ionizante, particularmente na infância (SCHLUMBERGER, 2000, WELKER & ORLOV *et al,* 2003; BOONE *et al,* 2003; CHOW *et al*, 2003; FIGGE, 1999; MACK *et al*, 2003; RON, 1996; PRESTON-MARTIN *et al*, 2003). O risco de apresentar um nódulo tiroidiano benigno ou maligno aumentou quase 10 vezes em crianças de menos de 10 anos expostas à radiação proveniente do bombardeio nuclear de Hiroshima e Nagasaki durante a 2ª Guerra Mundial (THOMPSON *et al,* 1994). O risco de desenvolver um tumor tiroidiano apresenta nítida correlação com a dose de radiação à qual crianças foram expostas na Bielorússia após a explosão do reator nuclear de Chernobyl (CARDIS *et al*, 2005).

A elevada freqüência de câncer em familiares de pacientes com câncer da tiróide sugere que um fator hereditário predisponente seja importante, mas não há dúvidas de que fatores de exposição ambiental devem contribuir para o aparecimento do tumor em determinados indivíduos e não em outros com o mesmo perfil genético (HALL & HOLM, 1998). Mais ainda, as variações de incidência do câncer da tiróide em diferentes áreas geográficas e em diferentes grupos étnicos sugerem que a influência de fatores ambientais no risco do desenvolvimento do câncer esporádico da tiróide seja bastante importante

(RON *et al*, 1987; MEMON *et al*, 2002; HASELKOM *et al*, 2003; MACK *et al* 2002; FRIEDMAN & URY, 1983). Assim, por exemplo, em certas regiões como no Oriente Médio, o câncer da tiróide é a segunda neoplasia mais freqüente entre as mulheres (MEMON *et al*, 2004). No Kuwait, ele responde por 8% de todos os cânceres (MEMON *et al*, 2002). Na Nova Caledônia, no sul do Pacífico, atinge 35 casos por 100.000 habitantes (TRUONG *et al*, 2005).

O CDT - Carcinoma diferenciado da tiróide é diagnosticado de 2 a 3 vezes mais freqüentemente em mulheres do que em homens, sugerindo que hormônios sexuais ou genes ligados ao cromossomo X possam estar envolvidos na patogenia da doença (WELKER & ORLOV, 2003; SCHLUMBERGER, 2000; BOONE *et al*, 2003; CHOW *et al*, 2003; FIGGE, 1999; MACK *et al*, 2003; RON, 1996; PRESTON-MARTIN *et al,* 2003; TRUONG *et al*, 2005). Realmente, vários compostos estrogênicos têm efeito direto sobre células foliculares; alguns podem produzir metabólitos que reagem com o DNA e, portanto, poderiam ser carcinogênicos (FURLANETO *et al*, 2000, YAGER, 2000). No entanto, estudos de caso-controle em populações de mulheres portadoras de câncer papilífero da tiróide não identificou o uso de hormônios exógenos como fator de risco (ROSSING *et al*, 1998; TRUONG *et al*, 2005). Entretanto, acredita-se que fatores reprodutivos e/ou hormonais estão relacionados à maior incidência de tumores tiroidianos entre as mulheres (TRUONG *et al*, 2005).

O carcinoma folicular é mais comum em regiões com aporte insuficiente em iodo, enquanto que áreas suficientes em iodo apresentam incidência muito maior de carcinomas papilíferos (WELKER & ORLOV, 2003; SCHLUMBERGER, 2000; BOONE *et al* 2003; CHOW *et al*, 2003; FIGGE, 1999; MACK *et al*, 2003; RON, 1996; PRESTON-MARTIN *et al,* 2003). O papel do iodo na etiopatogenia do câncer da tiróide pode estar relacionado com a sua influência sobre a expressão de fatores de crescimento, oncogenes ou genes supressores tumorais sensíveis ao iodo, ou ainda atuando na manutenção da estabilidade genética das células foliculares (ESZLINGER *et al*, 2001; ESZLINGER *et al*, 2004; VAISH *et al*, 2004).

Ao contrário de grande parte dos tumores no ser humano, o cigarro não é fator de risco para o câncer da tiróide (MACK *et al*, 2003).

História de bócio ou de nódulos benignos (em particular, o diagnóstico de adenoma prévio), principalmente em mulheres, ao contrário, parece ser fator de risco importante em grandes séries (CHOW *et al,* 2003; FIGGE, 1999; MELLEMGAARD *et al*, 1998; FROM *et al, 2000;* MACK *et al,* 2003; PRESTON-MARTIN *et al,* 2003).

Relatos de casos e pequenas casuísticas sugeriam que a presença de auto-anticorpos poderia representar fator de risco para câncer diferenciado da tiróide (STOCKER *et al*, 2002; ZARDO *et al*, 1999; SINGH *et al*, 1999). Entretanto, não existe evidencia de associação entre anticorpos destruidores ou estimuladores de tiróide e câncer, em nenhuma grande série. Ao contrário, nossos dados sugerem que anticorpos possam proteger os portadores de carcinomas diferenciados da tiróide, proporcionando-lhes melhor evolução clínica (SOUZA *et al*, 2003). História de hipertiroidismo prévio não eleva significantemente o risco para CDT e tampouco existem evidências de aumento de risco em mulheres usando terapia de reposição hormonal para menopausa (CHOW *et al*, 2003; FIGGE, 1999; MACK *et al*, 2003; RON, 1996; PRESTON-MARTIN *et al,* 2003).

São suspeitos todos os nódulos que crescem rapidamente ou que continuam crescendo mesmo sob terapia supressiva com levotiroxina, os nódulos muito duros e aderidos, os que são acompanhados de gânglios cervicais ou de sintomas de compressão (como dispnéia) ou infiltração de outros órgãos (como rouquidão ou tosse) (WELKER & ORLOV, 2003; SCHLUMBERGER, 2000; BOONE *et al*, 2003; CHOW *et al,* 2003; FIGGE, 1999; MACK *et al*, 2003; RON, 1996; PRESTON-MARTIN *et al,* 2003).

Agentes carcinogênicos e sistemas de defesa

Câncer é um processo evolutivo causado pela interação gene-meio ambiente (VINEIS, 2003). Somos constantemente expostos a uma crescente lista de compostos químicos carcinogênicos, vírus transformadores de células, UV e a radiação ionizante, entre outros agentes tóxicos encontrados no meio ambiente (SCHOTTENFELD & BEEBE-DIMMER, 2005). Além disso, compostos eletrofílicos, radicais livres e uma série de produtos de nosso próprio metabolismo podem causar danos a nossas células quando

inapropriadamente metabolizados, inadequadamente eliminados ou produzidos em excesso (VINEIS, 2004; CARBONE *et al*, 2004).

Presume-se que influências ambientais contribuam com mais de 80% dos fatores envolvidos no surgimento do câncer esporádico, podendo-se incluir nestas influências ambientais comportamentos sociais como tabagismo, consumo de alimentos e bebidas, ambiente de trabalho (poluição), agentes químicos industriais, exposição a raios UV, entre outros (PALLI *et al*, 2000). Acredita-se que seres humanos chegam a consumir 1,5 g de pesticidas naturais por dia, na forma de fenóis provenientes de plantas e flavonóides de alimentos, entre outras substâncias tóxicas. Esses compostos são reconhecidos como potentes carcinógenos em murinos (AMES *et al*, 1990; AMES & GOLD, 1990; GOLDMAN & SHIELDS, 2003; THILLY, 2003). O contato com esses agentes carcinogênicos é provavelmente responsável por uma elevada freqüência de mutações no DNA (NIELSEN *et al*, 1996; BODIWALA *et al*, 2003; GOLDMAN & SHIELDS, 2003; THILLY, 2003). Indivíduos expostos a poluentes do ar, como oficiais de polícia, motoristas de ônibus, vendedores de rua e residentes em áreas urbanizadas altamente poluídas tendem a apresentar elevada freqüência de modificações no DNA (NIELSEN et al, 1996). Alterações cromossômicas foram encontradas em tecidos de mucosa do cólon, bexiga, nariz, pulmão e em mama destes indivíduos, sugerindo que estes altos níveis de anormalidades possam ser preditivos para o aparecimento de câncer (PELUSO et al, 1997; PERERA et al, 1995; TANG et al, 1995).

Agentes químicos carcinogênicos são, geralmente, substâncias pouco solúveis em água sendo, portanto, dificilmente eliminadas pelos rins, fezes ou perspiração (AUTRUP, 2000). Para garantir a sobrevida das células forçadas a constante exposição a carcinógenos ambientais, muitos mecanismos de defesa foram evolutivamente selecionados pelos seres vivos. Uma série de sistemas enzimáticos estão encarregadas de metabolizar e eliminar estas substâncias, reconhecendo-se duas fases diferentes neste processo. Uma primeira fase, denominada de fase I, envolve oxidação inicial de compostos tóxicos pelo citocromo P450. Segue-se a chamada fase II, em que geralmente acontece uma reação de conjugação catalisada por uma série de enzimas, entre as quais, a família das glutationa S-transferase (GST) (MANNERVIK & DANIELSON, 1988).

A probabilidade de desenvolvimento do câncer depende da resposta natural de cada organismo às diferentes exposições a agentes agressores diversos. Os seres humanos possuem diferentes susceptibilidades a diferentes carcinógenos (LICHTENSTEIN et al, 2000; VINEIS et al 2003). A base bioquímica para tal variação de susceptibilidade aos diversos agressores ambientais está relacionada a polimorfismos genéticos que normalmente ocorrem na população, em especial nos genes envolvidos na predisposição específica para câncer, ativação metabólica ou detoxificação de agentes tóxicos ambientais, controle do reparo de DNA ou dano celular (VINEIS, 2003; LICHTENSTEIN *et al* 2000; VINEIS *et al* 2001; AUTRUP, 2000; CLAPPER, 2000).

Muitos polimorfismos de genes que codificam enzimas envolvidas na biotransformação de carcinógenos vêm sendo bem estudados em busca de uma possível associação com o risco para o desenvolvimento de câncer.

O sistema Glutationa S-Transferase

O sistema Glutationa S-Transferase (GST) consiste em um grande grupo multigênico de enzimas de detoxificação essenciais para a proteção celular e que agem através da conjugação dos compostos tóxicos com a glutationa (MANNERVIK, 1985). Seis classes das isoenzimas vem sendo consideradas como de grande importância em humanos: *alpha (GSTA), mu (GSTM), pi (GSTP), sigma (GSTS) e theta (GSTT)*. Indivíduos que possuem a deleção em homozigose das enzimas *GSTT1* e *GSTM1* não possuem as enzimas de detoxificação respectivas, mu e theta, o que os torna mais susceptíveis à ação deletéria de agentes ambientais carcinogênicos (CLAPPER, 2000; MANNERVIK, 1985; KNUDSEN *et al*, 2001). Nós demonstramos que a ausência de *GSTT1* e *GSTM1* aumentam o risco para o desenvolvimento de câncer de tiróide em 2,6 vezes (MORARI *et al*, 2002).

No entanto, a enzima considerada mais importante na detoxificação de carcinógenos em tecidos de cabeça e pescoço é a GSTP (MULDER *et al*, 1995). Os níveis de expressão de *GSTP1* são extensivamente estudados em relação à malignidade associada ao tabaco, tendo-se encontrado uma expressão aumentada de *GSTP* em várias lesões

neoplásicas e pré-neoplásicas, incluindo tumores de cabeça e pescoço, mama, próstata e pulmão (OUDE OPHUIS *et al*, 2003; GUDMUNDSDOTTIR *et al*, 2001; GSUR *et al*, 2001; STUCKER *et al,* 2002). Uma troca de bases, em que uma adenosina (A) é substituída por uma guanina (G) no éxon 5 do gene *GSTP1,* resulta na troca do aminoácido isoleucina por uma valina na posição 105 da seqüência gênica (Ile105 Val). Esta substituição seqüencial produz uma isoforma do gene *GSTP* denominada isoforma Val, a qual causa uma alteração da estabilidade e da especificidade da atividade enzimática (JOHANSSON *et al*, 1998). Assim, esta variante enzimática possui menor atividade e menor capacidades de detoxificação dos carcinógenos quando comparada com a enzima codificada pelo gene tipo selvagem, denominado *GSTP1AA* (JOHANSSON *et al*, 1998; HU *et al*, 1997).

Mais recentemente, uma nova classe de enzima dentre a família das GSTs humanas, nomeada de GST ômega (*GSTO*), foi identificada por análise de ESTs (Expressed Sequence Tag) a partir de um banco de dados e alinhamento de sequências (BOARD *et al*, 2000; YIN *et al*, 2001). A importância da GSTO ainda não está completamente elucidada. No entanto, Dulhunty e colaboradores demonstraram que a enzima GSTO1 modula os receptores de rianodina (RyR) que são os canais de cálcio no retículo endoplasmático de várias células, sugerindo que ela desempenharia um papel importante na proteção das células contendo RyR2 da indução a apoptose pela mobilização de Ca^{+2} (DULHUNTY *et al*, 2001). A enzima GSTO1 poderia, portanto, a exemplo de outras GSTs, proteger células cancerígenas contra a apoptose causada por mobilização de Ca^{2+} de reservas RyR-sensíveis (Xie *et al*, 2005).

Um polimorfismo genético na seqüência codificadora de *GSTO1* foi descrito na base 419. Este polimorfismo causa uma troca do aminoácido alanina pelo aspartato (A140D) na porção 140 do exon 4 (Ala 140 Asp) (WHITBREAD *et al,* 2003, TANAKA-KAGAWA *et al,* 2003). Esta variação cria uma troca de aminoácido hidrofóbico para um hidrofílico, resultando em uma menor atividade da enzima variante substrato dependente que deve estar implicada na variação entre indivíduos na susceptibilidade ao estresse oxidativo e ao metabolismo do arsênico inorgânico (WHITBREAD *et al*, 2003; TANAKA-KAGAWA *et al,* 2003). O arsênico é um reconhecido carcinógeno ambiental,

relacionado com o câncer de bexiga (CHEN *et al*, 2005), câncer de pulmão (AHSAN & THOMAS, 2004), câncer de pele (GAWKRODGER , 2004) e outros.

Se, por um lado, as GSTs são responsáveis por prover ao organismo uma proteção contra agentes tóxicos ambientais, por outro elas também conferem uma resistência celular a medicamentos e outros compostos químicos que eventualmente são utilizados no tratamento do câncer. Assim, a expressão maior das GSTs também pode contribuir à resistência a drogas anti-neoplásicas, como por exemplo o clorambucil, um quimioterápico empregado no tratamento de vários cânceres (HAYES *et al*, 1995). Portanto, o perfil das GSTs também pode ter um valor prognóstico, estando associado com a resposta terapêutica em muitas doenças humanas (REAVEY-CANTWELL *et al*, 2001; FERRUZZI *et al*, 2003; BUSER *et al*, 1997).

O Gene p53

Carcinógenos que não são eliminados pelos nossos sistemas de detoxificação podem danificar nosso material genético. A característica destes danos genéticos é que eles conferem uma vantagem de crescimento à célula danificada e lhe permitem transmitir às suas células filhas esta vantagem, originando um clone de células que escapa dos controles normais de crescimento e diferenciação (WARD, 1998). No entanto, como regra geral, um único gene alterado geralmente não é capaz de conferir um fenótipo tumoral. São necessários vários danos genéticos que se adicionam e sobrepõem até levar a célula danificada a tornar-se independente dos controles do ciclo celular e capaz de escapar dos vários mecanismos de controle que detectam e reparam ou eliminam células danificadas, impedindo-as de passar para as células filhas tais danos (WARD, 1998). No ser humano, medidas indiretas baseadas na prevalência de tumores em diferentes faixas etárias permite inferir que são necessárias cerca de cinco a seis mutações sucessivas para que uma célula se torne maligna e agressiva (WEINBERG, 1989).

No entanto, possuímos uma série de mecanismos de detecção e defesa contra estes danos a nível celular. Uma extensa e ativa rede de genes é capaz de detectar mínimas anormalidades, como mutações em uma única base na seqüência de DNA, corrigi-las ou, na impossibilidade de tal correção, impedir o prosseguimento do ciclo celular.

De modo geral, considera-se que as anomalias genéticas ocorrem, ou são fixadas, durante a replicação do DNA ou a mitose (O'NEILL, 2000). Todos os dias, milhões de células do organismo adulto normal se dividem. Uma mutação não detectada e/ou reparada adequadamente pode ser transmitida na divisão celular, iniciando a formação de um clone de células com vantagens sobre as demais. As barreiras mais primárias ao desenvolvimento do clone de células tumorais são os próprios pontos de controle do ciclo de divisão celular. Na Figura 1, esquematizamos alguns destes pontos de controle.

Pontos de controle do ciclo celular. O ciclo celular é composto de uma seqüência ordenada de fases. A célula diferenciada se encontra em G0, onde ela atingiu sua diferenciação terminal e está quiescente. Se a célula está destinada a proliferar, ela entra em G1, período em que aumenta de tamanho e prepara as proteínas de que necessita para a síntese de DNA. Durante esta fase, a célula é sensível às condições ambientais. Se elas não forem favoráveis, a divisão celular pára em G1. No entanto, se ultrapassar o ponto R (ponto de restrição), a divisão celular ocorrerá independente de condições ambientais. Na fase S sintetiza-se o DNA que será replicado durante a fase G2. No início de G2 existe outro ponto de controle importante, onde se verificará a qualidade do DNA replicado. Finalmente, na fase mitótica (M), o DNA duplicado será eqüitativamente dividido entre as duas células filhas. A mitose será impedida se, na checagem da mitose, forem constatadas anormalidades na divisão dos cromossomas.

A divisão celular normal é positivamente regulada ou estimulada através de vias sinalizadoras. Estas vias respondem a fatores extra-celulares os quais agem através de uma seqüência de sinais. Por exemplo: receptores → proteína G → proteíno-quinases → fatores de transcrição. A progressão pelo ciclo celular a seguir é, em parte, controlada, por uma série de proteínas chamadas "quinases dependentes de ciclinas" (CDKs), particularmente nas transições de fases, tanto de G1 para S quanto de G2 para M (ARNOLD *et al*, 1989). Os níveis de ciclinas oscilam durante as fases do ciclo, determinando o momento apropriado de sua ligação com as CDKs. Este grupo de enzimas, por sua vez, fosforila uma série de substratos chave que permitirão a progressão de uma fase à outra do ciclo celular (O'NEILL, 2000).

Mecanismo de controle do avanço do ciclo celular. A associação das ciclinas D ou E com suas respectiva quinases foram um complexo ativo que, por sua vez, promove a fosforilação da proteína Rb. Uma vez fosforilada, Rb libera um complexo fator de transcrição chamado E2F-DP1. Ao ser ativado, este fator, por sua vez, promove a transcrição de genes que serão importantes na fase de síntese (S) para produzir nucleotídeos e enzimas necessárias para a replicação do material genético. Isso permite o avanço do ciclo celular.

À semelhança dos fatores estimuladores que levam à produção de ciclinas/CDKs, os reguladores negativos ativarão inibidores das CDKs: as CDKIs. Podemos distinguir duas grandes famílias de CDKIs, de acordo com seu mecanismo de ação, homologia e CDK alvo: 1) o grupo do *p21, p27* e *p57* e 2) o grupo do *p16, p15, p18* e *p19* (O'NEILL, 2000).

Fatores de estímulo e bloqueio do ciclo celular. As ciclinas são reguladoras das subunidades das CDKs. Diferentes ciclinas se associam a diferentes CDKs, podendo associar-se a mais de uma CDK nas diferentes fases do ciclo celular. A atividade ciclina/CDK é bloqueada por uma série de inibidores específicos. Eles podem ser agrupados em famílias como a do *p21/p27/p57* que bloqueia múltiplos complexos ciclina/CDKs e na família *p16/p15/p18/p19* que inibe os complexos CDK4/CDK6. Alguns fatores podem parar o ciclo em G1, como os danos causados ao DNA que, ativando o *p53*, induzem a produção de *p21*. Outros fatores podem atuar através de diferentes grupos de inibidores do ciclo, como TGF-β que induz produção tanto de *p15* como de *p27*. Assim, o ciclo celular é bloqueado para permitir o reparo dos danos detectados e impedir que eles se propaguem para as células filhas e dêem origem a um clone de células tumorais.

O gene p53 é um dos atores principais no controle da correta divisão celular, funcionando, em parte, como regulador da transcrição celular (MENDOZA-RODRIGUEZ & CERBON, 2001). Existem evidencias de que p53 pode interferir na diferenciação da célula tiroidiana. A introdução de um gene p53 mutado dificulta de forma importante a expressão de genes de diferenciação nas células CPC13 de tiróide (BATTISTA *et al*, 1995). Ao contrário, a reintrodução do gene p53 normal em um carcinoma indiferenciado leva à re-expressão de marcadores característicos de diferenciação tiroidiana (FAGIN *et al,* 1996).

O gene p53 também é muito importante na destruição de células que não podem ser reparadas. O próprio gene induz uma cascata de sinais que levam estas células a apoptose (ATTARDI, 2005; BROWN & ATTARDI, 2005).

Mutações no gene *p53* ou inadequado funcionamento da proteína p53 vêm sendo observados em pacientes com vários tipos de malignidades, incluindo a glândula tiróide (LEVINE, 1997; FARID, 2001). Muitos estudos com imunohistoquímica e análises

genéticas tem demonstrado que mutações em p53 são altamente prevalentes em carcinomas de tiróide pouco diferenciados e indiferenciados, assim como em linhagens celulares de câncer de tiróide (FAGIN *et al*, 1993; JOSSART *et al*, 1996). Estas mesmas mutações não são encontradas em tumores benignos e não são freqüentes nos tumores bem diferenciados, sugerindo que a mutação inativadora de p53 ocorre em um estágio mais tardio da progressão dos tumores tiroidianos (DOBASHI *et al*, 1994; ASAKAWA & KOBAYASHI, 2002; HORIE *et al*, 2001).

As características estruturais de p53 (codons 61-94) tem sido bem preservados durante a evolução exceto no exon 4 onde um polimorfismo comum resulta em uma troca do aminoácido prolina pelo arginina na posição 72 (ARA *et al*, 1990). Este polimorfismo tem sido demonstrado em associação com vários tumores, mas o seu papel ainda é muito controverso. Como muitos polimorfismos, o do codon 72 possui distribuição geográfica e variabilidade étnica, sendo demonstrado como um fator genético de risco para o câncer cervico- uterino (SIFUENTES ALVAREZ & REYES ROMERO, 2003), câncer de mama (BUYRU *et al*, 2003), pulmão (PAPADAKIS *et al*, 2002), câncer de cabeça e pescoço (SHEN *et al*, 2002), entre outros. Entretanto, nem todas as investigações têm sido consistentes e sua influência na predisposição a tumores tem permanecido controversa (OREN, 2003; DRUMMOND *et al*, 2002). A variante arginina do codon 72 de p53 (CGC) induz a apoptose de forma mais rápida, suprimindo assim a transformação maligna de maneira mais eficiente que a variante prolina (CCC) (DUMONT *et al*, 2003). A presença da variante Arg em homozigoze é considerada um fator de risco para câncer cervical (QIE *et al*, 2002), enquanto os homozigotos prolina são relacionados ao aumento do risco de carcinomas nasofaringeais (TSAI *et al*, 2002), pulmonares (WANG *et al*, 1999) e carcinomas hepatocelulares (YU *et al*, 1999). O genótipo Arg/Pro está associado ao aumento da susceptibilidade em adenocarcinomas de pulmão induzidos pelo cigarro (FAN *et al*, 2000).

Boltze examinou pacientes caucasianos com carcinomas tiroidianos e concluiu que o genótipo Pro do códon 72 era um fator potencial no risco de desenvolvimento de tumores indiferenciados (BOLTZE *et al*,2002). Entretanto, por ter enfocado o papel do

genótipo em tumores agressivos, não incluiu em seu estudo pacientes com patologias benignas nem comparou a influência do genótipo em CP e CF (BOLTZE *et al,* 2002).

Hipóteses

Com base nas considerações acima, formulamos as seguintes hipóteses:

a) indivíduos que herdaram o gene *GSTP1* em uma de suas formas variantes (*GSTP1 AB* e *GSTP1 BB*) poderiam apresentar um risco aumentado para o câncer de tiróide em comparação com os indivíduos que possuem o gene de tipo selvagem *GSTP1AA*.

b) indivíduos que herdaram o gene *GSTO1* em uma de suas formas variantes (*GSTO*) poderiam apresentar um risco aumentado para o câncer de tiróide em comparação com os indivíduos que possuem o gene de tipo selvagem *GSTO1*.

c) indivíduos que herdaram o gene *p53* em uma de suas formas variantes para o codon 72 poderiam apresentar um risco aumentado para o câncer de tiróide em comparação com os indivíduos que possuem o gene *p53* de tipo selvagem.

d) estes polimorfismos poderiam estar associados de forma a atuar aditivamente, aumentando o risco para câncer de tiróide quando presentes em conjunto.

e) estes polimorfismos poderiam influir, em separado ou aditivamente, na resposta à terapia com radioiodo dos pacientes com carcinoma de tiróide.

OBJETIVOS

Relacionamos a influência de polimorfismos dos genes envolvidos na proteção contra agentes carcinogênicos externos e contra anormalidades produzidas por estes agentes no ciclo celular sobre o desencadeamento do câncer da tiróide.

1. Analisar a influência de polimorfismos dos genes envolvidos na proteção contra agentes carcinogênicos externos e contra anormalidades produzidas por estes agentes no ciclo celular sobre a resposta dos pacientes com câncer da tiróide à terapia.

2. Avaliar o uso do padrão de herança para estes genes na predição de risco para câncer da tiróide em portadores de nódulos tiroidianos de qualquer etiologia.

3. Avaliar o uso do padrão de herança para estes genes na predição de evolução dos diferentes tipos de câncer da tiróide.

MATERIAL E MÉTODOS

Conduzimos um estudo prospectivo caso-controle para compararmos a proporção dos genótipos de *GSTP1, GSTO1* e os polimorfismos do códon 72 de *p53* em pacientes com nódulos tiroidianos benignos e malignos.

Casuística

Pacientes

Os pacientes selecionados para este estudo foram consecutivamente atendidos na Disciplina de Endocrinologia do Hospital das Clínicas da UNICAMP durante os anos de 2001 a 2003, no Ambulatório de Câncer da Tiróide, sob coordenação da profa Dra. Ligia Vera Montalli da Assumpção. Os pacientes foram inscritos no estudo após concordarem em participar do mesmo e assinarem o Termo de Consentimento Informado, conforme as normas do Comitê de Ética em Pesquisa da FCM/ UNICAMP.

Utilizamos material coletado de 44 pacientes com diagnóstico de nódulos tiroidianos benignos (30 bócio e 14 adenomas foliculares) e de 98 pacientes com nódulos malignos (77 carcinomas papilíferos e 21 carcinomas foliculares). Patologistas experientes da UNICAMP, capitaneados pela Profa Dra Patrícia Sabino Matos, especialista em Patologia Endócrina do Departamento de Anatomia Patológica da FCM/UNICAMP, confirmaram todos os diagnósticos.

Todos os pacientes seguem um protocolo padrão implantado há mais de 25 anos no Ambulatório de Câncer da Tiróide, do qual constam, além dos dados de identificação, a idade ao diagnóstico, sexo, cor, dados clínicos pré-cirúrgicos, exames realizados (ultra–som, cintilografia da tiróide, biópsia aspirativa), dados referentes à cirurgia e dados do exame anátomo-patológico (medida do tumor, tipo histológico, grau de diferenciação, presença de linfonodos metastáticos). Nenhum dos pacientes possuía história de exposição acidental ou médica a radiação ionizante. Todos os dados, incluindo os diagnósticos de outras patologias concomitantes, foram confirmados nos prontuários dos pacientes.

Todos os indivíduos que procuram o Ambulatório de Câncer da Tiróide com diagnóstico de carcinoma da tiróide ou a suspeita do mesmo na citologia da punção aspirativa realizada com agulha fina são tratados de acordo com um protocolo padrão.

Todos os pacientes são submetidos a tiroidectomia total ou quase total. Os pacientes com diagnóstico pré-operatório de nódulos mestastáticos no pescoço ou nos quais gânglios suspeitos são visualmente identificados no intra-operatório são submetidos a dissecção regional do pescoço.

Seguimento

O tempo de seguimento dos pacientes incluídos neste estudo foi de 12 - 342 meses (31 - 67 meses). Todos os pacientes com câncer são seguidos com exames periódicos para a detecção precoce de metástases, TSH sérico e tireoglobulina medidos de acordo com a rotina do protocolo de seguimento que inclui raios-X, ultra-sonografia, tomografia computadorizada e outros eventuais procedimentos para a detecção de metástases à distância.

Depois de 4 a 6 semanas da cirurgia, os pacientes são submetidos ao rastreamento de corpo inteiro com I^{131} (PCI). Todos os pacientes recebem cerca de 100 mCi de radiodo após o que se realiza nova PCI. Em seguida, são prescritas doses supressivas de levotiroxina para manter os níveis de hormônio tirotrófico (TSH) suprimido. Cerca de 6 meses após a cirurgia, estes pacientes são revistos e pede-se dosagem de Tiroglobulina sérica (Tg). Cerca de 1 ano após a cirurgia, todos os pacientes são submetidos a uma nova PCI, desta vez após suspensão da levotiroxina, acompanhada de nova dosagem de Tg. Na suspeita de qualquer lesão recidivante ou na presença de níveis de Tg sérica elevados (>2mg/dl), os pacientes são amplamente investigados através de métodos de imagem que incluem ultra-sonografias, RX, tomografias computadorizadas, cintilografia com tálio, PET scan ou o que for mais apropriado de acordo com a suspeita clínica. Nós definimos a evolução como "livre de doença" em indivíduos que mantém níveis de Tg abaixo de 1ng/dL e não possuem qualquer evidência de recorrência, enquanto que os pacientes com recorrência são divididos naqueles com "recorrência local", quando se detectam restos tiroidianos ou recidivas no leito tiroidiano ou gânglios cervicais, e naqueles com "metástases" na presença de metástases à distância.

Estadio

O estadio e o grau de diferenciação dos tumores foram obtidos pelos dados patológicos obtidos do material cirúrgico. O estadiamento tumoral foi baseado no estadiamento clínico de De Groot (DeGROOT, 1995). Esta classificação se baseia no clássico método do TNM, isto é, no tamanho do tumor (T), acometimento de nódulos cervicais (N) e metástases a distancia (M), mas leva em conta a idade, classificando de forma diferente os pacientes abaixo e acima dos 45 anos. Esta classificação considera no estadio 1 os pacientes em que o tumor é de foco único ou múltiplo mas restrito à tiróide; estadio 2 aqueles em que o tumor apresenta acometimento restrito a gânglios cervicais; no estadio 3 o tumor apresenta metástases restritas à região cervical; no estadio 4 o tumor apresenta metástases à distância. A Tabela 1 mostra como esta classificação qualifica os pacientes com câncer da tiróide.

Tabela 1- Critérios clinicopatológicos utilizados no estadiamento dos Carcinomas Diferenciados da Tiróide pelo sistema do Estadio.

	Idade < 45 anos	**Idade ≥ 45 anos**
Estadio I	Qualquer T, qualquer N, M0	T1, N0, M0
Estádio II	Qualquer T, qualquer N, M1	T2, N0, M0
		T3, N0, M0
Estádio III		T4, N0, M0
		Qualquer T, N1, M0
Estádio IV		Qualquer T, qualquer N, M1

Coletamos sangue periférico de todos os pacientes. Adicionalmente, amostras de tecido tiroidiano foram obtidas de 83 portadores de neoplasia operados. O tecido foi obtido no momento cirúrgico e instantaneamente congelado em nitrogênio líquido para posterior armazenamento em freezer – 80°C até o seu processamento. Amostras de tecidos e de sangue periférico dos mesmos pacientes foram comparadas em 35 casos de tumores malignos para observar se havia concordância entre os dados. Além disso, obtivemos tecido tiroidiano contra-lateral ou de local não acometido por neoplasia em 27 casos.

Controles

Obtivemos um grupo controle composto por 157 indivíduos saudáveis (115 mulheres e 42 homens) com idade de 16 a 81 anos (47± 18 anos) selecionados da população da região de Campinas, doadores de sangue do Hemocentro e voluntários. Para obtermos um grupo controle comparável com o dos portadores de neoplasia tiroidiana, selecionamos 3 mulheres para cada 2 homens já que o câncer de tiróide é mais freqüente em mulheres que em homens.

Todos os controles, assim como os pacientes com patologias tiroidianas benignas e malignas, foram classificados em brancos e não-brancos. Dados das condições de saúde e história médica com ênfase em patologias tiroidianas anteriores ou correntes foram obtidos por entrevista usando um questionário padrão. O uso de determinados alimentos foi cuidadosamente avaliado, em particular o uso dos alimentos bociogênicos como os vegetais contendo glucosídeos cianogênicos (muitas formas de repolho, couve-flor, brócolis e outros membros da família dos crucíferas). Também se questionou o uso de drogas que podem interferir na função tiroidiana, assim como o uso de outras medicações para doenças concomitantes, consumo de álcool e hábitos tabagistas, incluindo a duração do tabagismo, o início do hábito, a quantidade de cigarros e a data de abandono do hábito. Os pacientes foram agrupados em não fumantes e fumantes pra fins estatísticos já que estes dados foram considerados pouco confiáveis.

Indivíduos com história prévia de doença tiroidiana, exposição à radiação ou outros antecedentes de malignidade foram excluídos.

Métodos

Análise dos polimorfismos das GSTs

As amostras de sangue coletadas dos 142 pacientes e 157 indivíduos saudáveis foram processadas e seu DNA genômico extraído após a lise de hemácias. Para obter a separação dos leucócitos, usamos o protocolo padrão fenol-clorofórmio-proteinase K. As amostras de tecido foram processadas de acordo com um protocolo padrão para extração de DNA também baseado no método do fenol-clorofórmio – proteinase K. Visualizamos os

polimorfismos do gene *GSTP1* e *GSTO1* usando uma PCR (polymerase chain reaction) seguido de SSCP (single strand conformation polymorphism analysis). As mutações assim rastreadas foram confirmadas pelo seqüenciamento de algumas amostras.

Os primers usados na reação de PCR idealizada para detectar o polimorfismo de *GSTP1* foram desenhados a partir de sua seqüência gênica - http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=Nucleotide&list_uids=02204206&dopt=GenBank (GRANJA *et al,* 2004-B). Para a análise dos polimorfismos de *GSTO1* utilizamos primers previamente descritos (WHITBREAD *et al*, 2003).

Tabela 2- Seqüências dos primers usados nas reações de PCR para GSTs

GSTP1	sense	5' – TCTATGGGAAGGACCAGCAGG – 3'
	anti sense	5' –GCCCAACCTGGTGCAGATG – 3'
GSTO1	sense	5' –TCTAGGTGCCATCCTTG – 3'
	anti sense	5' – TGATAGCTAGGAGAAATAATTACCTC– 3'

Em ambas as reações de PCR foram usados 100ng de DNA genômico, 50 nM de cada primer, 100mM Tris-HCL (ph8, 0), 100uM de dNTP (dATP, dTTP, dCTP, dGTP), 2,0 mM MgCl2 e 0,5 U Taq DNA polimerase para um volume final de 25µl. A amplificação foi feita em termociclador programado para realizar desnaturação a 92°C por 2 minutos, seguida por 35 ciclos. As temperaturas de anelamento utilizadas foram 63°C para os primers de *GSTP1* e 56°C para os primers de *GSTO1* por 50 segundos com extensão de 72 °C por 1 minuto e uma extensão final de 72°C por 7 min. Usamos o termociclador MJ PTC-200. Os produtos obtidos na reação de amplificação por PCR foram submetidos à eletroforese em gel de agarose a 2%, corados com brometo de etídeo e visualizados no transiluminador por um sistema Kodak de visualização e fotografia.

A seguir, submetemos os produtos de PCR a uma eletroforese em gel de poliacrilamida a 6%, misturamos uma solução de 95% de formamida, 0,05% de azul de bromofenol, 0,05% de xileno cianol e 50 mM de NaOH. Após uma desnaturação prévia a 94°C por 10 minutos, a eletroforese foi conduzida entre 2 a 5 Watts em temperatura

ambiente, "overnight". Os géis foram posteriormente corados com nitrato de prata e fotografados para catalogação. Dezessete amostras que apresentaram migrações suspeitas foram seqüenciadas usando o Kit ABI prism big dye sequencing (Perkin Elmer, Warrington, Cheshire UK) e os primers sense, em um seqüenciador ABI 377 Prism DNA sequencer (Perkin Elmer). Controles positivos e negativos foram incluídos em todas as reações de PCR e todas as corridas de SSCP para detectarmos possíveis problemas de contaminação.

Análise dos polimorfismos do códon 72 de p53

Para a determinação do polimorfismo do códon 72 do gene p53 utilizamos 2 pares de primers: um amplifica o alelo da arginina (Arg) e o outro o alelo da prolina (Pro), de acordo com o descrito por Storey (STOREY *et al*, 1998).

Tabela 3- Seqüências dos primers usados nas reações de PCR para o códon 72 de p53

p53Arg	sense	5' – TCCCCCTTGCCGTCCCAA – 3'
	anti sense	5' –CTGGCCAGGGGCCACGC – 3'
p53Pro	sense	5' – GCCAGAGGCTGCTCCCCC – 3'
	anti sense	5' –CGTGCAAGTCACAGACTT – 3'

A detecção dos polimorfismos é feita em duas reações de PCR diferentes. Em ambas as reações utilizamos 100ng de DNA genômico, 50 nM de cada primer, 100mM Tris-HCL (ph8, 0), 100uM de dNTP (dATP, dTTP, dCTP,dGTP), 2,0 mM MgCl2 e 0,5 U Taq DNA polimerase para um volume final de 25µl. Para amplificação, utilizamos uma desnaturação de 94°C por 3 minutos, 35 ciclos de 94°C por 45 segundos. As temperaturas de anelamento utilizadas foram 68°C para os primers de Arg e 53°C para os primers de Pro por 45 segundos e 72°C por 1 minuto com uma extensão final de 72°C por 10 min em um termociclador MJ PTC-200. Os produtos obtidos na reação de amplificação por PCR foram submetidos à eletroforese em gel de agarose a 2%, corados com brometo de etídeo e visualizados no transiluminador por um sistema Kodak de visualização e fotografia.

O produto de PCR do alelo Arg possui 141pb e o produto do alelo Pro possui 177pb. Os indivíduos heterozigotos possuem a amplificação dos dois produtos de PCR enquanto que as amostras homozigotas apresentam somente um dos dois produtos (figura 2).

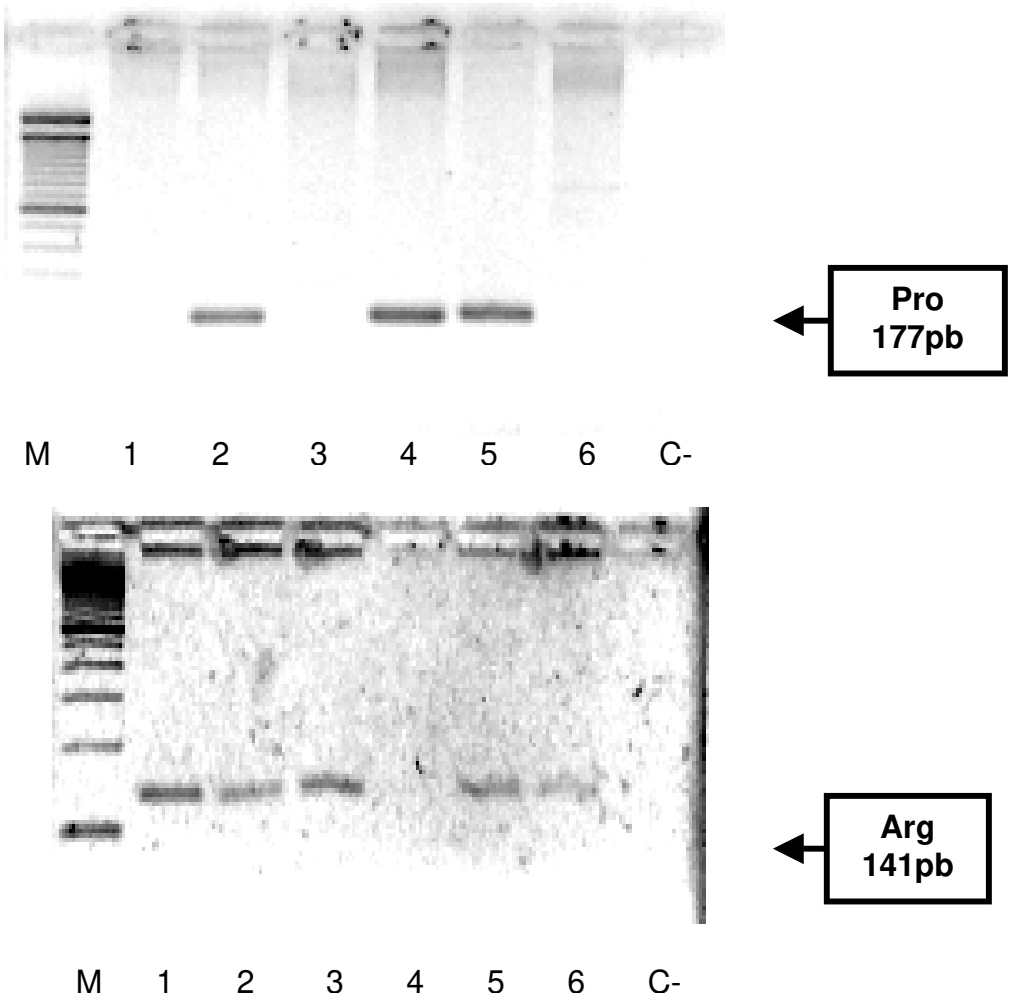


Figura 3- Gel de agarose a 2%, representando os resultados da PCR com os primers do alelo arginina (Arg) e um gel com os primers do alelo prolina (Pro). As amostras são pertencentes aos mesmos indivíduos. Observamos que nas amostras 1,3 e 6 há somente a amplificação do alelo arginina, já as amostras 2 e 5 os heterozigotos com a amplificação dos dois fragmentos e a amostra 4 só amplificou prolina, sendo portanto o paciente homozigoto para prolina; M marcador de peso molecular 100pb (Invitrogen- Brasil Ltda).

Selecionamos 4 amostras homozigotas Pro, 4 amostras homozigotas Arg e 10 amostras heterozigotas Arg/Pro para serem seqüenciadas e assim confirmamos os correspondentes genótipos. O seqüênciamento das amostras foi realizado utilizando-se um produto de PCR amplificado com os primers sense da arginina (5' – TCCCCCTTGCCGTCCCAA – 3') e o antisense da prolina (5' – CGTGCAAGTCACAGACTT – 3'), através dos quais amplificamos um fragmento de 279 pares de bases que abriga a região do polimorfismo. Todas as condições para a PCR foram semelhantes, assim como o ciclo, fazendo-se apenas uma modificação na temperatura de anelamento (60,8°C) (Figura 3).

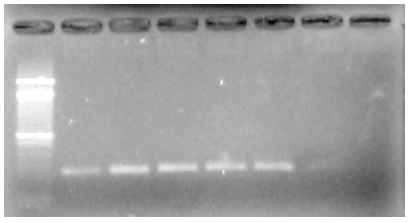


Figura 4- Gel de agarose 2% das amostras amplificadas do códon 72 de *p53* para posterior seqüênciamento das amostras. A PCR foi realizada com o primer p53Arg sense e o primer p53Pro anti sense.

Análise estatística

A análise estatística foi realizada utilizando o software SAS (Statistical Analyses System), versão 8.1, Cary, NC, USA, 1999-2000. Testes exatos de qui-quadrado (χ^2) e o teste de Fisher (F) foram usados para examinar a homogeneidade entre os casos e os controles com relação à raça, cor, doenças tiroidianas prévias, uso de medicações e fumo. A comparação entre o estadiamento dos carcinomas papilíferos e foliculares foi realizada usando-se o teste de Fisher. O teste de Kruskal-Wallis (KW) foi usado para comparar a idade entre os grupos. Os testes de Mann-Whitney ou Wilcoxon foram usados para comparar a idade entre diferentes grupos genotípicos. O odds ratio (OR) e o coeficiente de intervalo de 95% (confidence interval -CI) permitiram estimar a força de associação entre cada variável e o comportamento benigno ou maligno. Utilizamos uma

análise de regressão logística para avaliar os efeitos dos genótipos, depois de ajustados para idade, sexo, cor, fumo, consumo de álcool ou medicamentos na determinação de risco para malignidade e na determinação da resposta terapêutica e evolução á longo prazo. As interações entre as variáveis ambientais e os genótipos também foram avaliados. Todos os testes foram realizados com p=0,05 como nível de significância.

RESULTADOS

Não encontramos diferenças entre o grupo controle e os pacientes com doenças tiroidianas em relação à idade (47±21 anos *vs* 49±14 anos), sexo (42 homens e 115 mulheres *vs* 37 homens e 61 mulheres), cor (72 brancos e 60 não brancos *vs* 54 brancos e 44 não brancos), ingestão de medicamentos (39 controles *vs* 29 pacientes), fumo (61 controles *vs* 46 pacientes), consumo de álcool (61 controles *vs* 46 pacientes) e os diferentes tipos de dietas.

Na tabela 4 resumimos as características clínicas, os parâmetros de agressividade, o diagnóstico e o tempo de seguimento dos pacientes com câncer de tiróide.

Pacientes com carcinoma folicular (CF) são mais velhos que os pacientes com carcinoma papilífero (CP) (KW; p=0,0012) embora os grupos não sejam diferentes quanto à cor, raça, hábitos de fumo, uso de medicamentos ou doença benigna da tiróide preexistente.

A ocorrência de linfonodos cervicais acometidos já no momento do diagnóstico é mais freqüente em CP (40%), do que em CF (14%), (F; p<0,05). No entanto, pacientes com CF apresentam mais metástase à distância no momento do diagnóstico (48%) do que os CP (10%), (F; p<0,001). Os dados do seguimento dos pacientes não mostraram diferenças entre CP e CF no aparecimento de metástase à distância e/ou recorrência e tumor, embora pacientes com CF apresentem recorrência ou metástase em 48% dos casos e os pacientes com CP em apenas 26% dos casos (F; P= 0,11). Dois pacientes morreram durante o período de observação por condição relacionada diretamente ao câncer da tiróide.

Tabela 4- Distribuição dos pacientes com carcinoma de tiróide de acordo com a histologia, características clínicas incluindo idade (em anos), sexo (F, feminino; , masculino), cor (B, branco; NB, não branco), história prévia de doença benigna tiroidiana, tabagismo, uso de medicações, presença de linfonodo comprometido e metástase à distância no diagnóstico ou recorrência e/ou metástase durante o seguimento.

Características Clínicas								**Presença de Metástase ao Diagnóstico**		**Seguimento**
Idade	Sexo		Cor		Doença tiroidiana prévia	Tabagismo	Uso de medicamento	Linfonodo	Distância	Recorrência ou metástase à distância
	M	**F**	**B**	**NB**						
CP 44±15	28	66	61	16	18	37	21	30	08	20
CF 56±12	09	14	15	06	09	09	08	03	10	10

Tabela 5- Comparação estatística dos grupos e proporções dos genótipos de *GSTP1* na população controle e nos pacientes com doenças tiroidianas malignas e benignas.

	N.º de Casos	N (%)			OR (95% CI)	Valor de P
		AA	AB	BB		
Controles	157	148 (94.2%)	4 (2.5%)	5 (3.1%)		
Bócio	30	26 (86.6%)	1 (3.3%)	3 (10%)	AA *vs* AB 1.423 (0.1528 - 13.250) AA *vs* BB 3.415 (0.7688 – 15.172) AB *vs* BB 2.400 (0.1751 – 32.900) N^a *vs* A^B 2.530 (0.7251 – 8.827)	0.56 0.12 1.00 0.23
Adenoma folicular	14	11 (78.5%)	1 (7.1%)	2 (14.2%)	AA *vs* AB 3.364 (0.3455 – 32.750) AA *vs* BB 5.382 (0.9344 – 30.999) AB *vs* BB 1.600 (0.1036 – 24.720) N^a *vs* A^B 4.485 (1.059 – 18.994)	0.32 0.09 1.00 0.06
Carcinoma papilífero	77	57 (74%)	13 (16.8%)	7 (9%)	AA *vs* AB 8.439 (2.641 – 26.968) AA *vs* BB 3.635 (1.108 – 11.924) AB *vs* BB 0.430 (0.086 – 2.143) N^a *vs* A^B 7.092 (2.307 – 21.802)	<0.0001 0.0444 0.4223 <0.0001
Carcinoma folicular	21	14 (66.6%)	6 (28.5%)	1 (4.7%)	AA *vs* AB 15.85 (3.993 – 62.973) AA *vs* BB 2.114 (0.2305 – 19.397) AB *vs* BB 0.133 (0.01103 – 1.612) N^a *vs* A^B 9.625 (2.484 – 37.291)	0.0002 0.4345 0.1451 <0.0001

[a]N, Normal
[b]VA, Variantes alélicas

O gráfico 1 representa a comparação estatística da prevalência de *GSTP1* normal e de suas variantes alélicas entre os grupos. A presença das variantes alélicas de *GSTP1* não foi diferente entre os adenomas foliculares (21.3%) e os carcinomas foliculares (33.2%) (p=0.704).

O perfil genotípico de *GSTP1* demonstrou ser absolutamente idêntico nas amostras de tecidos normais e nos tumores, assim como nas amostras de sangue periférico.

Pacientes com nódulos benignos, e, sobretudo, pacientes com CP e CF demonstram uma presença significativamente maior das variantes alélicas de *GSTP1* quando comparados com a população controle (χ^2, p<0.0001). No grupo de pacientes com nódulos malignos da tiróide predominam os indivíduos heterozigotos para *GSTP1AB* (21%) sobre os homozigotos *GSTP1BB* (9%), enquanto que na população controle a prevalência de hetero e homozigotos é de 2,5 e 3%, respectivamente (χ^2, p<0.0001). O risco para desenvolver câncer de tiróide em indivíduos com as variantes de *GSTP1,* depois de ajustados para a raça, a idade, o fumo e o uso de drogas, aumenta 7 vezes (OR=7.092; CI 2.307 – 21.802) para carcinoma papilífero e mais de 9 vezes (OR=9.625; CI 2.484 – 37.291) para carcinoma folicular.

Não encontramos associação entre genótipo e as características clínicas dos pacientes, parâmetros tumorais, agressividade ao diagnóstico ou o comportamento durante o seguimento.

Tabela 6- Perfil dos genótipos de *GSTO1* dos nódulos benignos e dos carcinomas tiroidianos, quando comparados com a população controle.

	A140/A140	**A140/D140**	**D140/D140**
Nódulos benignos	41	8	3
N=52	(78.8%)	(15.4%)	(5.8%)
Carcinomas tiroidianos	74	17	2
N=93	(79.6%)	(18.3%)	(2.1%)
Controles	146	19	8
N=173	(84.4%)	(11%)	(4.6%)

Em relação ao gene *GSTO1,* não observamos diferenças entre a incidência de heterozigotos e homozigotos em nódulos benignos, malignos e na população controle.

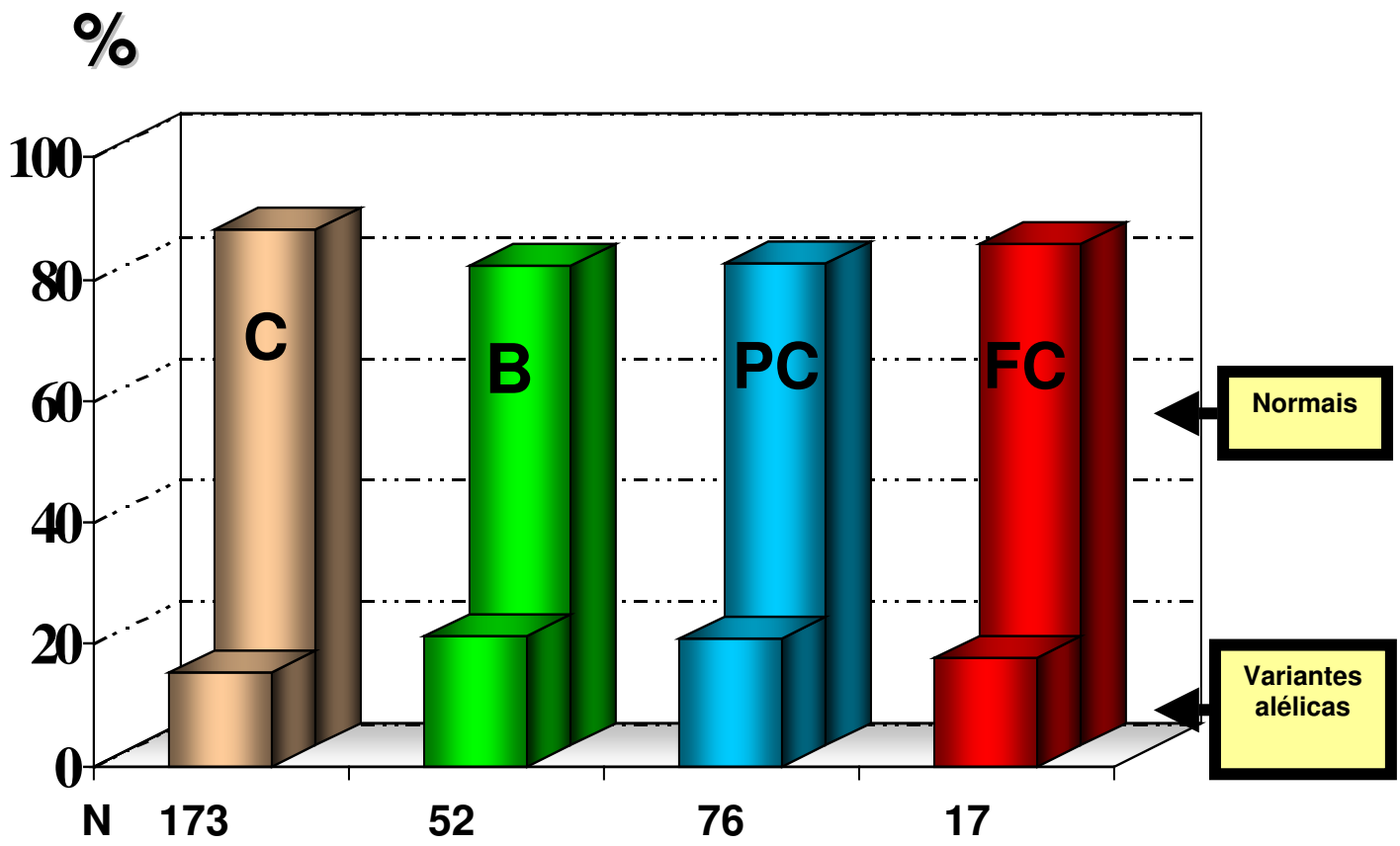


Gráfico 2- Proporções dos genótipos de *GSTO1* selvagem e variantes na população controle e nos pacientes com doenças tiroidianas benignas e malignas.

A freqüência das variantes de *GSTO1* encontrada em nossa população foi similar, a de outros estudos (f = 0.156) e à freqüência descrita em australianos (f= 0.335), japoneses (f= 0.118), chineses (f= 0.165), mexicanos (f= 0.118) e africanos (f= 0.081) (WHITBREAD *et al*, 2003; CERDA-FLORES *et al,* 2002).

Tabela 7- Perfil dos genótipos do códon 72 de p53 na população controle e em pacientes com doenças tiroidianas benignas e malignas.

	Controles	**Nódulos Benignos**		**Nódulos Malignos**	
	N (%)	**Bócio**	**Adenoma folicular**	**Carcinoma papilífero**	**Carcinoma folicular**
Arg/Arg	51 (33.3%)	18 (60%)	5 (35.7%)	28 (36.3%)	9 (42.9%)
Arg/Pro	99 (64.7%)	11 (36.6%)	9 (64.2%)	41 (53.2%)	8 (38%)
Pro/Pro	3 (1.9%)	1 (3.3%)	0	8 (10.3%)	4 (19%)
Total de casos	153	30	14	77	21

Em relação ao codon 72 do gene *p53,* apresentamos os dados resumidos de todas as proporções dos genótipos encontrados na tabela 7. Os pacientes com nódulos benignos e, em particular, os pacientes com CP e CF, demonstraram uma maior prevalência do genótipo Pro/Pro (χ^2, p=0.0015). O risco para o câncer de tiróide, depois do ajuste dos dados para raça, idade, fumo, consumo de álcool e medicamentos aumentou mais de 7 vezes (OR=7,023; CI 1,928 – 25.588) em indivíduos com o genótipo Pro/Pro quando comparado com os outros genótipos. O risco para carcinoma do tipo papilífero aumentou mais de 5 vezes (OR=5,299; CI 2,3344 – 40.436)(p=0.0074) e o risco para carcinoma do tipo folicular aumentou quase 10 vezes (OR=9,714; CI 2,334 – 39.425)(p=0.0043) em indivíduos que apresentam o genótipo Pro/Pro quando comparados com os outros genótipos.

O gráfico 3 representa a distribuição dos grupos estudados em relação à prevalência percentual do genótipo Pro/Pro. O genótipo Pro/Pro não apareceu em nenhum dos 14 casos de adenomas foliculares, mas ocorreu em 19% dos carcinomas foliculares. O genótipo Arg/Pro incide de maneira similar em todos os grupos.

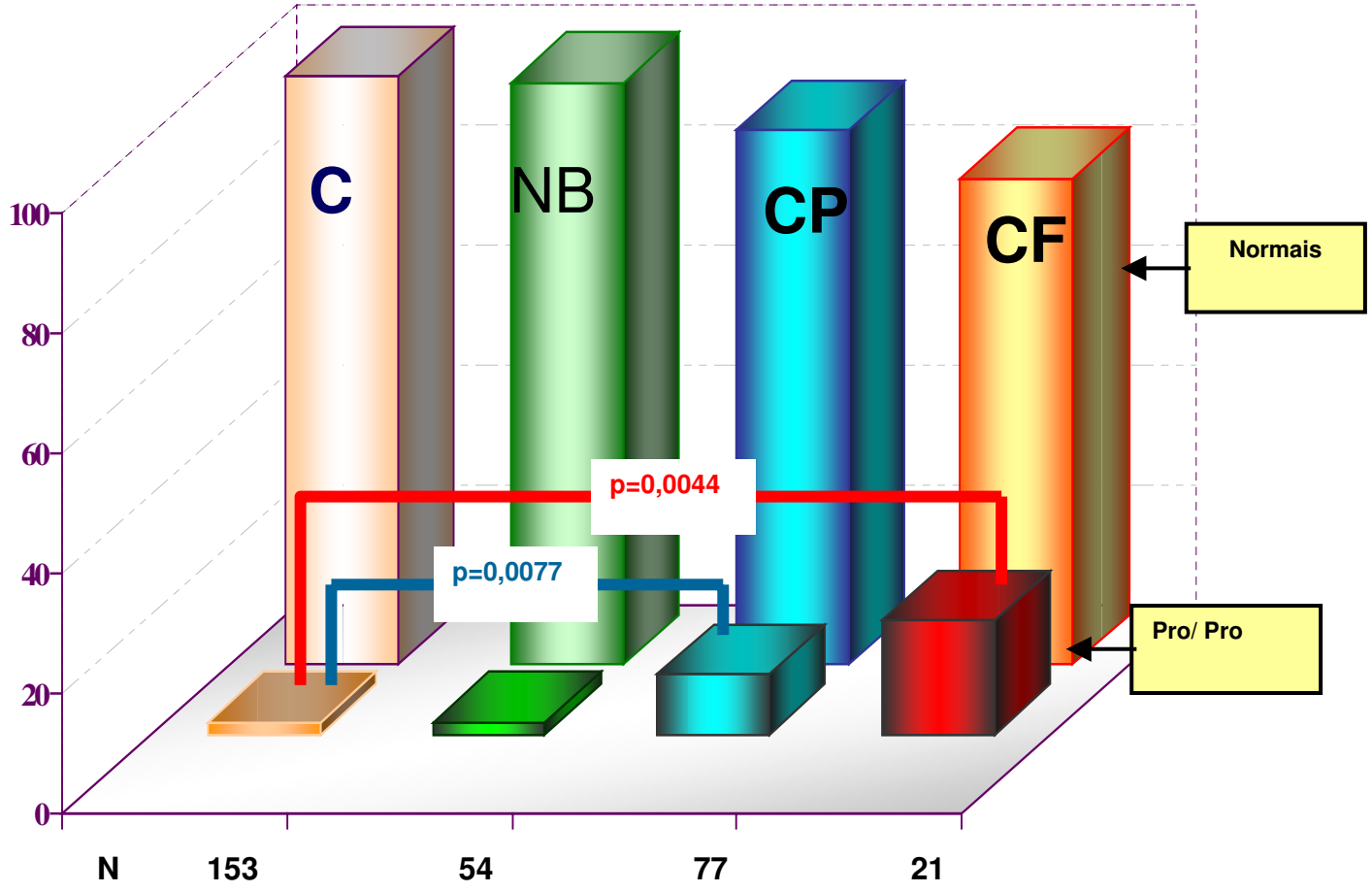


Gráfico 3- Análise estatística da prevalência do genótipo Pro/Pro do códon 72 de p53 (colunas da frente) em comparação com os outros genótipos (coluna de trás) na população controle (C) e nos pacientes com nódulos benignos (NB), carcinoma papilífero (CP) e carcinomas foliculares (CF).

Nossos dados, tanto de *GSTP* quanto de *GSTO* e *p53* não estão de acordo com o equilíbrio de Hardy-Weinberg, nem em pacientes com câncer nem na população controle.

Para que um a população esteja no equilíbrio de Hardy-Weinberg deve obedecer a algumas regras: os acasalamentos entre os indivíduos dessa população devem ser aleatórios e não seletivos; a população não deve estar sujeita a migrações nem a mutações constantes. Assim as freqüências e razões genotípicas nessa população serão constantes de geração para geração. Notemos que as suposições para a validade da lei são muito restritas, e que na maioria dos casos ela só pode ser aplicada a populações teóricas. (UZUANI & BINER,1997) No caso da população brasileira, com suas múltiplas e sucessivas ondas de imigração das mais variadas origens, o equilíbrio de Hardy-Weinberg deve levar ainda algum tempo para ser atingido.

DISCUSSÃO

Identificar os indivíduos que possuem um risco aumentado para o câncer é importante para planejar e implementar políticas de prevenção e estratégias de conduta não apenas em nível de saúde pública, mas também para cada paciente em particular. Assim como em outros países, temos verificado um grande aumento no número de indivíduos identificados como portadores de nódulos de tiróide graças ao maior acesso da população ao sistema de saúde e, sobretudo, a métodos diagnósticos não-invasivos, simples, rápidos e de custo relativamente baixo em nosso meio, como é a ultra-sonografia (CHOW *et al*, 2003; HEGEDUS, 2003; TAN & GHARIB, 1997). Esta crescente população faz prever que devamos ter, nos próximos anos, uma imensa massa de indivíduos buscando aconselhamento médico para seus nódulos de tiróide. O que faremos com estes pacientes?

O uso de marcadores moleculares de risco, identificados através de um simples exame em sangue periférico, poderia auxiliar no rastreamento de malignidade. Poderíamos, por exemplo, programar ultra-sonografias periódicas e/ou obter citologia de nódulos de maior risco. Ao contrário, pacientes com nódulos de baixo risco de malignidade poderiam ser acompanhados apenas clinicamente ou de forma menos intensa, poupando grandes somas de dinheiro ao sistema de saúde e diminuindo consideravelmente o impacto psicológico do risco de malignidade que pesa sobre os portadores de nódulos e seus médicos.

Uma série de fatores clínicos e epidemiológicos vem sendo utilizada na seleção dos pacientes de risco para câncer da tiróide entre os portadores de nódulos tiroidianos (WELKER & ORLOV, 2003; LIVOLSI, 1990; CHOW et al, 2003; FIGGE, 1999; MACK *et al*, 2003). Infelizmente, nenhum destes fatores é suficientemente confiável para afastar o risco de câncer ou predizê-lo com certeza.

Marcadores moleculares podem ser aplicados a um grande número de indivíduos em qualquer fase de sua vida. Poderiam assim, se tornar uma importante arma de rastreamento, identificando indivíduos com risco maior para o câncer dentre a vasta maioria de nódulos benignos. Tal rastreamento poderia baratear sensivelmente o custo do diagnóstico do câncer da tiróide e ser de grande impacto social. Mais ainda, poderia identificar indivíduos com maior risco de desenvolver câncer em situações médicas como o uso de radiação ionizante, além de prover aconselhamento adequado em relação a fatores de risco reconhecidos epidemiologicamente como a injestão de iodo.

Os estudos de marcadores de polimorfismos pontuais ou de uma única base (single nucleotide polymorphysms - SNPs) vêm se tornando populares graças às suas propriedades que os transformam em instrumentos simples e baratos para a análise genética de diferentes doenças (KIRK *et al*, 2002). A esmagadora quantidade de dados advindos de estudos com polimorfismos gênicos vem indicando a sua importância na compreensão dos principais fatores implicados no desenvolvimento do câncer (VINEIS *et al*, 2003). Os polimorfismos que existem nas enzimas responsáveis pelo metabolismo de drogas e de substâncias tóxicas ajudam a compreender a susceptibilidade individual aos carcinógenos químicos e ajudam a explicar as variações da incidência do câncer de tiróide em todo o mundo (STRANGE & FRYER, 1999).

Para podermos estudar a base genética para a susceptibilidade ao câncer da tiróide, deveríamos conhecer os fatores que podem desencadear a doença. Infelizmente, pouco se sabe sobre os fatores de susceptibilidade ao câncer de tiróide. A exposição à radiação ionizante, principalmente em crianças, é o único fator ambiental reconhecido como capaz de levar à formação de tumores benignos e malignos da tiróide, embora deficiência de iodo também tenha sido ligada ao desencadeamento de tumores por este mecanismo (SCHLUMBERGER et al, 1999; RONCKERS *et al*, 2005). Radicais livres produzidos pela irradiação ionizante aumentam o estresse oxidativo, formando produtos que reagem com o DNA e podem induzir mutações (SARASIN, 1999). Além disso, a exposição à radiação ionizante induz instabilidade genética que aumenta consideravelmente o risco de novas e sucessivas mutações poderem se acumular e levar a célula atingida a progredir em direção à formação de um clone de células tumorais (NIKIFOROV *et al*, 1998; BOUNACER *et al*, 2000; TALINI 2002). A exposição à radiação ionizante sem dúvida pode causar câncer. No entanto, não explica a maior parte dos casos de tumores esporádicos. Por outro lado, em populações expostas à mesma quantidade de radiação na mesma faixa etária, alguns indivíduos desenvolvem câncer enquanto que outros não. Seguramente outros fatores genético-ambientais influenciam a etiopatogenia dos tumores tiroidianos.

Infelizmente, os dados disponíveis sobre produtos carcinogênicos e a glândula da tiróide são relativamente escassos e bastante conflitantes. Muitos produtos são capazes de induzir neoplasia tiroidiana em roedores, alguns especificamente relacionados ao sexo

dos animais (MIYAWAKI *et al*, 2003). Especula-se que a ação destes produtos se deva ao estímulo prolongado da célula folicular pelo TSH, provavelmente envolvendo inativação da peroxidase tiroidiana por espécies reativas resultantes do metabolismo destes produtos (MIYAWAKI, 2003; TAMURA *et al*, 1999; O'BRIEN, 2000). Assim, estes produtos tóxicos formariam compostos intermediários reativos que se ligariam a aminoácidos críticos para a atividade da peroxidase (O'BRIEN, 2000). A inativação da ação da tireoperoxidase leva a queda na produção de hormônios tiroidianos e daí, a um aumento do TSH hipofisário, o qual causa aumento da tireoperoxidase e também da síntese de NADPH oxidase (O'BRIEN, 2000). Mais ainda é possível que estas espécies intermediárias reativas possam reagir com outras moléculas levando a produtos de peroxidação lipídica que reagem com o DNA (O'BRIEN, 2000). Com isso, a célula folicular recebe um estímulo de crescimento prolongado que pode selecionar células que já apresentam vantagens proliferativas, por exemplo, por apresentarem variantes gênicas que dificultam o reconhecimento de anormalidades no DNA ou impedem a adequada apoptose das células defeituosas.

Um amplo rastreamento com mais de 200 drogas revelou que apenas duas, a griseofulvina e a senna, estavam associadas com o aumento do risco de carcinomas tiroidianos em humanos (FRIEDMAN & URY, 1983). A ingestão de alimentos bociogênicos não está associada com o aumento do risco de carcinomas tiroidianos de acordo com um estudo caso–controle de Ron et al, 1987. No entanto, um estudo baseado no aumento da incidência de câncer de tiróide nas mulheres do sudeste da Ásia que vivem nos Estados Unidos, comparados com norte americanas de outras ascendências, concluiu que o consumo de carotenóides e isoflavonas na alimentação baseada em soja das primeiras contribui para a maior incidência de câncer da tiróide (HASELKORN *et al*, 2003; MACK *et al*, 2002).

Um dos mais importantes mecanismos de defesa contra os carcinógenos ambientais é uma família de genes que codificam enzimas diméricas que são provavelmente expressas em todas as formas de vida, as enzimas do sistema glutationa S-transferase (MANNERVIK, 1985). Indivíduos com as formas variantes do gene *GSTP1* produzem uma enzima com uma capacidade de detoxificação diminuída para uma extensa lista de carcinógenos ambientais. Indivíduos com ausência dos genes *GSTM1* e *GSTT1*

também são mais susceptíveis aos efeitos de uma grande série de carcinógenos. Infelizmente, dados da literatura a respeito do papel das enzimas do sistema GST em câncer de tiróide são bastante escassos. Um estudo recente não foi capaz de correlacionar polimorfismos dos genes *GSTM1*, *GSTT1* e *GSTP1* com o risco de câncer de tiróide (HERNANDEZ *et al*, 2003). Os autores usaram restrição enzimática para a análise dos polimorfismos mas, infelizmente, não pareararam casos e controles em relação à idade e ao sexo (HERNANDEZ *et al*, 2003). Ao contrário, com o auxílio de uma grande população controle e de bom número de casos, todos eles bem controlados, pareados para idade e sexo, nós demonstramos anteriormente que o genótipo nulo combinado de *GSTT1* e *GSTM1* aumenta o risco de lesões malignas da tiróide em (MORARI *et al*, 2002). Em adição, o presente estudo mostrou que a prevalência das variantes de *GSTP1* em lesões malignas, carcinomas foliculares 33% e em carcinomas papilíferos 26%, são significativamente mais alta que na população controle 5% (x^2 ;p<0,001). Deste modo, as variantes de *GSTP1* aumentam o risco estimado para carcinoma papilífero 5,7 vezes e para carcinoma folicular 8,2 vezes (GRANJA *et al*, 2004-B). Mais recentemente, Gaspar et al, 2004, corroborando nossos achados, mostrou que uma combinação destes 3 alelos de risco: a ausência de *GSTM* e de *GSTT*, combinada com a variante Ile/Ile de *GSTP1*, aumenta o risco de câncer diferenciado da tiróide quase 3 vezes (OR=2.91). Este aumento ocorre às custas de aumento de risco para o CP mas não para o CF nos dados de Gaspar, contrariando nossos achados que sugerem uma importância maior do perfil genotípico de *GSTP1* para o desenvolvimento de carcinomas foliculares (GASPAR *et al*, 2004; GRANJA *et al*, 2004-B).

Estudos de polimorfismos freqüentemente apresentam diferentes resultados relacionados ao sexo, à idade e à etnia das populações estudadas (GARTE *et al*, 2001). Em contraste com a população espanhola estudada por Hernandez et al, e a população portuguesa estudada por Gaspar et al, a população brasileira possui alta heterogeneidade já que é composta por imigrantes da Europa, África e Ásia mesclados com indígenas da população nativa. Nossos hábitos alimentares, baseado em arroz e feijão, também diferem dos europeus. Além disso, muitas condições sociais e culturais contribuem para a exposição a diferentes fatores ambientais que podem ser muito importantes e que podem explicar as

diferenças entre nossos resultados e os de Herandez e de Gaspar (HERNANDEZ *et al*, 2003; GASPAR *et al*, 2004).

É interessante notar que entre os portadores de câncer de tiróide, quando comparamos com a população controle, indivíduos com o genótipo heterozigoto AB são mais freqüentes que homozigotos BB do gene *GSTP1*. Os dois genótipos variantes de *GSTP1* (AB e BB) produzem enzimas com uma diminuída atividade específica e afinidade aos componentes eletrofílicos (ALI-OSMAN *et al*, 1997). No entanto "experimentos *in vitro*" demonstraram que esta afinidade e atividade diferem em muitos substratos eletrofílicos (ALI-OSMAN *et al,* 1997). Uma série de dados obtidos em amostras de tecidos e experimentos com cultura de células usando um considerável número de compostos diferentes mostra que realmente a ação tóxica é tecido e/ou célula específico (PAL *et al*, 2000; WATSON *et al*, 1998; VAN LIESHOUT *et al*, 1999). Assim, é possível que os heterozigotos AB de *GSTP1* sejam mais propensos a sofrer a ação de determinados produtos tóxicos que lesam particularmente a célula folicular tiroidiana.

Portanto, nós sugerimos que o polimorfismo de *GSTP1* é um importante fator de susceptibilidade dos indivíduos aos efeitos tóxicos de fatores ambientais relacionados ao processo de carcinogênese tiroidiana (GRANJA *et al*, 2004-B). Infelizmente, não encontramos nenhuma associação entre os fatores clínicos, histológicos ou os parâmetros de agressividade medidos por qualquer tipo de estadiamento ou sistema de classificação de agressividade ao diagnóstico ou durante o seguimento com os genótipos de *GSTP1*. Assim, presumimos que o perfil genotípico de *GSTP1* não deva ser útil como um indicador de prognóstico para carcinomas diferenciados da tiróide. Por outro lado, estes dados sugerem que uma genotipagem relativamente simples, como é a de *GSTP1*, pode ser utilizada para o rastreamento de malignidade dos nódulos tiroidianos.

Também não encontramos correlação entre os genótipos anteriormente já estudados por nós nestes pacientes (*GSTM* e *GSTT*) com o de *GSTP*, contrariamente aos dados Gaspar et al, o qual demonstrou que a combinação dos genótipos para *GSTM1, GSTT1* e *GSTP1* AA leva a um significante aumento do risco para tumores papilíferos (MORARI *et al,* 2002; GASPAR *et al,* 2004). Novamente, a explicação desta discrepância pode residir nas diferentes populações estudadas. Também não podemos excluir a

influência de outros polimorfismos de *GSTP1* ainda não estudados, ou a existência de "linkage" de *GSTP* com outros genes envolvidos em estágios mais tardios da carcinogênese tiroidiana.

A recente caracterização de uma nova classe de genes, a *GSTO*, também atraiu nossa atenção (BOARD *et al*,2000). As variantes gênicas da *GSTO1* codificam enzimas que apresentam reduzida atividade enzimática e baixa capacidade de detoxificação do arsênico inorgânico (WHITBREAD *et al*, 2003). O arsênico é um carcinógeno ambiental bem conhecido e a contaminação da água potável com o arsênico inorgânico é um problema de saúde mundial (WHITBREAD *et al*, 2003). A detoxificação do arsênico inorgânico é feita principalmente através do processo de metilação (WHITBREAD *et al*, 2003). Variações na capacidade individual de metilação são bem correlacionadas com a intolerância ou a maior susceptibilidade aos efeitos tóxicos do arsênico (HIRAKAWA *et al*, 2002). Infelizmente, nossos dados, mostram que o gene *GSTO* e as suas variantes não apresentam correlação com o risco de malignidade em nódulos tiroidianos ou com as características clínicas e patológicas do câncer da tiróide (GRANJA *et al,* 2005). Por outro lado, nosso estudo do *GSTO* mostrou que a população brasileira possui uma freqüência das variantes do gene similar (f=0.156) àquelas freqüências descritas em australianos (f= 0.335), Japoneses (f= 0.118), chineses (f= 0.165), mexicanos (f= 0.118) a africanos (f= 0.081) (WHITBREAD *et al*, 2003; CERDA-FLORES *et al,* 2002). Estes dados de distribuição genotípica em nossa população vêm ajudando a traçar um perfil que poderá ser útil para o cálculo do risco para diferentes condições médicas e para propor ações preventivas em indivíduos expostos aos agentes tóxicos ambientais.

Finalmente, estudamos o polimorfismo do códon 72 de *p53.* Esse polimorfismo é particularmente interessante desde que se vem demonstrando que a variante Pro 72 possui uma habilidade marcadamente reduzida de induzir a apoptose em comparação com a variante Arg 72. Uma das causas desta redução na capacidade apoptótica advém do fato de que a variante Pro não é capaz de se localizar na mitocôndria, ao contrário da variante Arg. Com isso, a variante Pro não deve ser capaz de promover uma liberação eficiente de citocromo c oxidase para o citosol (DUMONT *et al*, 2003). Esta liberação, essencial para o processo da apoptose, protegeria da destruição células que podem apresentar uma vantagem de crescimento sobre as demais (DUMONT *et al*, 2003). A associação do polimorfismo do

códon 72 de p53 com maior susceptibilidade a muitos tipos de câncer vem sendo bem documentada na literatura (BUYRU *et al*, 2003; PAPADAKIS *et al,* 2002; SHEN *et al*, 2002; DRUMMOND *et al*, 2002; DONG *et al*, 2003; WU *et al,* 1995; WANG *et al*, 1999; QIE *et al*, 2002; TSAI *et al*, 2002; FAN *et al*, 2000; YU *et al*, 1999). No entanto, existe um único estudo da relação entre o polimorfismo do códon 72 de p53 e o câncer de tiróide (BOLTZE *et al*, 2002). Boltze conseguiu reunir 22 carcinomas anaplásicos que foram comparados com 21 carcinomas papilíferos. Os seus dados, de forma similar aos nossos, mostram que o homozigoto prolina é um potente fator de risco para câncer de tiróide. No entanto, em contraste com o trabalho de Boltze, nós encontramos a variante Pro 72 também em carcinomas tiroidianos bem diferenciados e mesmo em um caso de hiperplasia benigna. Além disso, estudamos um número consideravelmente maior de pacientes com carcinomas diferenciados da tiróide, o que nos permitiu avaliar o diferente impacto da variante Pro 72 em carcinomas papilíferos e foliculares. Mostramos que o genótipo Pro/Pro confere um risco bem maior para o desenvolvimento de carcinoma folicular (OR=9,714) do que para carcinoma papilífero (OR=5,299) (GRANJA *et al*, 2004-A).

Infelizmente, novamente não fomos capazes de identificar qualquer associação entre o perfil genotípico destas variantes com qualquer característica clínica ou histológica de agressividade do tumor nem tampouco com sua evolução ou sua resposta ao tratamento (GRANJA *et al,* 2004-A).

Nossos dados, tanto de GSTP quanto de GSTO e p53 não estão em equilíbrio alélico na população, ou seja, não estão de acordo com o princípio de equilíbrio de Hardy-Weinberg, nem em pacientes com câncer nem na população controle. Evidentemente, este desequilíbrio pode indicar a existência de relação entre estes genes e o desenvolvimento do câncer da tiróide, apoiando nossas hipóteses. Entretanto, outros 2 fatores importantes podem concorrer para este fato: o tamanho relativamente pequeno do grupo estudado e a alta heterogenidade da população brasileira, composta de imigrantes europeus, africanos, asiáticos e a população indígena. Temos uma população relativamente jovem, em que sucessivas migrações e imigrações influenciam o perfil genético. O fato de não haver equilíbrio alélico nem na população controle favorece esta última possibilidade.

CONCLUSÃO

Conduzimos um estudo prospectivo de tipo caso-controle para comparar a proporção de variantes conhecidas dos genótipos de *GSTP1, GSTO1* e o polimorfismo do códon 72 de *p53* entre pacientes com nódulos tiroidianos benignos, malignos e um grupo controle pareado para idade, sexo, raça, hábitos, uso de medicamentos e proveniência. Nossos dados sugerem que o perfil genotípico de *p53* e de *GSTP1*, mas não o de *GSTO1,* pode ser usado para o rastreamento de malignidade em pacientes com nódulos tiroidianos.

Grandes estudos e/ou meta-análises de dados provenientes de outras populações deverão confirmar nossos dados e possibilitar uma compreensão melhor dos fatores envolvidos na etiopatogenia do câncer da tiróide.

Estudos populacionais são necessários para avaliar o custo-benefício dos métodos que utilizam o sangue periférico, empregando estes SNPs como marcadores de malignidade para nódulos tiroidianos.

Concluímos a partir dos dados obtidos neste trabalho:

- Sugerimos que o polimorfismo (I105V) de *GSTP1* está associado com uma susceptibilidade aumentada ao desenvolvimento do câncer de tiróide.
- Sugerimos que o polimorfismo (Ala140Asp) de *GSTO1* não está relacionado com o aumento da susceptibilidade ao desenvolvimento do câncer de tiróide. Nossos dados são semelhantes aos de outros estudos deste polimorfismo realizados na população Australiana, Japonesa, Chinesa, Mexicana e Africanos estudado por Whitbread e colaboradores,2003.
- Sugerimos que o genótipo Pro/ Pro do codon 72 de *p53* está associado com uma susceptibilidade aumentada ao desenvolvimento do câncer de tiróide.
- Quando relacionamos e associamos os diferentes genótipos do sistema GST e genótipo Pro/ Pro de *p53* entre si, não observamos associação entre os genótipos com a susceptibilidade aumentada ao desenvolvimento do câncer de tiróide. Quando relacionamos e associamos os diferentes genótipos do sistema GST e genótipo Pro/ Pro de *p53* não observamos associação com os fatores prognósticos, o grau de agressividade e nem com a resposta terapêutica destes pacientes.

Em conclusão, nós sugerimos que uma combinação desses SNP´s com as características clínico-epidemiológicas pode ser muito interessante para identificar risco de malignidade para câncer da tiróide. Esta pode ser uma arma interessante para rastrear a presença de câncer entre os indivíduos portadores de nódulos tiroidianos, diagnosticando o câncer mais precocemente, definindo grupos de maior e de menor risco que poderão ser acompanhado de mais perto ou com maior frequência, com ultra-sonografias periódicas ou mais punções aspirativas.

REFERÊNCIAS BIBLIOGRÁFICAS

AHSAN H, THOMAS DC. Lung cancer etiology: independent and joint effects of genetics, tobacco, and arsenic. **JAMA.** 292 (24): 3026-9, 2004.

ALI-OSMAN F, AKANDE O, ANTOUN G, MAO JX, BUOLAMWINI J. Molecular cloning, characterization, and expression in Escherichia coli of full-length cDNAs of three human glutathione S-transferase Pi gene variants. Evidence for differential catalytic activity of the encoded proteins. **J Biol Chem.** *272*:10004-10012, 1997.

American Cancer Society. Cancer facts and figures 2003. Disponível em: http://www.cancer.org/docroot/

AMES BN, GOLD LS. Carcinogenesis debate. **Science.** 14; 250(4987): 1498-9,1990.

AMES BN, PROFET M, GOLD L S. Dietary pesticides (99,99% all natural). **Proc. Natl. Acad. Sci. U.S.A.** 87: 7777-7781, 1990.

ARA S, LEE PS, HANSEN MF, SAYA H. Codon 72 polymorphism of the TP53 gene. **Nucleic Acids Res.** 18:4961,1990.

ARNOLD A, KIM HG, GAZ RD, EDDY RL, FUKUSHIMA Y, BYERS MG, SHOWS TB, KRONENBERG HM. Molecular cloning and chromosomal mapping of DNA rearranged with the parathyroid hormone gene in a parathyroid adenoma. **Journal of Clinincal Investigation** 83(6): 2034-40, 1989.

ASAKAWA H, KOBAYASHI T. Multistep carcinogenesis in anaplastic thyroid carcinoma: a case report. **Pathology.** 34: 94-97,2002.

ATTARDI LD. The role of p53-mediated apoptosis as a crucial anti-tumor response to genomic instability: lessons from mouse models. **Mutat Res.** 6; 569(1-2): 145-57,2005.

AUTRUP H. Genetic polymorphisms in human xenobiotica metabolizing enzymes as susceptibility factors in toxic response. **Mutat Res.** 464: 65–76, 2000.

BATTISTA S, MARTELLI ML, FEDELE M, CHIAPPETTA G, TRAPASSO F, DE VITA G, BATTAGLIA C, SANTORO M, VIGLIETTO G, FAGIN JA, FUSCO A. A mutated p53 alters differentiation of thyroid cells. **Oncogene** 72: 2029-2037,1995.

BISI H, RUGGERI GB, LONGATTO FILHO A, DE CAMARGO RY, FERNANDES VS, ABDO AH. Neoplastic and non-neoplastic thyroid lesions in autopsy material: historical review of six decades in Sao Paulo, Brazil. **Tumori** 4:499-503,1998.

BOARD PG, COGGAN M, CHELVANAYAGAM G, EASTEAL S, JERMIIN LS, SCHULTE GK, DANLEY DE, HOLTH LR, GRIFFOR MC, KAMATH AV, ROSNER MH, CHRUNYK BA, PERRAGAUX DE, GABEL CA, GEOGHEGAN KF, PANDIT J. Identification, characterization and crystal structure of the omega class of glutathione transferases. **J Biol Chem**. 275: 24798-24806,2000.

BODIWALA D, LUSCOMBE CJ, FRENCH ME, LIU S, SAXBY MF, JONES PW, RAMACHANDRAN S, FRYER AA, STRANGE RC. Susceptibility to prostate cancer: studies on interactions between UVR exposure and skin type**. Carcinogenesis**.24: 711-717, 2003.

BOLTZE C, ROESSNER A, LANDT O, SZIBOR E, PETERS B, SCHNEIDER-STOCK R. Homozygous proline at codon 72 of p53 as a potential risk factor favoring the development of undifferentiated thyroid carcinoma. **Int J Cancer.** 21:1151-1154,2002.

BOONE RT, FAN CY, HANNA EY. Well-differentiated carcinoma of the thyroid. **Otolaryngol Clin North Am** 36:73-90,2003.

BOUNACER A, SCHLUMBERGER M, CAILLOU B, SARASIN A, SUAREZ HG. Radiation-induced thyroid cancers. **Ann Endocrinol** 61(2): 113-8, 2000.

BRENNA SM, SILVA ID, ZEFERINO LC, PEREIRA JS, MARTINEZ EZ, SYRJANEN KJ. Prognostic value of P53 codon 72 polymorphism in invasive cervical cancer in Brazil. **Gynecol Oncol.** 93 (2): 374-80,2004.

BROWN JM, ATTARDI LD. The role of apoptosis in cancer development and treatment response. **Nat Rev Cancer** 5(3): 231-7,2005.

BUSER K, JONCOURT F, ALTERMATT HJ, BACCHI M, OBERLI A, CERNY T. Breast cancer: pretreatment drug resistance parameters (GSH-system, ATase, P-glycoprotein) in tumor tissue and their correlation with clinical and prognostic characteristics. **Ann. Oncol.** 8: 335-341, 1997.

BUYRU N, TIGLI H, DALAY H. P53 codon 72 polymorphism in breast câncer. **Oncol Rep.** 10: 711-714,2003.

CARBONE M, PASS HI. Multistep and multifactorial carcinogenesis: when does a contributing factor become a carcinogen? **Semin Cancer Biol.** 14(6): 399-405,2004.

CARDIS E, KESMINIENE A, IVANOV V, MALAKHOVA I, SHIBATA Y , KHROUCH V, DROZDOVITCH V , MACEIKA E , ZVONOVA I, VLASSOV O, BOUVILLE A , GOULKO G, HOSHI M, ABROSIMOV A, ANOSHKO J, ASTAKHOVA L, CHEKIN S, DEMIDCHIK E , GALANTI R , ITO M, KOROBOVA E, LUSHNIKOV E,MAKSIOUTOV M, MASYAKIN V , NEROVNIA A, PARSHIN V, PARSHKOV E , PILIPTSEVICH N, PINCHERA A , POLYAKOV S , SHABEKA N , SUONIO E , TENET V , TSYB A, YAMASHITA S , WILLIAMS D. Risk of Thyroid Cancer After Exposure to 131 I in Childhood. **Journal of the National Cancer Institute** 97: 10, 2005.

CASELLA C, FUSCO M. Thyroid cancer. **Epidemiol Prev** 28:88-91,2004.

CASTRO MR, GHARIB H. Thyroid nodules and cancer. When to wait and watch, when to refer. **Postgrad Med**. 107(1):113-6, 119-20, 123-4,2000.

CASTRO MR,GHARIB H, 2000 Thyroid nodules and cancer when to wait and watch, when to refer . **Postgrad.Med** 107: 113-116,2000.

CERDA-FLORES RM, BUDOWLE B, JIN L, BARTON SA, DEKA R, CHAKRABORTY R. Maximum likelihood estimates of admixture in Northeastern Mexico using 13 short tandem repeat loci. **Am J Human Biol.** 14: 429-439,2002.

CHEN YC, SU HJ, GUO YL, HOUSEMAN EA, CHRISTIANI DC. Interaction between environmental tobacco smoke and arsenic methylation ability on the risk of bladder cancer. **Cancer Causes Control.** 16(2): 75-81,2005.

CHOW SM, LAW SC, AU SK, MANG O, YAU S, YUEN KT, LAU WH. Changes in clinical presentation, management and outcome in 1348 patients with differentiated thyroid carcinoma: experience in a single institute in Hong Kong, 1960-2000. **Clin Oncol** 15:329-36,2003.

CLAPPER ML. Genetic polymorphism and cancer risk. **Curr Oncol Rep.** 2(3): 251-6, 2000. Review.

DE OLIVEIRA WR, RADY PL, GRADY J, HUGHES TK, NETO CF, RIVITTI EA, TYRING SK. Association of p53 arginine polymorphism with skin cancer. **Int J Dermatol.** 43(7): 489-93,2004.

DEGROOT LJ, KAPLAN EL, SHUKLA MS, SALTI G, STRAUS FH. Morbidity and mortality in follicular thyroid cancer. **J Clin Endocrinol Metab.** 80(10): 2946-53,1995.

DOBASHI Y, SUGIMURA H, SAKAMOTO A, MERNYEI M, MORI M, OYAMA T, MACHINAMI R. Stepwise participation of p53 gene mutation during dedifferentiation of human thyroid carcinomas. **Diagn Mol Pathol.** 3: 9-14,1994.

DONG M, NIO Y, YAMASAWA K, TOGA T, YUE L, HARADA T. p53 alteration is not an independent prognostic indicator, but affects the efficacy of adjuvant chemotherapy in human pancreatic cancer. **J Surg Oncol.** 82: 111-120,2003.

DRUMMOND SN, DE MARCO L, IDE AP, BARBOSA AA, GOMEZ RZ. TP53 codon 72 polymorphism in oral squamous cell carcinoma. **Anticancer Res.** 22: 3379-3381,2002.

DRUMMOND SN, DE MARCO L, NORONHA JC, GOMEZ RS. GSTM1 polymorphism and oral squamous cell carcinoma. **Oral Oncol.** 40(1): 52-5,2004.

DULHUNTY A, GAGE P, CURTIS S, CHELVANAYAGAM G, BOARD P. The glutathione transferase structural family includes a nuclear chloride channel and a ryanodine receptor calcium release channel modulator. **J Biol Chem.** 276: 3319-3323,2001.

DUMONT P, LEU JI, DELLA PIETRA AC, GEORGE DL, MURPHY M. The codon 72 polymorphic variants of p53 have markedly different apoptotic potential, **Nat Genet.** 33:357-365,2003.

ESZLINGER M, KROHN K, FRENZEL R, KROPF S, TONJES A, PASCHKE R. Gene expression analysis reveals evidence for inactivation of the TGF-beta signaling cascade in autonomously functioning thyroid nodules. **Oncogene.** 22; 23(3):795-804,2004.

ESZLINGER M, KROHN K, KRATZSCH J, VOIGT C, PASCHKE R. Growth factor expression in cold and hot thyroid nodules. **Thyroid.** 11(2): 125-35, 2001.

FAGIN JA, MATSUO K, KARMAKAR A, CHEN DL, TANG SH, KOEFFLER HP. High prevalence of mutations of the p53 gene in poorly differentiated human thyroid carcinomas. **J Clin Invest.** 91: 179-84,1993.

FAGIN JA, TANG S-H, ZEKI K, DI LAURO R, FUSCO A, GONSKY R. Reexpression of thyroid peroxidase in a derivative of an undifferentiated thyroid carcinoma cell line by introduction of wild-type p53. **Cancer Res** 56: 765-771,1996.

FAN R, WU MT, MILLER D, WAIN JC, KELSEY KT, WIENCKE JK, CHRISTIANI DC. The p53 codon 72 polymorphism and lung cancer risk, **Cancer Epidemiol Biomarkers Prev.** 9: 1037-1042,2000.

FARID NR. p53 mutations in thyroid carcinoma: tidings from an old foe, **J. Endocrinol. Invest.** 24: 536-545,2001.

FERRUZZI E, FRANCESCHINI R, CAZZOLATO G, GERONI C, FOWST C, PASTORINO U, TRADATI N, TURSI J, DITTADI R, GION M. Blood glutathione as a surrogate marker of cancer tissue glutathione-transferase activity in non-small cell lung cancer and squamous cell carcinoma of the head and neck. **Eur. J. Cancer** 39:1019-1029, 2003.

FIGGE J. Epidemiology of thyroid cancer. In: WARTOFSKY L, **Thyroid Cancer: a comprehensive guide to clinical management.** Towota: Humana Press, 1999; 77-83.

FRANSSILA K O, HARACH H R. Occult papillary carcinoma of the thyroid in children and young adults: A systemic autopsy study in Finland. **Cancer** 58: 715-719,1986.

FRIEDMAN GD, URY HK. Screening for possible drug carcinogenicity: second report of findings. **J. Natl. Cancer Inst.** 71: 1165-1175,1983.

FROM G, MELLEMGAARD A, KNUDSEN N, JORGENSEN T, PERRILD H. Review of thyroid cancer cases among patients with previous benign thyroid disorders. **Thyroid** 10:697-700,2000.

FURLANETTO TW, PECCIN S, DE O SCHNEIDER MA, DOS S ZIMMER A, DOS REIS PS, GENRO SK, FERREIRA EV, BITTELBRUM F, MULLER AS, SILVA RW, SIQUEIRA II, DA SILVEIRA MF. Prevalence of thyroid nodules in 40 years-old or old women. **Rev Assoc Med Bras** 46: 331-4,2000.

GARTE S, GASPARI L, ALEXANDRIE AK, AMBROSONE C, AUTRUP H, AUTRUP JL, BARANOVA H, BATHUM L, BENHAMOU S, BOFFETTA P, BOUCHARDY C, BRESKVAR K, BROCKMOLLER J, CASCORBI I, CLAPPER ML, COUTELLE C, DALY A, DELL'OMO M, DOLZAN V, DRESLER CM, FRYER A, HAUGEN A, HEIN DW, HILDESHEIM A, HIRVONEN A, HSIEH LL, INGELMAN-SUNDBERG M, KALINA I, KANG D, KIHARA M, KIYOHARA C, KREMERS P, LAZARUS P, LE MARCHAND L, LECHNER MC, VAN LIESHOUT EM, LONDON S, MANNI JJ, MAUGARD CM, MORITA S, NAZAR-STEWART V, NODA K, ODA Y, PARL FF, PASTORELLI R, PERSSON I, PETERS WH, RANNUG A, REBBECK T, RISCH A, ROELANDT L, ROMKES M, RYBERG D, SALAGOVIC J, SCHOKET B, SEIDEGARD J, SHIELDS PG, SIM E, SINNET D, STRANGE RC, STUCKER I, SUGIMURA H, TO-FIGUERAS J, VINEIS P, YU MC, TAIOLI E. Metabolic gene polymorphism frequencies in control populations. **Cancer Epidemiol Biomarkers Prev**. 10 (12): 1239-48, 2001

GASPAR J, RODRIGUES S, GIL OM, MANITA I, FERREIRA TC, LIMBERT E, GONCALVES L, PINA JE, RUEFF J. Combined effects of glutathione S-transferase polymorphisms and thyroid cancer risk. **Cancer Genet Cytogenet.**151 (1): 60-7,2004.

GAWKRODGER DJ. Occupational skin cancers. **Occup Med** 54(7):458-63,2004.

GOLDMAN R, SHIELDS PG. Food mutagens. **J Nutr.** 3:965S-973S, 2003.

GRANJA F, MORARI EC, ASSUMPÇÃO LVM, WARD LS. GSTO polymorphism analysis in thyroid nodules suggest that gsto1 variants do not influence the risk for malignancy. **Eur J Cancer Prev** 3(14), 2005 (in press).

GRANJA F, MORARI J, MORARI EC, CORREA LA, ASSUMPCAO LV, WARD LS. Proline homozygosity in codon 72 of p53 is a factor of susceptibility for thyroid cancer. **Cancer** Lett. 16; 210 (2): 151-7,2004(A).

GRANJA F, MORARI J, MORARI EC, CORREA LA, ASSUMPCAO LV, WARD LS. GST profiling may be useful in the screening for thyroid nodule malignancy. **Cancer Lett.** 25; 209(2): 129-37,2004 (B).

GSUR A, HAIDINGER G, HINTEREGGER G, BERNHOFER G, SCHATZL G, MADERSBACHER S, MARBERGER M, VUTUC C, MICKSCHE M. Polymorphisms of glutathione-S-transferase genes (GSTP1, GSTM1 and GSTT1) and prostate-cancer risk. **Int. J. Cancer** 95: 152-155, 2001.

GUDMUNDSDOTTIR K, TRYGGVADOTTIR L, EYFJORD JE. GSTM1, GSTT1, and GSTP1 genotypes in relation to breast cancer risk and frequency of mutations in the p53 gene. **Cancer Epidemiol., Biomark. Prev.** 10: 1169-1173, 2001.

HACKEL C, OLIVEIRA LE, FERRAZ LF, TONINI MM, SILVA DN, TORALLES MB, STUCHI-PEREZ EG, GUERRA-JUNIOR G. New mutations, hotspots, and founder effects in Brazilian patients with steroid 5alpha-reductase deficiency type 2. **J Mol Med.**16, 2005.

HALL P, HOLM L. Radiation-associated thyroid cancer- facts and fiction. **Acta Oncol** 37:325-30,1998.

HARACH H R, FRANSSILA K O, WASENIUS V. Occult papillary carcinoma of the thyroid. A “normal” finding in Finland. A systemic autopsy study. **Cancer** 56: 531-38,1985.

HASELKORN T, STEWART SL, HORN-ROSS PL. Why are thyroid cancer rates so high in Southeast Asian women living in the United States? The Bay Area thyroid cancer study. **Cancer Epidemiol. Biomark. Prev** 12: 144-150, 2003.

HAYES JD, PULFORD DJ. The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. **Crit Rev Biochem Mol Biol.** 30(6):445-600,1995

HEFER T H, JOACHIMS H Z, HASHINONAI M, BEM-ARIEH Y, BROWN J. Highly aggressive behaviour of occult papillary thyroid carcinoma. **J Laryngol Otol** 109: 1109-1112,1995.

HEGEDUS L, BONNENA SJ, BENNEDBAEK FN. Management of Simple Nodular Goiter: Current Status and Future Perspectives. **Endocr Rev** 24:102-32,2003.

HERNANDEZ A, CÉSPEDES W, XAMENA N, SURRALLES J, CREUS A, GALOFRE P, MARCOS R. Glutathione S-transferase polymorphisms in thyroid cancer patients. **Cancer Letters** 190: 37-44, 2003.

HIRAKAWA M, TANAKA T, HASHIMOTO Y, KURODA M, TAKAGI T, NAKAMURA Y. JSNP: a database of common gene variations in the Japanese population. **Nucleic Acids Res.** 30: 158-62,2002.

HORIE S, MAETA H, ENDO K, UETA T, TAKASHIMA K, TERADA T. Overexpression of p53 protein and MDM2 in papillary carcinomas of the thyroid: Correlations with clinicopathologic features. **Pathol Int.** 51:11-15, 2001.

HU X, JI X, SRIVASTAVA SK, XIA H, AWASTHI S, NANDURI B, AWASTHI YC, ZIMNIAK P, SINGH SV. Mechanism of differential catalytic efficiency of two polymorphic forms of human glutathione S-transferase P1-1 in the glutathione conjugation of carcinogenic diol epoxide of chrysene. **Arch. Biochem. Biophys.** 345: 32-38, 1997.

ITO Y, TOMODA C, URUNO T, TAKAMURA Y, MIYA A, KOBAYASHI K, MATSUZUKA F, KUMA K, MIYAUCHI A. Papillary microcarcinoma of the thyroid: how should it be treated? **World J Surg**. 28(11): 1115-21,2004.

JEMAL A, TIWARI RC, MURRAY T, GHAFOOR A, SAMUELS A, WARD E, FEUER EJ, THUN MJ. American Cancer Society. Cancer statistics 2004. **CA Cancer J Clin** 54:8-29,2004.

JOHANSSON AS, STENBERG G, WIDERSTEN M, MANNERVIK B. Structure-activity relationships and thermal stability of human glutathione transferase P1-1 governed by the H-site residue 105. **J. Mol. Biol.** 278: 687-698, 1998.

JOSSART GH, EPSTEIN HD, SHAVER JK, WEIER HU, GREULICH KM, TEZELMAN S, GROSSMAN RF, SIPERSTEIN AE, DUH QY, CLARK OH. Immunocytochemical detection of p53 in human thyroid carcinomas is associated with mutation and immortalization of cell lines. **J Clin Endocrinol Metab.** 81:3498-3504,1996.

KIRK BW, FEINSOD M, FAVIS R, KLIMAN RM, BARANY F. Single nucleotide polymorphism seeking long term association with complex disease. **Nucleic Acids Res.** 30: 3295-3311,2002.

KNOBEL M, MEDEIROS-NETO G. Disorders associated to chronic iodine deficiency. **Arq Bras Endocrinol Metabol**. 48(1): 53-61,2004.

KNUDSEN LE, LOFT SH, AUTRUP H. Risk assessment: the importance of genetic polymorphisms in man. **Mutat. Res.** 482: 83-88, 2001.

KUSUNOKI T, MURATA K. A giant cervical lymph node metastasis from thyroid papillary microcarcinoma considered as a parotid gland tumor in preoperative diagnosis. **Thyroid** 14(12): 1103-4, 2004.

LANG W, BORRUSCH H, BAUER L. Occult carcinomas of the thyroid: Evaluation of 1020 sequential autopsies. Am J Clin Pathol 40: 72-6,1998.

LEVINE AJ. p53 the cellular gatekeeper for growth and division. **Cell.** 88: 323-331,1997.

LICHTENSTEIN P, HOLM NV, VERKASALO PK, ILIADOU A, KAPRIO J, KOSKENVUO M, PUKKALA E, SKYTTHE A, HEMMINKI K. Environmental and heritable factors in the causation of cancer--analyses of cohorts of twins from Sweden, Denmark, and Finland. **N Engl J Med.** 343(2):78-85,2000.

LIVOLSI VA. Highly aggressive behaviour of occult papillary thyroid carcinoma. **J Laryngol Otol** 110:710, 1996.

LIVOLSI VA. **Surgical pathology of the thyroid.** Vol 5. Philadelphia: WB Saunders Company, 1990.

MACK WJ, PRESTON-MARTIN S, BERNSTEIN L, QIAN D. Lifestyle and other risk factors for thyroid cancer in Los Angeles County females. **Ann. Epidemiol.** 12: 395-40,2002.

MACK WJ, PRESTON-MARTIN S, DAL MASO L, GALANTI R, XIANG M, FRANCESCHI S, HALLQUIST A, JIN F, KOLONEL L, LA VECCHIA C, LEVI F, LINOS A, LUND E, MCTIERNAN A, MABUCHI K, NEGRI E, WINGREN G, RON E. A pooled analysis of case-control studies of thyroid cancer: cigarette smoking and consumption of alcohol, coffee, and tea. **Cancer Causes Control** 14:773-85, 2003.

MANNERVIK B, DANIELSON U H. Glutathione transferases- structure and catalytic activity, **Crit.Rev. Biochem.** 23, 283-337, 1988.

MANNERVIK B, The isozymes of glutathione S-transferase. **Adv. Enzymol.** *57*: 357-417, 1985.

MARTINEZ-TELLO FJ, MARTINEZ-CABRUJA R, FERNANDEZ-MARTIN J, LASSO-ORIA C, BALLESTIN-CARCAVILLA C. Occult carcinoma of the thyroid. A systematic autopsy study from Spain of two series performed with two different methods. **Cancer** 71: 4022-9,1993.

MELLEMGAARD A, FROM G, JORGENSEN T, JOHANSEN C, OLSEN JH, PERRILD H. Cancer risk in individuals with benign thyroid disorders. **Thyroid** 8:751-4,1998.

MEMON A, BERRINGTON DE GONZALEZ A, LUQMANI Y, SURESH A. Family history of benign thyroid disease and cancer and risk of thyroid cancer. **Eur J Cancer** 40:754-60, 2004.

MEMON A, VARGHESE A, SURESH A. Benign thyroid disease and dietary factors in thyroid cancer: a case-control study in Kuwait. Br. **J. Cancer** 86: 1745-1750, 2002.

MENDOZA-RODRIGUEZ CA, CERBON MA. Tumor suppressor gene p53: mechanisms of action in cell proliferation and death. **Rev Invest Cl.** 53: 266-273, 2001.

MITSELOU A, VOUGIOUKLAKIS T, PESCHOS D, DALLAS P, AGNANTIS NJ. Occult thyroid carcinoma. A study of 160 autopsy cases. The first report for the region of Epirus-Greece. **Anticancer Res.** 22:427-32, 2002.

MIYAWAKI I, MORIYASU M, FUNABASHI H, YASUBA M, MATSUOKA N. Mechanism of clobazam-induced thyroidal oncogenesis in male rats. **Toxicol Lett.** 145(3): 291-301,2003.

MORARI EC, LEITE JPL, GRANJA F, ASSUMPÇÃO LVM, WARD LS. The null genotype of glutathione s-transferase M1 and T1 locus increases the risk for thyroid cancer. **Cancer Epidemiol. Biomark. Prev**. 11:1485-1488, 2002.

MORTENSEN JD, WOOLNER LB, BENNETT WA. Gross and microscopic findings in clinically normal thyroid glands. **J Clin Endocrinol.** 15:1270-80,1955.

MULDER TPJ, MANNI JJ, ROELOFS HMJ, PETERS WHM, WIERSMA A. Glutathione S-transferases and glutathione in human head and neck cancer. **Carcinogenesis** 16: 619-624, 1995.

National Cancer Data, CDC- Centers for Disease Control and Prevention: http://www.cdc.gov/cancer/natlcancerdata.htm. Acessado em 06/2005.

NIELSEN PS, DE PATER N, OKKELS H, AUTRUP H. Environmental air pollution and DNA adducts in Copenhagen bus drivers-effect of GSTM1 and NAT2 genotypes on adduct levels. **Carcinogenesis,** 17: 1021-1027, 1996.

NIKIFOROV YE, NIKIFOROVA M, FAGIN JA. Prevalence of minisatellite and microsatellite instability in radiation-induced post-Chernobyl pediatric thyroid carcinomas. **Oncogene**. 17(15): 1983-8,1998.

NOGUCHI S, YAMASHITA H, MURAKAMI N, NAKAYAMA I, TODA M, KAWAMOTO H. Small carcinomas of the thyroid. A long-term follow-up of 867 patients. **Arch Surg** 131:187-91,1996.

O'BRIEN P. Peroxidases. **Chem Biol Interact** 129:113-119, 2000.

O'NEILL JP. DNA damage, DNA repair, cell proliferation, and DNA replication: how do gene mutations result? **Proceedings of the National Academy of Science USA** 97:11137-9, 2000.

OREN M. Decision making by p53: life, death and cancer. **Cell Death Differ.** 10: 431-442,2003.

OUDE OPHUIS MB, ROELOFS HMJ, BRANDT PA, PETERS WHM, MANNI JJ. Polymorphisms of the glutathione S-transferase P1 gene and head and neck cancer susceptibility. **Head Neck** 25: 37-43, 2003.

PAL A, HU X, ZIMNIAK P, SINGH SV. Catalytic efficiencies of allelic variants of human glutathione S-transferase Pi in the glutathione conjugation of alpha, beta-unsaturated aldehydes. **Cancer Lett.** 154:39-43, 2000.

PALLI D, VIENIS P, RUSSO A, BERRINO F, KROGH V, MASSALA G, MUNNIA A, PANICO S, TAIOLI E, TUMINO R, GARTE S, PELUSO M. Diet, metabolic polymorphisms and DNA adducts: the epi-Italy cross-sectional study. **Int. J. Cancer.** 87: 444-451, 2000.

PAPADAKIS ED, SOULITZIS N, SPANDIDOS DA. Association of p53 codon 72 polymorphism with advanced lung cancer: the Arg allele is preferentially retained in tumours arising in Arg/Pro germline heterozygotes, **Br J Cancer.** 87:1013-1018,2002.

PELUSO M, AMASIO E, BONASSI S, MUNNIA A, ALTRUPA F, PARODI S. Detection of DNA adducts in human nasal mucosa tissue by 32P-postlabeling analysis. Carcinogenesis. 18(2): 339-44,1997.

PERERA FP. Molecular epidemiology and prevention of cancer. Environ Health Perspect. 103 Suppl 8:233-6, 1995.

PRESTON-MARTIN S, FRANCESCHI S, RON E, NEGRI E. Thyroid cancer pooled analysis from 14 case-control studies: what have we learned? **Cancer Causes Control** 14:787-9,2003.

QIE M, ZHANG Y, WU J. Study on the relationship between cervical cancer and p53 codon 72 polymorphism. **Hua Xi Yi Ke Da Xue Xue Bao.** 33: 274-275,2002.

REAVEY-CANTWELL JF, HAROUN RI, ZAHURAK M, CLATTERBUCK RE, PARKER RJ, MEHTA R, FRUEHAUF JP, BREM H. The prognostic value of tumor markers in patients with glioblastoma multiforme: analysis of 32 patients and review of the literature. **J. Neurooncol**, 55:195-204, 2001.

RON E, KLEINERMAN RA, BOICE JR JD, LIVOLSI VA, FLANNERY JT, FRAUMENI JR JF. A population-based case-control study of thyroid cancer. **J. Natl. Cancer Inst** 79:1-12,1987.

RON E. Epidemiology of thyroid cancer. In: SCHOTTENFELD D, FRAUMENI JR. **Cancer Epidemiology and Prevention**. Oxford: Oxford University Press, 1996,1000-102.

RONCKERS CM, MCCARRON P, RON E. Thyroid cancer and multiple primary tumors in the SEER cancer registries1. **Int J Cancer**. 4, 2005

ROSSING M, VOIGT L, WICKLUND K, WILLIAMS M, DALING J. Use of exogenous hormones and the risk of papillary thyroid cancer. **Cancer Causes Control** 9:341-9, 1998.

SARASIN A, BOUNA NCER A, LEPAGE F, SCHLUMBERGER M, SUAREZ H. Mechanisms of mutagenesis in mammalian cells. Applications to human thyroid tumors. **CR Acad Sci Paris III** 322: 143-9, 1999.

SCHLUMBERGER M, CAILLEUX AF, SUAREZ HG, DE VATHAIRE F. Irradiation and second cancers. The thyroid as a case in point. **C R Acad Sci III.** 322:205-13,1999.

SCHLUMBERGER MJ. Papillary and follicular thyroid carcinoma. **Baillieres Best Pract. Res. Clin. Endo. Metab.** 14:601-613,2000.

SCHOTTENFELD D, BEEBE-DIMMER JL. Advances in cancer epidemiology: understanding causal mechanisms and the evidence for implementing interventions. **Annual Reviews Public Health** 26:37-60, 2005.

SHEN H, ZHENG Y, STURGIS EM, SPITZ MR, WEI Q. P53 codon 72 polymorphism and risk of squamous cell carcinoma of the head and neck: a case-control study. **Cancer Lett.** 183: 123-130,2002.

SHEN H, ZHENG Y, STURGIS EM, SPITZ MR, WEI Q. P53 codon 72 polymorphism and risk of squamous cell carcinoma of the head and neck: a case-control study. **Cancer Lett.** 183:123-130,2002.

SIFUENTES ALVAREZ A, REYES ROMERO M. Risk factors for cervico-uterine cancer associated to HPV: p53 codon 72 polymorphism in women attending hospital care **Ginecol Obstet Mex.** 71: 12-15,2003.

SINGH B, SHAHA AR, TRIVEDI H, CAREW JF, POLURI A, SHAH JP. Coexistent Hashimoto's thyroiditis with papillary thyroid carcinoma: impact on presentation, management, and outcome. **Surgery** 126:1070-6; discussion 1076-7,1999.

SOUZA SL, ASSUMPÇÃO LVM AND WARD LS. Impact of previous thyroid autoimmune diseases on prognosis of patients with well-differentiated thyroid cancer. **Thyroid** 13(5): 491-495, 2003.

STOCKER DJ, FOSTER SS, SOLOMON BL, SHRIVER CD, BURCH HB.Thyroid cancer yield in patients with Graves' disease selected for surgery on the basis of cold scintiscan defects. **Thyroid** 12:305-311,2002.

STOREY A, THOMAS M, KALITA A, HARWOOD C, GARDIOL D, MANTOVANI F, BREUER J, LEIGH IM, MATLASHEWSKI G, BANKS L. Role of p53 polymorphism in the development of human papilloma-virus-associated cancer. **Nature.** 21: 229-234,1998.

STRANGE RC, FRYER AA. The glutathione S-transferases: influence of polymorphism on cancer susceptibility. **IARC Sci Publ.** 148: 231-249,1999.

STUCKER I, HIRVONEN A, DE WAZIERS I, CABELGUENNE A, MITRUNEN K, CENEE S, KOUM-BESSON E, HEMON D, BEAUNE P, LORIOT MA. Genetic polymorphisms of glutathione S-transferases as modulators of lung cancer susceptibility. **Carcinogenesis** 23: 1475-1481, 2002.

TALLINI G. Molecular pathobiology of thyroid neoplasms. **Endocr Pathol**. 13(4): 271-88,2002.

TAMIMI D. The association between chronic lymphocytic thyroiditis and thyroid tumors. **Int J Surg Pathol** 10:141-146,2002.

TAMURA T, MITSUMORI K, ONODERA H, TAKAHASHI M, FUNAKOSHI T, YASUHARA K, TAKEGAWA K, TAKAGI H, HIROSE M. Time course observation of thyroid proliferative lesions and serum levels of related hormones in rats treated with kojic acid after DHPN initiation. **J Toxicol Sci.** 24(3): 145-55,1999.

TAN GH, GHARIB H. Thyroid incidentalomas: management approaches to nonpalpable nodules discovered incidentally on thyroid imaging. **Ann Intern Med.** 1; 126(3):226-31, 1997.

TANAKA-KAGAWA T, JINNO H, HASEGAWA T, MAKINO Y, SEKO Y, HANIOKA N, ANDO M. Functional characterization of two variant human GSTO1-1s (Ala140Asp and Thr217Asn). **Biochem Biophys Res Commun.** 301: 516-520,2003.

THE´RE`SE TRUONG, LAURENT ORSI, DOMINIQUE DUBOURDIEU, YANNICK ROUGIER, DENIS HE´MON, PASCAL GUE´NEL. Role of Goiter and of Menstrual and Reproductive Factors in Thyroid Cancer: A Population-based Case-Control Study in New Caledonia (South Pacific), a Very High Incidence Area. **Am J Epidemiol** 161:1056–1065,2005.

THILLY WG. Have environmental mutagens caused oncomutations in people? **Nat Genet.** 34(3):255-9, 2003.

THOMPSON DE, MABUCHI K, RON E, SODA M, TOKUNAGA M, OCHIKUBO S, SUGIMOTO S, IKEDA T, TERASAKI M, IZUMI S, et al. Cancer incidence in atomic bomb survivors. Part II: Solid tumors, 1958-1987. **Radiat Res.** 37:17-67,1994. Review. Erratum in: **Radiat Res** 139(1):129,1994.

TOMIMORI E, PEDRINOLA F, CAVALIERE H, KNOBEL M, MEDEIROS-NETO G. Prevalence of incidental thyroid disease in a relatively low iodine intake area. **Thyroid** 5:273-6,1995.

TSAI MH, LIN CD, HSIEH YY, CHANG FC, TSAI FJ, CHEN WC, TSAI CH. Prognostic Significance of the proline form of the p53 codon 72 polymorphism in nasopharyngeal carcinoma. **Laryngoscope.** 112:116-119, 2002.

UZUANI A, BINER E. **Biologia**. São Paulo : Ed Habra,1997.v.3

VAISH M, MISHRA A, KAUSHAL M, MISHRA SK, MITTAL B. Microsatellite instability and its correlation with clinicopathological features in a series of thyroid tumors prevalent in iodine deficient areas. **Exp Mol Med.** 30; 36(2): 122-9,2004.

VAN LIESHOUT EM, ROELOFS HM, DEKKER S, MULDER CJ, WOBBES T, JANSEN JB, PETERS WH. Polymorphic expression of the glutathione S-transferase P1 gene and its susceptibility to Barrett's esophagus and esophageal carcinoma. **Cancer Res.** 59:586-589, 1999.

VANDER JB, GASTON EA, DAWBER TR. Significance of solitary nontoxic thyroid nodules. **Preliminary report. N Engl J Med** 251:970,1954.

VINEIS P, MATULLO G, MANUGUERRA M. An evolutionary paradigm for carcinogenesis? **J Epidemiol Community Health.** 57(2) :89-95, 2003.

VINEIS P. Individual susceptibility to carcinogens. **Oncogene.** 23(38):6477-83, 2004.

VINEIS P. Cancer as an evolutionary process at the cell level: an epidemiological perspective. **Carcinogenesis** 24:1-6, 2003.

VINEIS P. Diet, genetic susceptibility and carcinogenesis. **Public Health Nutr.**4: 485-91,2001.

WANG YC, LEE HS, CHEN SK, CHANG YY, CHEN CY. Prognostic significance of p53 codon 72 polymorphism in lung carcinomas. **Eur J Cancer.** 35: 226-230,1999.

WARD E, JEMAL A, COKKINIDES V, SINGH GK, CARDINEZ C, HAFOOR A, THUN M. Cancer disparities by race/ethnicity and socioeconomic status. **CA Cancer J Clin.** 54 (2):78-93,2004.

WARD LS, CARNEIRO MCO, TOLEDO AJO, FURLANETTO RP E MACIEL RMB. A citologia do material obtido por punção aspirativa da tiróide como método único indicativo de cirurgia: análise de custo-benefício. **Arquivos Brasileiros de Endocrinologia e Metabologia** 37 (1): 18-22, 1993.

WARD LS, FAGIN JA. Molecular genetics of thyroid cancer: evidence that inactivation of tumor supressor genes occurs at late stages of tumor progression. In: Molecular and cellular pediatric endocrinology. **Humana Press Inc**, Totowa, 1998: 201-211.

WATSON MA, STEWART RK, SMITH GB, MASSEY TE, BELL DA. Human glutathione S-transferase P1 polymorphisms: relationship to lung tissue enzyme activity and population frequency distribution. **Carcinogenesis** 19:275-280, 1998.

WEINBERG R. Oncogenes, antioncogenes, and the molecular bases of multistep carcinogenesis. **Cancer Research** 49: 3713-21, 1989.

WELKER MJ, ORLOV D. Thyroid nodules. **Am. Fam. Physician** 67:559-66, 2003.

WHITBREAD AK, TETLOW N, EYRE HJ, SUTHERLAND GR AND BOARD PG. Characterization of the human omega class glutathione transferase genes and associated polymorphisms. **Pharmacogenetics** 13: 131-44,2003.

WU WJ, KAKEHI Y, HABUCHI T, KINOSHITA H, OGAWA O, TERACHI T, HUANG CH, CHIANG CP, YOSHIDA O. Allelic frequency of p53 gene codon 72 polymorphism in urologic cancers. **Jpn J Cancer Res.** 86: 730-736,1995.

XIE J, SHULTS K, FLYE L, JIANG F, HEAD DR, BRIGGS RC. Overexpression of GSTA2 protects against cell cycle arrest and apoptosis induced by the DNA inter-strand crosslinking nitrogen mustard, mechlorethamine. **J Cell Biochem.** 15;95(2):339-51,2005.

YIN ZL, DAHLSTROM JE, LE COUTEUR DG, BOARD PG. Immunohistochemistry of omega class glutathione S-transferase in human tissues. **J Histochem Cytochem.** 49: 983-987,2001.

YU MW, YANG SY, CHIU YH, CHIANG YC, LIAW YF, CHEN CJ. A p53 genetic polymorphism as a modulator of hepatocellular carcinoma risk in relation to chronic liver disease, familial tendency, and cigarette smoking in hepatitis B carriers. **Hepatology.** 29: 697-702, 1999.

ZARDO F, SOLDO P, ALTISSIMI G, PARNASI E, BERTIN S. Hashimoto's thyroiditis and thyroid cancer: a report of a clinical case and a review of the literature. G Chir. 20:174-6,1999.

ANEXOS

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Cancer Letters 209 (2004) 129–137
CANCER Letters
www.elsevier.com/locate/canlet

GST profiling may be useful in the screening for thyroid nodule malignancy

Fabiana Granja[a], Joseane Morari[a], Elaine C. Morari[a], Luiz A.C. Correa[b], Lígia V.M. Assumpção[a], Laura S. Ward[a,*]

[a]*Laboratory of Cancer Molecular Genetics, Department of Medicine, State University of Campinas, São Paulo, Olympio Pattaro 45, 13085-857. Brazil*
[b]*Heliópolis Hospital, São Paulo, Brazil*

Received 6 October 2003; received in revised form 14 December 2003; accepted 16 December 2003

Abstract

Screening tools are of utmost necessity in order to identify individuals at risk for thyroid nodule cancer. The polymorphic inheritance of human drug-metabolizing enzymes, such as those encoded by the Glutathione-S-Transferase (GST) system, plays an important role in the development of most human cancers. GSTP1 enzyme is the most important detoxification enzyme in human head and neck tissues. An aminoacid substitution (1105V) in the *GSTP1* gene result in two genotypes, *GSTP1AB* and GSTP1BB. Those produce a variant enzyme with lower activity and less capability of effective detoxification of carcinogens than the wild type GSTP1AA. In order to look for the influence of GSTP1 enzymes inheritance pattern on thyroid cancer risk we used a PCR-SSCP-sequencing approach to compare the genotypes of 98 malignant nodules, including 77 papillary carcinomas (PC) and 21 follicular carcinomas (FC), to 44 benign nodules and to 157 healthy control individuals. Individuals with history of previous thyroid disease, exposure to radiation and antecedents of malignancy were excluded. Patients with PC and FC showed a significant over-representation of the variants of *GSTP1* allele compared to the control population ($p < 0.0001$). The risk for thyroid cancer in individuals with the variant GSTP1 enzymes, after adjusting for gender, age, tobacco and drugs use, increased 7,092 (CI: 2,307-21,802) and 9,625 (CI: 2.484-37.291) times for PC and FC, respectively. We suggest that *GST* genotype may be associated with an increased susceptibility to thyroid cancer. *GSTP1* profiling from peripheral blood may be a simple and useful tool in the screening for thyroid nodule malignancy. Glutathione-S-Transferase system; GSTP; Thyroid cancer; Screening.

Keywords: GST, glutathione S-transferase; GSTP1, glutathione S-transferase *pi* 1; *GSTP1*, glutathione S-transferase *pi* 1 locus; PCR, polymerase chain reaction; SSCP, single strand conformation polymorphism analysis; PC, papillary carcinomas; FC, follicular carcinomas; CI, confidence interval; M, mu; T, theta; TSH, thyrotropin stimulating hormone; ECM, Elaine Cristina Morari; LVMA, Ligia Vera Montalli Assumpção; LSW, Laura Sterian Ward; Tg, thyroglobulin; SAS, statistical analysis system; χ^2, Chi-square test; F, Fisher test; KW; Kruskal–Wallis, OR; Odds ratio

1. Introduction

Thyroid nodules are found in 5–7% of the population by palpation and are almost tenfold more

* Corresponding author. Address: GEMOCA Med. Interna, Clinica Medica, FCM-UNICAMP, Rua Tessalia Vieira de Camargo 126, Campinas SP 13081-970, Brazil. Tel/fax: +55-19-3289-4107.
E-mail address: ward@unicamp.br (L.S. Ward).

0304-3835/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.canlet.2003.12.013

frequent at ultrasonographic screening, mainly in iodine-deficient communities. Malignant nodules, however, are a small minority among them, making the choice to address all nodules to fine-needle biopsy impractical and cost ineffective [1,2]. On the other hand, despite the low incidence and favorable prognosis, thyroid cancer can be a mortal disease: mortality ranges from 10% of well-differentiated tumors, to 50% of poorly differentiated and medullary and to 100% of anaplastic tumors, respectively [3]. Although clinical and epidemiological features are helpful, there are still no accepted serological markers that can guide the identification of patients at risk for malignancy among individuals with thyroid nodules.

Cancer is an evolutionary process caused by a gene-environment interaction [4]. We are constantly exposed to an increasing list of chemical carcinogens, cell-transforming viruses, UV and ionizing radiation, among many other environmental mutagens. However, the likelihood of developing cancer in response to natural hazards varies considerably. Individual differences in the susceptibility to carcinogens play an essential role in the development of sporadic cancer [5,6]. The biochemical basis for this susceptibility is related to genetic polymorphisms that normally occur in the general population, regarding genes involved in predisposition to a specific cancer, in the metabolic activation or detoxification of environmental genotoxins, and in controlling DNA repair or cellular damage [4–8].

Several polymorphic genes, encoding for enzymes involved in the biotransformation of carcinogens, have been studied as possible cancer risk modifiers. The Glutathione-S-Transferase (GST) system consists of a large multigenic group of detoxifying enzymes whose activity, catalyzing the conjugation of toxic and mutagenic compounds with glutathione, is essential for cell protection [9]. Five classes of isoenzymes have been considered important in humans: alpha, mu (GSTM1), pi (GSTP1), sigma and theta (GSTT1). At present, genetic polymorphisms have been demonstrated for *GSTM1*, *GSTT1*, and *GSTP1* genes. Individuals who are deletion homozygotes, classified as *GSTM1* null or *GSTT1* null, exhibit absence of enzymatic activity and are hypothesized to be at increased risk for the carcinogenic effects of a wide variety of environmental exposures [8–10]. In a recent publication, we demonstrated that the combined null *GSTM1* and *GSTT1* genotypes increase the risk for thyroid cancer 2.6 times [11].

The most important detoxification enzyme in head and neck tissues, in a quantitative sense, is GSTP1 [12]. GSTP1 enzyme level has been extensively studied in relation to tobacco-associated malignancies and found overexpressed in many preneoplastic and neoplastic lesions, including head and neck, breast, prostate and lung tumors [13–16]. Regarding the gene, a functional significance has been demonstrated for an exon 5 A–G transition resulting in a codon Ile105Val amino acid substitution, which modifies heat stability and specific activity of the Val containing isoform [17]. The resulting enzyme variants present lower activity and less capability of effective detoxification of carcinogens than the wild type *GSTP1 AA* [17,18]. In addition, *GST* profile may have a prognostic value since it is associated with the response to therapy in many human malignancies [19–21].

The primary objective of this study was to test the hypothesis that individuals with an inherited *GSTP1* variant (*GSTP1AB* and *GSTP1BB*) are at increased risk of thyroid cancer in comparison to the wild-type *GSTP* profile. We also aimed to evaluate a possible utility of *GST* profiling in the outcome prediction for thyroid cancer patients. For these purposes, we conducted a prospective case control study in which we compared the proportion of *GSTP1* genotypes between a group of patients with benign and malignant thyroid nodules and a control group. Heterogeneity of risk according to clinical and morphological subtypes of thyroid tumors and their correspondent genotype was also explored.

2. Material and methods

2.1. Subjects

The study was approved by the Ethics Committee of the University Hospital-School of Medicine of the State University of Campinas, and informed written consent was obtained from all individuals. A control group of 157 healthy individuals (42 males and 115 females, 16–81 years old, 47 ± 18 years old) was selected from the general population of our region.

There were 115 blood donors and 42 volunteers recruited among co-workers and volunteers from the State University of Campinas. In order to obtain a comparable control group with respect to gender proportion and age range, we selected 2–3 women for every man presenting to donate blood, because thyroid cancer occurs more frequently in women than in men. Data on lifetime occupational history, smoking history, general health conditions, previous diseases and other anamnestic data were obtained through interviews. Individuals with history of previous thyroid disease, radiation exposure and antecedents of malignancy were excluded.

One hundred forty-two patients that consecutively sought medical attention for thyroid disease evaluation at the outpatient clinic of the University Hospital, during the years 2001–2003, were enrolled in the study after they agreed to participate. The study population included 44 benign thyroid nodules-30 goiters and 14 follicular adenomas-and 98 malignant nodules, including 77 papillary carcinomas and 21 follicular carcinomas. Stage and grade of differentiation of the tumors were obtained from surgical and pathological records. Experienced pathologists of the University 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 that were referred to surgery because of 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. Four to six weeks after the operations, whole body ^{131}I scans were performed. All patients received 30–100 mCi ^{131}I. Long-term levothyroxine suppressive doses were given following a whole body scan, in order to keep serum thyrotropin (TSH) levels at low normal.

Patients were classified into whites and non-whites. Data on general health conditions and medical history with emphasis on previous and/or current thyroid diseases were obtained through interviews, using a structured questionnaire administered by the same interviewers (ECM, LVMA, LSW). The use of drugs and certain foods was also carefully assessed, in particular nutritional goitrogens like vegetables containing cyanogenic glucosides (most forms of cabbage, cauliflower, broccoli, and other members of the cruciferous family) and drugs that could interfere with thyroid function, as well as medication for other concomitant diseases. Cigarette smoking habit was recorded but, because of the few 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 each pack) for 1 year during the previous 5 years. None of the patients had a history of accidental or medical radiation exposure. All data, including pathological diagnoses, were confirmed in the patients' records. A peripheral blood sample was collected from all 142 patients. Also, thyroid tissue samples were obtained at surgery from 83 out of these patients, snap frozen in liquid N_2 immediately after surgery and kept at -80° C until processed. We were able to obtain cancer tissue samples and autologous blood specimens and/or normal thyroid tissue from the contralateral lobe in 35 cases of malignant tumors.

2.2. Follow-up

Cancer patients were followed with periodic whole 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 12–342 months (31 ± 67 months). Patients with high serum Tg levels (>2 mg/dl) and/or suspicious whole 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.

2.3. Polymorphism analysis

A peripheral blood sample was collected from all 142 patients Genomic DNA was extracted from leukocytes separated from whole blood using a standard proteinase K-phenol-chloroform protocol. *GSTP1* variants were studied using a PCR-SSCP-sequencing approach. The primers used were forward

5′- TCTATGGGAAGGACCAGCAGG-3′ and reverse 5′-GCCCAACCTGGTGCAGATG -3′. 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 uM of each dinucleotide triphosphate, 2.0 mM MgCl2 and 0.5 U Taq DNA polymerase. Amplifications were carried out for 35 cycles of 94 °C for 45 s, annealing temperatures 63 °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. The PCR products were mixed with 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol and 50 mM NaOH, denatured at 94 °C for 10 min, and loaded onto 0.4 mm/30/45 cm polyacrylamide gels. The electrophoresis was conducted at 2–5 W at room temperature overnight. The gels were then stained with silver nitrate. Forty-five samples suspected of presenting aberrant migrating bands, as exemplified in Fig. 1, were directly sequenced using the ABI prism big dye sequencing kit (Perkin Elmer, Warrington, Cheshire, UK) using the ABI 377 Prism DNA Sequencer (Perkin Elmer). Also 10 samples, four from the control group and six samples from patients with a normal banding pattern, were directly sequenced. Forty three out of the 45 suspicious samples confirmed to be variants of the wild type GSTP sequence that was present in all 10 control sequenced samples. Positive and negative control samples were included in all PCRs and SSCPs runs to detect possible contamination problems, gel loading and typing inconsistencies.

2.4. 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) or Fisher's (F) exact tests were used to examine homogeneity between cases and controls regarding gender, color, previous thyroid disease, use of medication and cigarette smoking. Also, extent of disease was compared between papillary and follicular carcinomas using Fisher's test. Kruskal–Wallis (KW) test was used to compare age among groups. Mann–Whitney or Wilcoxon tests were used to compare age among different genotype groups. The odds ratio (OR) and 95% CI provide a measure of the strength of association, e.g. indicating the increase in odds of a given benign or malignant thyroid nodule demonstrating a particular genotype compared to the control population. Logistic regression was used to evaluate the effect of genotypes, after adjusting for other potential confounders like age, sex, color, tobacco, alcohol and medication consumption. Interactions between environmental variables and genotypes were also assessed. All tests were conducted at the $P = 0.05$ level of significance.

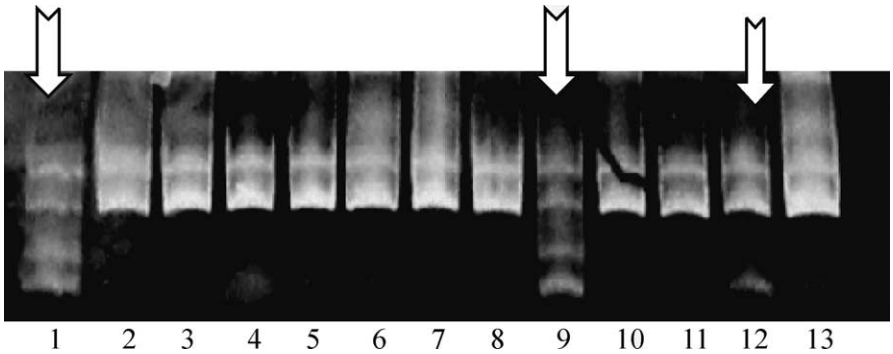


Fig. 1. Polyacrylamide gel electrophoresis for single-strand conformation polymorphysm (SSCP) analysis, representative of our results for GSTP1 gene screening for polymorphisms. The gel was loaded with 6 samples from PC - lanes 1–6, and 7 samples from FC patients—lanes 7–13. The arrows indicate samples that display an aberrant eletrophoretic run in lanes 1, 9 and 12. All samples were sequenced directly and lanes 1 and 9 confirmed to be heterozygous GSTP1 variants while lane 12 was an homozygous GSTP1 variant.

3. Results

There were no differences between the control and the thyroid disease patients regarding age (47 ± 21 years vs. 49 ± 14 years); gender (42 males and 115 females vs. 37 males and 61 females); color (72 white and 60 non-white vs. 54 white and 44 non-white individuals); drug ingestion (39 control individuals vs. 29 patients); smoking (61 control individuals vs. 46 patients); alcohol consumption (61 control individuals vs. 46 patients) and dietary patterns.

Table 1 summarizes clinical characteristics and parameters of aggressiveness at diagnosis and during follow-up of the thyroid cancer patients. Patients with FC were older than the patients with PC (KW; $P = 0.0012$). Nevertheless, there were no differences among the patients regarding color, gender distribution, smoking habit, the use of drugs or preexisting benign thyroid diseases. The occurrence of lymph node involvement at the time of diagnosis was more frequent among PC (40%) than FC (14%) (F; $P < 0.05$). However, these last tumors already presented long distance metastasis at the time of diagnosis (48%) more frequently than papillary carcinomas (10%) (F; $P < 0.001$). Follow-up data did not evidence differences between PC and FC concerning distant metastasis and/or recurrence of the tumor although FC patients presented evidence of recurrence and/or metastasis in 48% of the cases against 26% of the PC ones (F; $P = 0.11$). Two patients died during the period of observation. Table 2 summarizes data of the overall proportions of the *GSTP1* genotypes in the control population and in the benign and malignant thyroid disease patients. Fig. 2 represents the statistic comparison among groups regarding the prevalence of *GSTP1* normal and variant alleles. The presence of *GSTP1* variant alleles did not differ in FA (21.3%) and in FC (33.2%) ($P = 0.704$). *GSTP1* gene profile proved to be exactly the same in the tumor and normal tissue samples, as well as in the corresponding peripheral blood samples in all tested samples.

Patients with benign nodules and, in particular, PC and FC patients, showed a significant over-representation of the variants of *GSTP1* genotype compared to the control population (χ^2, $P < 0.0001$). The incidence of heterozygous *GSTP1AB* individuals (21%) was higher than that of homozygous *GSTP1BB* patients (9%) in the group of patients with malignant thyroid nodules than in the control population (2.5 and 3%, respectively) (χ^2, $P < 0.0001$). The risk for thyroid cancer in individuals with the variant GSTP1 enzymes, after adjusting for gender, age, tobacco and drugs, was increased 7,092 (OR; CI: 2,307–21,802) and 9,625 (OR; CI: 2.484–37.291) times for PC and FC, respectively. There was no association between genotype and the patients' clinical features, tumor parameters of aggressiveness at diagnosis or behavior during follow-up.

4. Discussion

Most of the nodules diagnosed by ultrasonography or palpation will prove to be benign since thyroid cancer is responsible for only 0.6–1.6%, respectively, of all kinds of cancers that occur in men and women in the USA [1,2]. However, variations of thyroid cancer

Table 1
Distribution of thyroid carcinoma patients according to their histology, clinical features including age (X ± SD in years), gender (F, female; M, male), color (W, white, NW, non-white), history of previous thyroid benign diseases, smoking habits, use of medication, presence of lymph node involvement and distant metastasis at the time of diagnosis and diagnosis of recurrence and/or distant metastasis during follow-up

	Clinical characteristics								Diagnosis (Presence of metastasis)		Follow-up
	Age (X ± SD)	Sex		Color		Previous thyroid disease	Smokers	Use of medication	Lymph node	Distant	Recurrence and/or distant metastasis
		M	F	W	NW						
PC	44 ± 15	28	49	42	35	18	37	21	30	8	20
FC	56 ± 12	9	12	12	9	9	9	8	3	10	10

Table 2
Comparison of *GSTP1* genotype distribution in the normal population and in benign (goiter and follicular adenoma) and malignant (papillary and follicular) thyroid patients

	No. of. cases	N (%)			OR (95%CI)	*P*-value
		AA	AB	BB		
Controls	157	148 (94.2%)	4 (2.5%)	5 (3.1%)		
Benign nodules						
Goiter	30	26 (86.6%)	1 (3.3%)	3 (10%)	AA vs. AB 1.423 (0.1528–13.250)	0.56
					AA vs. BB 3.415 (0.7688–15.172)	0.12
					AB vs. BB 2.400 (0.1751–32.900)	1.00
					N[a] vs. A[b] 2.530 (0.7251–8.827)	0.23
Follicular adenoma	14	11 (78.5%)	1 (7.1%)	2 (14.2%)	AA vs. AB 3.364 (0.3455–32.750)	0.32
					AA vs. BB 5.382 (0.9344–30.999)	0.09
					AB vs. BB 1.600 (0.1036–24.720)	1.00
					N[a] vs. VA[b] 4.485 (1.059–18.994)	0.06
Malignant nodules						
Papillar carcinoma	77	57 (74%)	13 (16.8%)	7 (9%)	AA vs. AB 8.439 (2.641–26.968)	<0.0001
					AA vs. BB 3.635 (1.108–11.924)	0.0444
					AB vs. BB 0.430 (0.086–2.143)	0.4223
					N[a] vs. VA[b] 7.092 (2.307–21.802)	<0.0001
Follicular carcinoma	21	14 (66.6%)	6 (28.5%)	1 (4.7%)	AA vs. AB 15.85 (3.993–62.973)	0.0002
					AA vs. BB 2.114 (0.2305–19.397)	0.4345
					AB vs. BB 0.133 (0.01103–1.612)	0.1451
					N[a] vs. VA[b] 9.625 (2.484–37.291)	<0.0001

[a] N, Normal.
[b] VA, Variant alleles.

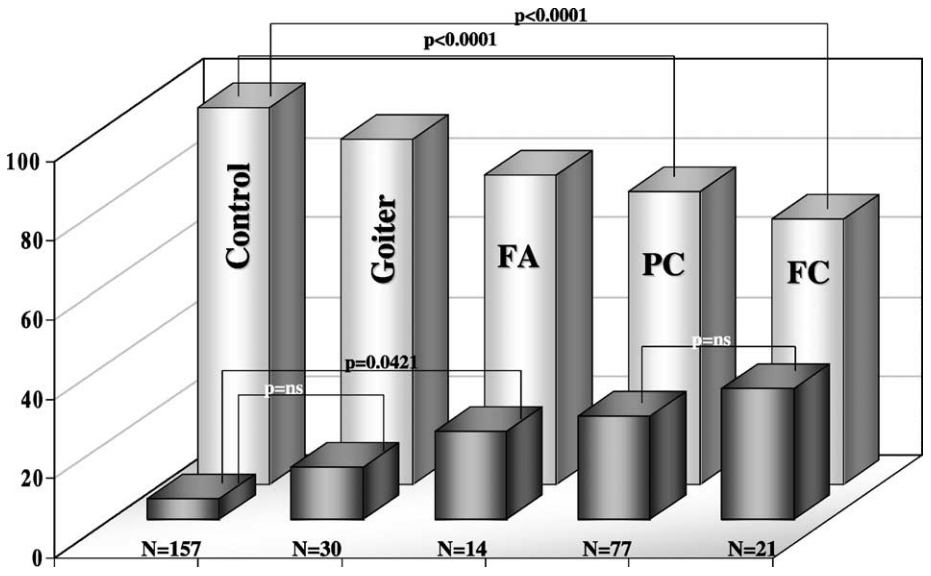


Fig. 2. Graphic representation of the statistic analysis of the prevalence of the wild type (lighter columns) and the variant alleles (darker columns) of GSTP1 gene in the control population, in the patients with goiter, follicular adenomas (FA), papillary (PC) and follicular (FC) carcinomas.

incidence in different geographic and ethnic groups suggest that environmental factors may influence the thyroid tumorigenesis process. For instance, thyroid cancer is the second most common neoplasm among women in several countries in the Middle West, accounting for more than 8% of all cancers in Kuwait [22]. Indeed, the environment has the principal role in causing human sporadic cancer [4–6]. Numerous specific rearrangements are being discovered in thyroid cancer suggesting activation of DNA repair programs after environmentally induced genetic alterations [23]. Unfortunately, available data regarding carcinogenic products and the thyroid gland are conflicting. Several chemicals produce thyroid neoplasia in rodents, but a broad screening of more than 200 drugs revealed that just two, griseofulvin and senna, were associated with increased risk of thyroid carcinoma in humans [24]. Nutritional goitrogens intake was not associated with increased risk of thyroid carcinoma in humans in a population-based case-control study published earlier [25]. However, a recent study designed to understand why thyroid cancer incidence rates are higher among Southeast Asian women living in the United States than among other United States women concluded that consumption of carotenoids and of isoflavones from soy-based foods may contribute to the rate differences, corroborating other data that suggest a role for dietary variables in thyroid carcinogenesis [26,27]. There is no epidemiological evidence of increased risk of thyroid cancer in smokers. On the contrary, recent data suggest a protective effect of smoking and alcohol consumption, perhaps involving an effect on thyroid stimulating hormone, estrogen metabolism, or other mechanisms. [25,28].

Individuals with *GSTP1* variant genes produce enzymes with diminished ability to detoxify a wide range of environmental carcinogens. Also, individuals lacking *GSTM1* or *GSTT1* genes are more susceptible to the effects of a large series of carcinogens. However, literature data regarding the role of detoxifying enzymes in thyroid tumors are scarce. A recent study was not able to relate polymorphisms of *GSTM1, GSTT1* and *GSTP1* genes to cancer risk using restriction length polymorphism analysis [29]. In contrast, we demonstrated earlier a high prevalence of the combined null genotype for *GSTT1* and *GSTM1* in malignant lesions compared to benign nodules and a large control population [11]. In addition, the present study demonstrates that *GSTP1* variants also play a determinant independent role in the susceptibility to thyroid cancer since an estimated 7.0 fold greater risk of PC and 9.6 fold risk of FC was observed in individuals with *GSTP1* variants. The different results we obtained may be due, in part, to our genotyping protocol since we used a different methodological approach, PCR-SSCP-sequencing. Also, there are major differences in the ethnic populations studied. In contrast to the Spanish population studied by Hernandez et al, Brazilian population is of high heterogeneity, composed of immigrants from Europe, Africa, Asia, and indigenous populations. Our alimentary habits, based on rice and beans, also differ from European ones. Likewise, many social and cultural conditions may contribute to different exposition to distinct environmental factors that may be important, since very little is known concerning the susceptibility factors for thyroid cancer.

Interestingly, in the group of patients with thyroid cancer, among patients presenting variants of the *GSTP1* gene, individuals with heterozygous *AB* genotype were more frequent than *BB* genotype, compared to the control population. Both GSTP1 variants produce enzymes with decreased specific activity and affinity for eletrophilic compounds [30]. However, 'in vitro experiments' demonstrated their activity and affinity to differ from several electrophilic substrates [30]. These data were corroborated by many studies in tissue samples and cell culture experiments using a considerable number of different toxic compounds [31–33]. Therefore, we may hypothesize that *GSTP1* polymorphism is an important factor in differential susceptibility of individuals to the toxic effects of some environmental pollutant related to the process of thyroid carcinogenesis.

Our data indicate a prevalence of *GSTP1* variants in malignant lesions, FC (33%) and PC (26%), significantly higher than in the control population (5%) (χ^2; $P < 0.001$). Since we were not able to find any association between clinical features, histology, and parameters of aggressiveness at diagnosis or during follow-up and the *GSTP1* genotype, we presume that *GSTP1* profiling may not be useful as a prognostic factor for nodules that are already diagnosed as carcinomas. On the other hand, these

data suggest that a relatively simple *GSTP1* profile may be useful in the screening for thyroid nodule malignancy. Larger ongoing studies may confirm these preliminary data and are necessary to ascertain the cost-effectiveness of this serological screening method.

References

[1] M.J. Welker, D. Orlov, Thyroid nodules, Am. Fam. Physician 67 (2003) 559–566.

[2] M.R. Castro, H. Gharib, Thyroid nodules and cancer when to wait and watch, when to refer, Postgrad. Med. 107 (2000) 113–116. see also pages:119, 120, 123, 124.

[3] N. Bhattacharya, Population-based analysis of survival factors in differentiated and medullary thyroid carcinoma, Otolaryngol. Head Neck Surg. 128 (2003) 115–123.

[4] P. Vineis, Cancer as an evolutionary process at the cell level: an epidemiological perspective, Carcinogenesis 24 (2003) 1–6.

[5] P. Lichtenstein, N.V. Holm, P.K. Verkasalo, A. Iliadou, J. Kaprio, M. Koskenvuo, et al., Environmental and heritable factors in the causation of cancer, N. Engl. J. Med. 343 (2000) 78–85.

[6] P. Vineis, G. Matullo, M. Manuguerra, An evolutionary paradigm for carcinogenesis?, J. Epidemiol. Community Health 57 (2001) 89–95.

[7] H. Autrup, Genetic polymorphisms in human xenobiotic metabolizing enzymes as susceptibility factors in toxic response, Mutat. Res. 464 (2000) 65–76.

[8] M.L. Clapper, Genetic polymorphism and cancer risk, Curr. Oncol. Rep. 2 (2000) 251–256.

[9] B. Mannervik, The isozymes of glutathione S-transferase, Adv. Enzymol. 57 (1985) 357–417.

[10] L.E. Knudsen, S.H. Loft, H. Autrup, Risk assessment: the importance of genetic polymorphisms in man, Mutat. Res. 482 (2001) 83–88.

[11] E.C. Morari, J.L.P. Leite, F. Granja, L.V.M. Assumpção, L.S. Ward, The null genotype of glutathione s-transferase M1 and T1 locus increases the risk for thyroid cancer, Cancer Epidemiol. Biomark. Prev. 11 (2002) 1485–1488.

[12] T.P.J. Mulder, J.J. Manni, H.M.J. Roelofs, W.H.M. Peters, A. Wiersma, Glutathione S-transferases and glutathione in human head and neck cancer, Carcinogenesis 16 (1995) 619–624.

[13] M.B. Oude Ophuis, H.M.J. Roelofs, P.A. Brandt, W.H.M. Peters, J.J. Manni, Polymorphisms of the glutathione S-transferase P1 gene and head and neck cancer susceptibility, Head Neck 25 (2003) 37–43.

[14] K. Gudmundsdottir, L. Tryggvadottir, J.E. Eyfjord, GSTM1, GSTT1, and GSTP1 genotypes in relation to breast cancer risk and frequency of mutations in the p53 gene, Cancer Epidemiol. Biomark. Prev. 10 (2001) 1169–1173.

[15] A. Gsur, G. Haidinger, S. Hinteregger, G. Bernhofer, G. Schatzl, S. Madersbacher, et al., Polymorphisms of glutathione-S-transferase genes (GSTP1, GSTM1 and GSTT1) and prostate-cancer risk, Int. J. Cancer 95 (2001) 152–155.

[16] I. Stucker, A. Hirvonen, I. de Waziers, A. Cabelguenne, K. Mitrunen, S. Cenee, et al., Genetic polymorphisms of glutathione S-transferases as modulators of lung cancer susceptibility, Carcinogenesis 23 (2002) 1475–1481.

[17] A.S. Johansson, G. Stenberg, M. Widersten, B. Mannervik, Structure-activity relationships and thermal stability of human glutathione transferase P1-1 governed by the H-site residue 105, J. Mol. Biol. 278 (1998) 687–698.

[18] X. Hu, X. Ji, S.K. Srivastava, H. Xia, S. Awasthi, B. Nanduri, et al., Mechanism of differential catalytic efficiency of two polymorphic forms of human glutathione S-transferase P1-1 in the glutathione conjugation of carcinogenic diol epoxide of chrysene, Arch. Biochem. Biophys. 345 (1997) 32–38.

[19] J. Reavey-Cantwell, R.I. Haroun, M. Zahurak, R.E. Clatterbuck, R.J. Parker, R. Mehta, et al., The prognostic value of tumor markers in patients with glioblastoma multiforme: analysis of 32 patients and review of the literature, J. Neurooncol. 55 (2001) 195–204.

[20] E. Ferruzzi, R. Franceschini, G. Cazzolato, C. Geroni, C. Fowst, U. Pastorino, et al., Blood glutathione as a surrogate marker of cancer tissue glutathione-transferase activity in non-small cell lung cancer and squamous cell carcinoma of the head and neck, Eur. J. Cancer 39 (2003) 1019–1029.

[21] K. Buser, F. Joncourt, H.J. Altermatt, M. Bacchi, A. Oberli, T. Cerny, Breast cancer: pretreatment drug resistance parameters (GSH-system, ATase, P-glycoprotein) in tumor tissue and their correlation with clinical and prognostic characteristics, Ann. Oncol. 8 (1997) 335–341.

[22] A. Memon, A. Varghese, A. Suresh, Benign thyroid disease and dietary factors in thyroid cancer: a case-control study in Kuwait, Br. J. Cancer 86 (2002) 1745–1750.

[23] G. Tallini, Molecular pathobiology of thyroid neoplasms, Endocr. Pathol. 13 (2002) 271–288.

[24] G.D. Friedman, H.K. Ury, Screening for possible drug carcinogenicity: second report of findings, J. Natl Cancer Inst. 71 (1983) 1165–1175.

[25] E. Ron, R.A. Kleinerman, J.D. Boice Jr., V. LiVolsi, J.T. Flannery, J.F. Fraumeni Jr., A population-based case-control study of thyroid cancer, J. Natl. Cancer Inst. 79 (1987) 1–12.

[26] T. Haselkorn, S.L. Stewart, P.L. Horn-Ross, Why are thyroid cancer rates so high in Southeast Asian women living in the United States? The Bay Area thyroid cancer study, Cancer Epidemiol. Biomark. Prev. 12 (2003) 144–150.

[27] W.J. Mack, S. Preston-Martin, L. Bernstein, D. Qian, Lifestyle and other risk factors for thyroid cancer in Los Angeles County females, Ann. Epidemiol. 12 (2002) 395–401.

[28] M.A. Rossing, K.L. Cushing, L.F. Voigt, K.G. Wicklund, J.R. Daling, Risk of papillary thyroid cancer in women in relation to smoking and alcohol consumption, Epidemiology 11 (2000) 49–54.

[29] A. Hernandez, W. Céspedes, N. Xamena, J. Surralles, A. Creus, P. Galofre, R. Marcos, Glutathione S-transferase polymorphisms in thyroid cancer patients, Cancer Lett. 19 (2003) 37–44.
[30] F. Ali-Osman, O. Akande, G. Antoun, J.X. Mao, J. Buolamwini, Molecular cloning, characterization, and expression in *Escherichia coli* of full-length cDNAs of three human glutathione S-transferase Pi gene variants. Evidence for differential catalytic activity of the encoded proteins, J. Biol. Chem. 272 (1997) 10004–10012.
[31] A. Pal, X. Hu, P. Zimniak, S.V. Singh, Catalytic efficiencies of allelic variants of human glutathione S-transferase Pi in the glutathione conjugation of alpha, beta-unsaturated aldehydes, Cancer Lett. 154 (2000) 39–43.
[32] M.A. Watson, R.K. Stewart, G.B. Smith, T.E. Massey, D.A. Bell, Human glutathione S-transferase P1 polymorphisms: relationship to lung tissue enzyme activity and population frequency distribution, Carcinogenesis 19 (1998) 275–280.
[33] E.M. Van Lieshout, H.M. Roelofs, S. Dekker, T. Wobbes, J.B. Jansen, W.H. Peters, Polymorphic expression of the glutathione S-transferase P1 gene and its susceptibility to Barrett's esophagus and esophageal carcinoma, Cancer Res. 59 (1999) 586–589.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Cancer Letters 210 (2004) 151–157
CANCER Letters
www.elsevier.com/locate/canlet

Proline homozygosity in codon 72 of p53 is a factor of susceptibility for thyroid cancer

Fabiana Granja[a], Joseane Morari[a], Elaine C. Morari[a], Luiz A.C. Correa[b], Lígia V.M. Assumpção[a], Laura S. Ward[a,*]

[a]*Laboratory of Cancer Molecular Genetics, Department of Medicine, State University of Campinas, Olympio Pattaro 45, Campinas, São Paulo, Brazil*
[b]*Heliópolis Hospital, São Paulo, Brazil*

Received 6 September 2003; received in revised form 25 January 2004; accepted 27 January 2004

Abstract

A common germline polymorphism of *p53* gene produces an Arginine to Proline change at aminoacid position 72. The resulting codon 72 variants have been reported associated with tumor susceptibility since they reduce *p53* ability to activate apoptosis. Codon 72 polymorphism may play a role in subside vulnerability to different carcinogens and might account for ethnic variations in cancer frequency. Using an allele-specific polymerase chain reaction (PCR), we tested peripheral blood samples from 98 patients with thyroid cancer, including 21 follicular (FC) and 77 papillary carcinomas (PC), 44 patients with benign nodules, including 14 follicular adenomas and 30 goiters and 153 healthy individuals from the same geographical region. Data on lifetime occupational history, smoking history, general health conditions, previous diseases and other anamnestic data were obtained through interviews. Patients with FC (*Pro/Pro* = 19.0%, *Arg/Arg* = 42.9%, *Arg/Pro* = 38%) and with PC (*Pro/Pro* = 10.3%, *Arg/Arg* = 36.36%, *Arg/Pro* = 53.24%) showed a significant overrepresentation of codon 72 variants compared to the control population (*Pro/Pro* = 1.9%, *Arg/Arg* = 33.3%, *Arg/Pro* = 64.7%) ($P = 0.0015$). The *Pro/Pro* genotype, after adjusting for gender, age, tobacco and drugs, was associated with a markedly higher risk of FC (OR = 9.714; CI: 2.334–40.436) and of PC (OR = 5.299; CI: 2.334–40.436). These results provide evidence that p53 polymorphism is implicated in thyroid carcinogenesis and that individuals harboring the *Pro/Pro* genotype have an increased risk of developing thyroid cancer.

Keywords: p53 codon 72; Polymorphism; Thyroid cancer

1. Introduction

A mutated *p53* gene or a malfunctioning p53 protein has been observed in patients with most types of malignancies, including the thyroid gland [1,2]. The structural features of p53 (codons 61–94) have been well preserved throughout evolution except at

Abbreviations: PCR, Polymerase chain reaction; Arg, Arginine; Pro, Proline; PC, Papillary carcinomas; FC, Follicular carcinomas; FA, Follicular adenomas; CI, Confidence interval; TSH, Thyrotropin stimulating hormone; Tg, Thyroglobulin; SAS, Statistical analysis system; χ^2, Chi-square test; F, Fisher's test; KW, Kruskal–Wallis test; OR, Odds ratio.

* Corresponding author. Address: GEMOCA, Med. Interna, Clinica Medica, FCM-UNICAMP, Rua Tessalia Vieira de Camargo 126, Campinas, SP 13081 970, Brazil. Tel./fax: +55-19-3289-4107.
E-mail address: ward@unicamp.br (L.S. Ward).

0304-3835/$ - see front matter © 2004 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.canlet.2004.01.016

exon 4, where a common polymorphism results in either proline or arginine at amino-acid position 72 [3]. This polymorphism occurs in the proline-rich domain of exon 4, which is necessary for the protein to fully induce apoptosis [4].

Many studies, both with immunocytochemical and genetic analyses, have shown that *p53* mutations are highly prevalent in poorly differentiated and undifferentiated thyroid carcinomas, as well as thyroid cancer cell lines [5,6]. However, they 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 [7–9]. These data suggest that mutational inactivation of the *p53* gene may be a key event in the progression from differentiated to anaplastic carcinoma [4,8,9]. There is also evidence that *p53* may interfere with thyroid cell differentiation. Introduction of a mutated *p53* markedly impairs the differentiated gene expression of PCC13 thyroid cells [10]. By contrast, wild-type *p53* reintroduction into poorly differentiated thyroid carcinoma cell line leads to cell growth arrest and re-expression of the characteristic differentiated markers of the thyroid cell [11,12].

The role of *p53* polymorphism in various tumors is still controversial. This polymorphism has been shown to have varying ethnic and geographical distribution. It has been reported to be a potential genetic risk factor for some types of cancer, like cervico-uterine [13], breast cancer [14], lung [15], head and neck cancer [16], among others. However, not all investigations have been consistent and this hypothesized association remains controversial [17,18]. The role of this single nucleotide polymorphism (SNP) in the prognosis of these 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 [19]. 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 [20]. However, Wang et al. demonstrated *p53* polymorphism to be related to the prognosis of lung cancer [21]. Also, he found different *p53* genotypes to be associated with increased susceptibility for different subgroups of lung cancer [21]. In fact, *p53 Arg* homozygosity is considered a risk factor for cervical cancer [22], while proline homozygotes were related to a higher risk of nasopharyngeal carcinomas [23], lung [21], and hepatocellular carcinomas [25], and the *Arg/Pro* genotype was associated to an increased susceptibility for smoke-induced lung adenocarcinoma [24].

In the unique report on codon 72 polymorphism we were able to find regarding thyroid tumors, Botze et al. examined Caucasian thyroid carcinoma patients and concluded that Pro72 genotype was a potential risk factor favoring the development of undifferentiated thyroid carcinomas [26]. In order to further investigate the role of codon 72 of *p53* polymorphism in thyroid tumorigenesis, especially in the well-differentiated types, we conducted a prospective case-control study in Brazilian patients with thyroid nodules including 98 cases of well-differentiated tumors and 44 benign thyroid nodules. We also aimed to evaluate a possible utility of *p53* polymorphism genotyping in the prediction of thyroid cancer patient's outcome.

2. Material and methods

2.1. Subjects

The study was approved by the Ethics Committee of the University Hospital, School of Medicine of the State University of Campinas (HC-FCM/UNICAMP), and informed written consent was obtained from all individuals. Because of the highly heterogeneous ethnic composition of the Brazilian population we included a control group of 153 healthy individuals (52 males and 101 females, 16–81 years old, 47 ± 21 years old) selected from the general population of our region, considered to have a normal iodine intake. There were 115 blood donors and 42 volunteers recruited among co-workers and volunteers from the State University of Campinas (UNICAMP). All individuals were classified into white and non-white. In order to obtain a comparable control group with respect to gender proportion and the range of ages, we selected 2 to 3 women for every man that presented himself to donate blood, because thyroid cancer occurs more frequently in women than in men. Also, we selected some more individuals classified as white. Data on lifetime

Table 1
Distribution of thyroid carcinoma patients according to their histology, clinical features including age (X ± SD in years), gender (F, female; M, male), color (W, white; NW, non-white), the history of previous thyroid benign diseases, smoke habits, use of medicines, the presence of lymph node involvement and distant metastasis by the time of the diagnosis and the diagnosis of recurrence and/or distant metastasis during the follow-up

	Clinical characteristics								Diagnosis (Presence of metastasis)		Follow-up
	Age (X ± SD)	Sex		Color		Previous thyroid disease	Smokers	Use of medicines	Lymph node	Distant	Recurrence and/or distant metastasis
		M	F	W	NW						
PC	44 ± 15	11	66	61	16	18	37	21	30	8	20
FC	56 ± 12	5	14	15	6	9	9	8	3	10	10

occupational history, smoking history, general health conditions, previous diseases and other anamnestic data were obtained through interviews. Individuals with history of previous thyroid disease, exposure to radiation and antecedents of malignancy were excluded.

One hundred forty two patients consecutively referred to the outpatient clinic of the University Hospital (HC-FCM/UNICAMP) for thyroid disease evaluation, during the years of 2001–2003, that agreed to participate, were enrolled in the study. The study population was composed of 44 benign thyroid nodules including 30 goiters and 14 follicular adenomas (FA) and 98 malignant nodules, including 77 papillary carcinomas (PC) and 21 follicular carcinomas (FC). Experienced pathologists of the University Hospital confirmed all diagnoses. All cases were managed according to a standard protocol. The diagnosis of thyroid carcinoma was established or suspected by fine-needle aspiration cytological study and/or by the histological analysis of thyroid tissues from patients that were sent to surgery because of thyroid nodules that presented clinical or epidemiological suspicion of cancer. All patients were submitted to total or near-total thyroidectomy. Patients with neck node metastases palpable preoperatively or intraoperatively underwent regional neck dissection. Four to six weeks after the operations, whole body ^{131}I scans were performed. All patients received 30–100 mCi ^{131}I. Long-term levothyroxine suppressive doses were given after the whole body scans in order to keep serum thyrotropin (TSH) levels at low normal.

Hospital records were reviewed and clinical data like age, gender, fine-needle aspiration cytological results, thyroid function tests, primary tumor size and operative findings were compiled. Tumor stage and degree of differentiation were obtained from surgical and pathological records. Tumor staging was based on clinical staging of DeGroot (1995). Stage 1 is a tumor with single or multiple intrathyroidal foci. Stage 2 is a tumor with limited cervical metastasis only. Histopathologically proven cervical lymph node metastases were identified in all the patients. Stage 3 is a thyroid tumor with local cervical metastasis or fixed cervical metastasis. Stage 4 is a lesion metastasis outside the neck. Clinical and pathological features of the patients with thyroid carcinomas are detailed in Table 1.

Data on general health conditions and medical history with emphasis on previous and/or current thyroid diseases were obtained through interviews. The use of drugs was also carefully assessed, in particular nutritional goitrogens, drugs that could interfere with thyroid function, as well as medicines for other concomitant diseases. Cigarette smoking habit was recorded but, because of the few 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. None of the patients had a history of accidental or medical radiation exposure. All data, including pathological diagnoses, were confirmed in the patients' records.

2.2. Follow-up

Cancer patients were followed with periodic whole body scans, serum TSH and Tg measurements according to a routine follow-up protocol that includes X-ray, ultrasonography, computer tomography scan and other eventual procedures to detect distant metastasis for a period of 12–382 months (28 ± 57 months). Patients with high serum thyroglobulin levels >2 mg/dL) and/or suspicious whole 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.

2.3. Polymorphism analysis

A peripheral blood sample was collected from all patients Genomic DNA was extracted from leukocytes separated from whole blood using a standard proteinase K-phenol–chloroform protocol. 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. [27] (Fig. 1). The detection of the two polymorphic variants was done in two different tubes. 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 uM of each dinucleotide triphosphate, 2.0 mM MgCl2 and 0.5 U Platinum® Taq DNA polymerase. Amplifications were carried out for 35 cycles of 94 °C for 45 s, annealing temperatures 68 °C for the Arg allele and 53 °C for the Pro allele for 45 s and 72 °C for 1 min, with an initial denaturation step of 94 °C for 3 min and a final extension step of 72 °C for 10 min using a MJ PTC-200 PCR system. The PCR fragments were analyzed by electrophoresis in a 2% agarose gel stained with ethidium bromide and visualized on a UV light transilluminator. The PCR product of the Arg allele was 141 bp, while the product of the Pro allele was 177 bp. Heterozygous specimens had both PCR products, while homozygous samples exhibited only one of the two products. Positive and negative control samples were included in all PCRs to detect possible contamination problems. PCR procedures were repeated at least two times in all doubtful or unclear bands. Four samples that presented pattern runs of homozygozity for *Arg*, four samples *Pro* homozygous and five *Arg/Pro* heterozygous were directly sequenced employing the ABI prism big dye sequencing kit (Perkin Elmer, Warrington, Cheshire, UK) using the ABI 377 Prism DNA Sequencer (Perkin Elmer) in order to confirm the corresponding genotype. Sequencing controls were run on PCR products obtained with primers forward 5′-TCCCCCTTGCCGTCCCAA-3′ (ArgF) and reverse 5′-CGTGCAAGTCACAGACTT-3′ (ProR) that amplified a 279 bp fragment harboring the region of the polymorphism at 60.8 °C (figure not showed).

2.4. Statistical analysis

Statistical analysis was conducted using SAS statistical software (Statistical Analysis System, version 8.1 (SAS Institute Inc, Cary, NC, USA, 1999–2000) Chi-square (χ^2) or Fisher's (F) exact tests were used to examine homogeneity between cases and controls regarding gender, color, previous thyroid disease, use of medicines and cigarette smoking. Also, extent of disease was compared

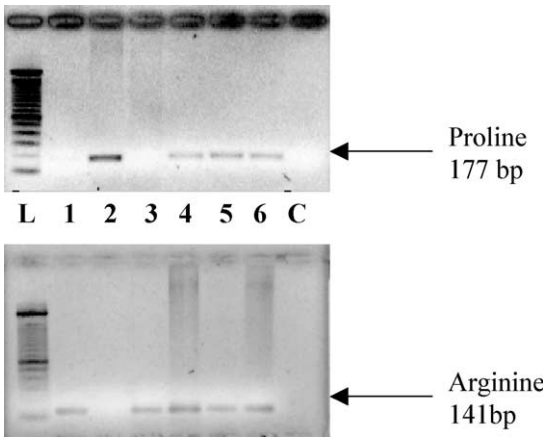


Fig. 1. 2% agarose gel electrophoresis representative of our results for the p53 gene screening for polymorphisms. The gel was loaded with two samples from FC—lanes 1 and 2, and 4 samples from PC patients—lanes 3–6. Lane 1 (L) was loaded with a molecular size marker. Lane 7 (C) was loaded with a PCR mixture that did not include DNA (negative control). The arrows indicate samples that display an eletrophoretic run suggestive of Pro/Pro homozygosis in lane 2, Arg/Arg homozygosis in lanes 1 and 3 and Arg/Pro heterozygosis in lanes 4, 5 and 6. The six samples were sequenced directly and confirmed to be the predicted p53 variants for codon 72.

Table 2
Camparison among the different p53 codon 72 genotypes in the control population and in the benign and malignant thyroid disease patients

	Controls	Benign nodules		Malignant nodules	
	N (%)	Goiter	FA	PC	FC
Arg/Arg	51 (33.3%)	18 (60%)	5 (35.7%)	28 (36.3%)	9 (42.9%)
Arg/Pro	99 (64.7%)	11 (36.6%)	9 (64.2%)	41 (53.2%)	8 (38%)
Pro/Pro	3 (1.9%)	1 (3.3%)	0	8 (10.3%)	4 (19%)
Total of cases	153	30	14	77	21

between papillary and follicular carcinomas using Fisher's test. Kruskal–Wallis (KW) test was used to compare age among groups. Mann–Whitney or Wilcoxon tests were used to compare age among different genotype groups. The odds ratio (OR) and 95% confidence interval (CI) provide a measure of the strength of association, e.g. indicating the increase in odds of a given benign or malignant thyroid nodule demonstrating a particular genotype compared to the control population. All tests were conducted at the $P = 0.05$ level of significance.

3. Results

There were no differences between the control and the thyroid disease patients regarding age (47 ± 21 years versus 49 ± 14 years); gender (52 males and 101 females versus 37 males and 61 females); color (72 white and 60 non-white versus 54 white and 44 non-white individuals); drug ingestion (39 control individuals versus 29 patients); smoking (61 control individuals versus 46 patients); alcohol consumption (61 control individuals versus 46 patients) and dietary patterns.

Table 1 summarizes clinical characteristics and parameters of aggressiveness at diagnosis and during follow-up of the thyroid cancer patients. Patients with FC were older than the patients with PC (KW; $P = 0.0012$). Nevertheless, there were no differences among the patients regarding color, gender distribution, smoking habit, the use of drugs or preexisting benign thyroid diseases. The occurrence of lymph node involvement at the time of the diagnosis was more frequent among PC (40%) than FC (14%) patients (F; $P < 0.05$). However, these last tumors already presented long distance metastasis at the time of the diagnosis (48%) more frequently than papillary carcinomas (10%) (F; $P < 0.001$). Follow-up data did not evidence differences between PC and FC concerning distant metastasis and/or recurrence of the tumor although FC patients presented evidence of recurrence and/or metastasis in 48% of the cases against 26% of the PC ones (F; $P = 0.11$). Two patients died during the period of observation.

Table 2 summarizes data of the overall proportions of the p53 codon 72 genotypes in the control population and in the benign and malignant thyroid disease patients.

Patients with benign nodules and, in particular, PC and FC patients, showed a significant over-representation of the *Pro/Pro* genotypes (χ^2, $P = 0.0015$). The risk for thyroid cancer, after adjusting for gender, age, tobacco, alcohol and medicines, was more than seven times higher (Odds ratio = 7.023, 95% CI: 1.928–25.588) in individuals with the *Pro/Pro* genotype compared to the other genotypes.

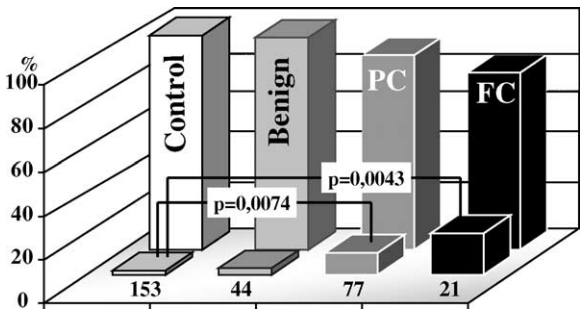


Fig. 2. Graphic representation of the statistic analysis of the prevalence of the Pro homozygous (front columns) and the other alleles (columns in the back) of codon 72 of the *p53* gene in the control population, in patients with benign nodules, papillary (PC) and follicular (FC) carcinomas.

An estimated 5.299 fold greater risk of PC (OR; CI: 2.334–40.436) ($P = 0.0074$) and 9.714 fold of FC (OR; CI: 2.334–40.436) ($P = 0.0043$) was observed in individuals that presented the *Pro/Pro* genotypes compared to the other genotypes. Fig. 2 represents the percentage of *Pro/Pro* cases in the groups studied. *Pro/Pro* genotype was never demonstrated among the 14 FA cases examined, but occurred in 19% of the FC. The incidence of individuals with the *Arg/Pro* genotype did not differ among groups.

We were not able to find any correlation between genotype and other possible risk factors, like age, gender, smoke habits, use of medicine or antecedents. Also, there was no association between genotype and the patients' clinical features, tumor parameters of aggressiveness at diagnosis or behavior during follow-up.

4. Discussion

Single nucleotide polymorphisms are the most abundant types of DNA sequence variation in the human genome, and as heritable variable landmarks they are useful markers for genome mapping [28]. The SNP marker has gained increasing popularity for its quick, accurate, and inexpensive properties for genetic analysis of different diseases [29]. Indeed, a overwhelming amount of data have been indicating the importance of hosts factors in cancer development [30]. The polymorphism of *p53* at codon 72 is very interesting since it has been demonstrated that Pro72 variants have an ability to induce apoptosis markedly poorer than does the Arg 72 variant. One source of this inferior apoptotic potential is the greater ability of the Arg variant to localize to themitocondria; this localization is accompanied by release of cytochrome c into cytosol [4].

An association of the *p53* codon 72 polymorphism with several cancers susceptibilities has been reported [13–16,18–25]. However, there is only one report about the relationship between the *p53* codon 72 polymorphism and thyroid cancer [26]. In this paper, Boltze et al. focused on the undifferentiated carcinomas studying just 21 papillary carcinomas, the most frequent type of differentiated thyroid tumors. Our data confirm that homozygous proline, but not heterozygous proline/arginine at codon 72 of *p53*, is a potential risk factor for thyroid cancer. In contrast to Boltze et al. we also found Pro72 variants in well-differentiated thyroid carcinomas and even in one benign goiter. In addition, Boltze et al. did not present data on patients follow-up, preventing any conclusion on eventual correlations between genotype, the effect on pathology and prognostic of the thyroid well-differentiated carcinomas. Our data suggest that there correlations do not exist.

Thyroid cancer is responsible for only 0.6–1.6%, respectively, of all kinds of cancers that occur in men and women in the USA; in contrast, thyroid nodules occur in more than 5% of the population [31]. Although clinical and epidemiological features are helpful, there are still no accepted serological markers that can guide the identification of patients at risk for malignancy among individuals with thyroid nodules [31,32].

Acting in concert with individual susceptibility, environmental factors such as smoking, diet, and pollutants play the principal role in causing sporadic role in most human cancers [30,33]. The etiology of thyroid cancer is markedly uncertain. Exposure to ionizing radiation, especially in childhood, remains the only factor clearly associated with benign and malignant thyroid tumors in humans [33]. However, there is strong epidemiological evidence pointing toward the involvement of geographic, ethnic and dietary factors in the risk of sporadic thyroid cancer [33]. We demonstrated previously that *GSTT1* and *GSTM1* combined null inheritance was associated with a 2.6 fold increase in the susceptibility to thyroid cancer [34]. The homozygous Pro 72 variant of *p53* increased the risk of PC more than five times and of FC more than nine times. We suggest that *p53* genotype profiling, together with other susceptibility factors, may be useful in the screening for thyroid nodule malignancy.

References

[1] A.J. Levine, p53 the cellular gatekeeper for growth and division, Cell 88 (1997) 323–331.
[2] N.R. Farid, p53 mutations in thyroid carcinoma: tidings from an old foe, J. Endocrinol. Invest. 24 (2001) 536–545.
[3] S. Ara, P.S. Lee, M.F. Hansen, H. Saya, Codon 72 polymorphism of the TP53 gene, Nucleic Acids Res. 18 (1990) 4961.

[4] P. Dumont, J.I. Leu, A.C. Della Pietra, D.L. George, M. Murphy, The codon 72 polymorphic variants of p53 have markedly different apoptotic potential, Nat. Genet. 33 (2003) 357–365.
[5] J.A. Fagin, K. Matsuo, A. Karmakar, D.L. Chen, S.H. Tang, H.P. Koeffler, High prevalence of mutations of the p53 gene in poorly differentiated human thyroid carcinomas, J. Clin. Invest. 91 (1993) 179–184.
[6] G.H. Jossart, H.D. Epstein, J.K. Shaver, H. Weier, K.M. Greulich, S. Tezelman, et al., Immunocytochemical detection of p53 in human thyroid carcinomas is associated with mutation and immortalization of cell lines, J. Clin. Endocrinol. Metab. 81 (1996) 3498–3504.
[7] Y. Dobashi, H. Sugimura, A. Sakamoto, M. Mernyei, M. Mori, T. Oyama, R. Machinami, Stepwise participation of p53 gene mutation during dedifferentiation of human thyroid carcinomas, Diagn. Mol. Pathol. 3 (1994) 9–14.
[8] H. Asakawa, T. Kobayashi, Multistep carcinogenesis in anaplastic thyroid carcinoma: a case report, Pathology 34 (2002) 94–97.
[9] S. Horie, H. Maeta, K. Endo, T. Ueta, K. Takashima, T. Terada, Overexpression of p53 protein and MDM2 in papillary carcinomas of the thyroid: correlations with clinicopathologic features, Pathol. Int. 51 (2001) 11–15.
[10] S. Battista, M.L. Martelli, M. Fedele, G. Chiappetta, F. Trapasso, G. de Vita, et al., A mutated p53 alters differentiation of thyroid cells, Oncogene 72 (1995) 2029–2037.
[11] M. Narimatsu, Y. Nagayama, K. Akino, M. Yasuda, T. Yamamoto, A. Yang, et al., Therapeutic usefulness of wild-type p53 gene introduction in a p53-null anaplastic thyroid carcinoma cell line, J. Clin. Endocrinol. Metab. 83 (1998) 3668–3672.
[12] Y. Nagayama, K. Shigematsu, H. Namba, K. Zeki, S. Yamashita, M. Niwa, Inhibition of angiogenesis and tumorigenesis, and induction of dormancy by p53 in a p53-null thyroid carcinoma cell line in vivo, Anticancer Res. 20 (2000) 2723–2728.
[13] A. Sifuentes Alvarez, M. Reyes Romero, Risk factors for cervico-uterine cancer associated to HPV: p53 codon 72 polymorphism in women attending hospital care, Ginecol. Obstet. Mex. 71 (2003) 12–15.
[14] N. Buyru, H. Tigli, N. Dalay, P53 codon 72 polymorphism in breast câncer, Oncol. Rep. 10 (2003) 711–714.
[15] E.D. Papadakis, N. Soulitzis, D.A. Spandidos, Association of p53 codon 72 polymorphism with advanced lung cancer: the Arg allele is preferentially retained in tumours arising in Arg/Pro germline heterozygotes, Br. J. Cancer 87 (2002) 1013–1018.
[16] H. Shen, Y. Zheng, E.M. Sturgis, M.R. Spitz, Q. Wei, P53 codon 72 polymorphism and risk of squamous cell carcinoma of the head and neck: a case-control study, Cancer Lett. 183 (2002) 123–130.
[17] M. Oren, Decision making by p53: life, death and cancer, Cell Death Differ. 10 (2003) 431–442.
[18] S.N. Drummond, L. De Marco, A.P. Ide, A.A. Barbosa, R.S. Gomez, TP53 codon 72 polymorphism in oral squamous cell carcinoma, Anticancer Res. 22 (2002) 3379–3381.
[19] M. Dong, Y. Nio, K. Yamasawa, T. Toga, L. Yue, T. Harada, p53 alteration is not an independent prognostic indicator, but affects the efficacy of adjuvant chemotherapy in human pancreatic cancer, J. Surg. Oncol. 82 (2003) 111–120.
[20] W.J. Wu, Y. Kakehi, T. Habuchi, H. Kinoshita, O. Ogawa, T. Terachi, et al., Allelic frequency of p53 gene codon 72 polymorphism in urologic cancers, Jpn. J. Cancer Res. 86 (1995) 730–736.
[21] Y.C. Wang, H.S. Lee, S.K. Chen, Y.Y. Chang, C.Y. Chen, Prognostic significance of p53 codon 72 polymorphism in lung carcinomas, Eur. J. Cancer 35 (1999) 226–230.
[22] M. Qie, Y. Zhang, J. Wu, Study on the relationship between cervical cancer and p53 codon 72 polymorphism, Hua Xi Yi Ke Da Xue Xue Bao. 33 (2002) 274–275.
[23] M.H. Tsai, C.D. Lin, Y.Y. Hsieh, F.C. Chang, F.J. Tsai, W.C. Chen, C.H. Tsai, Prognostic Significance of the proline form of the p53 codon 72 polymorphism in nasopharyngeal carcinoma, Laryngoscope 112 (2002) 116–119.
[24] R. Fan, M.T. Wu, D. Miller, J.C. Wain, K.T. Kelsey, J.K. Wiencke, D.C. Christiani, The p53 codon 72 polymorphism and lung cancer risk, Cancer Epidemiol. Biomarkers Prev. 9 (2000) 1037–1042.
[25] M.W. Yu, S.Y. Yang, Y.H. Chiu, Y.C. Chiang, Y.F. Liaw, C.J. Chen, A p53 genetic polymorphism as a modulator of hepatocellular carcinoma risk in relation to chronic liver disease, familial tendency, and cigarette smoking in hepatitis B carriers, Hepatology 29 (1999) 697–702.
[26] C. Boltze, A. Roessner, O. Landt, E. Szibor, B. Peters, R. Schneider-stock, Homozygous proline at codon 72 of p53 as a potential risk factor favoring the development of undifferentiated thyroid carcinoma, Int. J. Cancer 21 (2002) 1151–1154.
[27] A. Storey, M. Thomas, A. Kalita, C. Harwood, D. Gardiol, F. Mantovani, et al., Role of p53 polymorphism in the development of human papilloma-virus-associated cancer, Nature 21 (1998) 229–234.
[28] P.Y. Kwok, Genomics. Genetic association by whole-genome analysis?, Science 294 (2001) 1669–1670.
[29] B.W. Kirk, M. Feinsod, R. Favis, R.M. Kliman, F. Barany, Single nucleotide polymorphism seeking long term association with complex disease, Nucleic Acids Res. 30 (2002) 3295–3311.
[30] P. Vineis, Cancer as an evolutionary process at the cell level: an epidemiological perspective, Carcinogenesis 24 (2003) 1–6.
[31] M.J. Welker, D. Orlov, Thyroid nodules, Am. Fam. Physician 67 (2003) 559–566.
[32] M.J. Schlumberger, Papillary and follicular thyroid carcinoma, Baillieres Best. Pract. Res. Clin. Endo. Metab. 14 (2000) 601–613.
[33] P. Lichtenstein, N.V. Holm, P.K. Verkasalo, A. Iliadou, J. Kaprio, M. Koskenvuo, et al., Environmental and heritable factors in the causation of cancer, N. Engl. J. Med. 343 (2000) 78–85.
[34] E.C. Morari, J.L. Leite, F. Granja, L.V. da Assumpcao, L.S. Ward, The null genotype of glutathione s-transferase M1 and T1 locus increases the risk for thyroid cancer, Cancer Epidemiol. Biomarkers Prev. 11 (2002) 1485–1488.

GSTO polymorphism analysis in thyroid nodules suggest that *GSTO1* variants do not influence the risk for malignancy

F Granja, E C Morari, L V M Assumpção and L S Ward

A new class of glutathione *S*-transferase enzymes named omega (GSTO) has been recently identified and shown to be expressed in a wide range of human tissues. A genetic polymorphism of the *GSTO1* gene causing an alanine-to-aspartate (A140D) substitution in amino acid 140 produces a variant with lowered enzyme activities in the biotransformation of inorganic arsenic, a common contaminant of drinking water in many regions of the world and a well-known carcinogen. In order to investigate the role of *GSTO1* inheritance pattern on thyroid cancer risk we used a polymerase chain reaction–single strand conformation polymorphism (PCR-SSCP)-sequencing approach to compare the genotypes of 173 (87 women, 86 men; 18–81 years old; 47 ± 18 years old) healthy control individuals with those of 145 patients with thyroid nodules (84 women, 61 men; 17–81 years old; 49 ± 14 years old) including 17 follicular carcinomas, 76 papillary carcinomas, 21 follicular adenomas and 31 multinodular goiters. The incidence of *GSTO1* variants was similar in the control population and population with the benign and malignant nodules. There was no association between genotype and the patients' clinical features, tumour parameters of aggressiveness at diagnosis or behaviour during follow-up. We conclude that *GSTO1* variants do not influence the risk for thyroid nodules or their pathologic and clinical characteristics. *European Journal of Cancer Prevention* 14:000–000 © 2005 Lippincott Williams & Wilkins.

European Journal of Cancer Prevention 2005, **14**:000–000

Keywords: Cancer, GSTO1, susceptibility

Laboratory of Cancer Molecular Genetics, Department of Medicine, State University of Campinas, Olympio Pattaro 45, 13085–045 Campinas, São Paulo, Brazil.

Correspondence to: L S Ward.
Fax: (+55) 19 3788 8877. E-mail: ward@unicamp.br

Received 20 June 2004 **Accepted** 27 September 2004

Introduction

The aetiology of thyroid cancer is mostly uncertain. The only factor clearly associated with benign and malignant thyroid tumours in humans is the exposure to ionizing radiation (Schlumberger, 2000). However, variations of thyroid cancer incidence in different geographic and ethnic groups suggest that environmental factors may influence the risk of sporadic thyroid cancer (Friedman and Ury, 1983; Ron *et al.*, 1987; Mack *et al.*, 2002; Haselkorn *et al.*, 2003). Although no increase in the risk of human thyroid cancer has ever been consistently observed with any chemical or drug, a wide variety of drugs, pesticides, goitrogenic xenobiotics and chemicals have been shown to increase the incidence of thyroid tumours in rodents (McClain, 1992; Hayes and Strange, 1995). Genetic polymorphisms of genes encoding for enzymes involved in the biotransformation of carcinogens form the biochemical basis for cancer susceptibility.

One of the most important mechanisms of defence against environmental carcinogens is a supergene family of dimeric enzymes that are expressed in probably all life forms (Mannervik, 1985). These enzymes catalyse the conjugation of glutathione to a variety of electophiles, including arene oxides, unsaturated carbonyls, organic hialides and other substrates (Strange and Fryer, 1999). Three classes of isoenzymes have been studied in the Brazilian population with thyroid nodules: *GSTM1*, *GSTT1* and *GSTP1*. We demonstrated previously that *GSTT1* and *GSTM1* null inheritance was associated with a 2.6-fold increase in the susceptibility to thyroid cancer (Morari *et al.*, 2002). More recently, we found a relatively large odds ratio for *GSTP1* variants association with thyroid cancer, implying that the allelic variants of *GSTP1* may exert a substantial biological effect (Granja *et al.*, 2004).

Recently, a new class of human GST, named GST omega (GSTO) was identified by analysis of the expressed sequence tag (EST) database and by sequence alignments (Board *et al.*, 2000; Yin *et al.*, 2001). The physiological importance of GSTO has not yet been fully elucidated. However, Dulhunty *et al.* (2001) report that GSTO1 enzyme modulates ryanodine receptors (RyR) which are calcium channels in the endoplasmic reticulum of various cells, and suggest a novel role in protecting cells containing RyR2 from apoptosis induced by Ca^{2+} mobilization. A genetic polymorphism of the *GSTO1* gene was described at base 419 causing an alanine-to-aspartate (A140D) substitution in amino acid 140 of exon 4 (Ala140Asp) (Tanaka-Kagawa *et al.*, 2003; Whitbread *et al.*, 2003). This variation creates a non-conservative amino acid change from a hydrophobic to a hydrophilic residue that results in 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).

The primary objective of this study was to verify a possible role of the GSTO gene and its variants in the susceptibility to thyroid cancer, its clinical and pathological characteristics and its response to therapy. For these purposes, we conducted a prospective case control study in which we compared the proportion of *GSTO1* genotypes in patients with benign and malignant thyroid nodules with a control population group. Heterogeneity of risk according to clinical and morphological subtypes of thyroid tumours and their correspondent genotype was also explored.

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, and informed written consent was obtained from all individuals. A control group of 173 healthy individuals (86 men and 87 women, 18–81 years old, 47 ± 18 years old) was selected from the general population of our region. There were 131 blood donors and 42 volunteers recruited among co-workers and volunteers from the State University of Campinas. Data on lifetime occupational history, smoking history, general health conditions, previous diseases, alcohol and medication consumption and other anamnestic data were obtained through interviews. Individuals with history of previous thyroid disease, radiation exposure and antecedents of malignancy were excluded.

The study population included 52 benign thyroid nodules (31 multinodular goitres and 21 follicular adenomas) and 93 malignant nodules, including 76 papillary carcinomas and 17 follicular carcinomas. Stage and grade of differentiation of the tumours were obtained from surgical and pathological records. Experienced pathologists of the University 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 that were referred to surgery because of 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. Four to six weeks after the operations, whole body ^{131}I scans were performed. All patients received 30–100 mCi iodine-131. Long-term levothyroxine suppressive doses were given following a whole body scan, in order to keep serum thyrotropin (TSH) levels at low normal.

Patients were classified into white and non-white groups. Data on general health conditions and medical history with emphasis on previous and/or current thyroid diseases were obtained through interviews, using a structured questionnaire. Cigarette smoking habit was recorded and the patients were grouped in never-smokers and ever-smokers categories. None of the patients had a history of accidental or medical radiation exposure. All data, including pathological diagnoses, were confirmed in the patients' records. A peripheral blood sample was collected from all 142 patients. Also, thyroid tissue samples were obtained at surgery from 83 out of these patients, snap frozen in liquid N_2 immediately after surgery and kept at $-80^{\circ}C$ until processed. We were able to obtain cancer tissue samples and autologous blood specimens and/or normal thyroid tissue from the contralateral lobe in 35 cases of malignant tumours.

Follow-up

Cancer patients were followed with periodic whole 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 12–342 months (31 ± 67 months). Patients with high serum Tg levels (> 2 mg/dl) and/or suspicious whole body scans were submitted to a thorough image search. We defined tumours as recurrent and/or presenting long distance metastasis according to the above parameters.

Polymorphism analysis

Genomic DNA was extracted using a standard proteinase K and phenol-chloroform protocol. *GSTO1* variants were studied using a polymerase chain reaction–single strand conformation polymorphism (PCR-SSCP)-sequencing approach. The corresponding primers used were previously described (Whitbread *et al.*, 2003). PCR was performed in 25 µl volumes of a mixture containing 100 ng DNA, 50 nmol/l of each primer, 10 mmol/l Tris–HCl (pH 8.0), 100 µmol/l of each dinucleotide triphosphate, 2.0 mmol/l $MgCl_2$ and 0.5 U Taq DNA polymerase. Amplifications were carried out for 35 cycles of 94°C for 30 s, annealing temperature was 56°C seconds 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 an MJ PTC–200 PCR system. The PCR products were mixed with 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol and 50 mmol/l NaOH, denatured at 94°C for 10°min, and loaded onto 0.4 mm/30 cm/45 cm polyacrylamide gels. The electrophoresis was conducted at 2–5 W at room temperature overnight. The gels were then stained with silver nitrate. Forty-five samples suspected of presenting aberrant migrating AQ1

bands were directly sequenced using the ABI prism big dye sequencing kit (Perkin Elmer, Warrington, Cheshire, UK) using the ABI 377 Prism DNA Sequencer (Perkin Elmer). Also 10 samples, four from the control group and six from patients with a normal banding pattern, were directly sequenced. Positive and negative control samples were included in all PCR and SSCP runs to detect possible contamination problems, gel loading and typing inconsistencies.

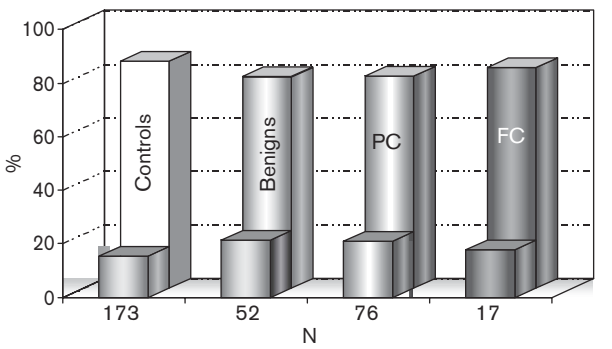
Statistical analysis

Statistical analysis was conducted using SAS statistical software (Statistical Analysis System, version 8.1, Cary, NC, USA, 1999–2000). Based on the allele frequency of *GSTO1* previously described, we estimated a size sample of 95 cases to be enough to retain high statistical power. Chi-squared or Fisher's exact tests were used to examine homogeneity between cases and controls regarding gender, colour, previous thyroid disease, use of medication and cigarette smoking. Also, extent of disease was compared between papillary and follicular carcinomas using Fisher's test. Kruskal–Wallis test was used to compare age among groups. Mann–Whitney or Wilcoxon tests were used to compare age among different genotype groups. The odds ratio (OR) and 95% confidence interval (95% CI) provide a measure of the strength of association, e.g. indicating the increase in odds of a given benign or malignant thyroid nodule demonstrating a particular genotype compared with the control population. Logistic regression was used to evaluate the effect of genotypes, after adjusting for other potential confounders like age, sex, colour, tobacco, and alcohol and medication consumption. Interactions between environmental variables and genotypes were also assessed. All tests were conducted at the $P = 0.05$ level of significance.

Results

There were no differences between the control and the thyroid disease patients regarding age (47 ± 18 years versus 49 ± 14 years), gender (86 men and 87 women versus 61 men and 84 women and colour (113 white and 60 non-white versus 98 white and 47 non-white individuals). Patients with benign nodules (A140/D140 = 15.4%, A140/A140 = 78.8%, D140/D140 = 5.8%), thyroid carcinomas (A140/D140 = 18.3%, A140/A140 = 79.6%, D140/D140 = 2.1%) did not show a significant over-representation of the variants of *GSTO1* genotype compared with the control population (A140/D140 = 11%, A140/A140 = 84.4%, D140/D140 = 4.6%). There were no differences between the incidences of heterozygous *GSTO1* or homozygous *GSTO1* genotypes in the benign, the malignant nodules and the control population. Figure 1 represents data on the overall proportions of the *GSTO1* genotypes in the control population and in the benign and malignant thyroid disease patients. There was no association between genotype and the patients' clinical features, tumour parameters of aggressiveness at diagnosis or behaviour during follow-up.

Fig. 1



Graphic representation of the prevalence of *GSTO1* gene variant alleles (first row columns), and *GSTO1* gene wild type (second row columns) in the control population, in the patients with benign thyroid diseases, papillary (PC) and follicular (FC) carcinomas.

Discussion

Thyroid nodules are very frequent among the general population, in contrast to thyroid cancer (Schlumberger, 2000). Identification of those individuals who are at an increased risk for cancer is important for planning and implementing proper prevention and management strategies. Although diagnostic tools such as fine-needle aspiration cytology are available for identifying malignancy, this procedure is not appropriate to large populations. Conversely, molecular markers could be applied to large number of individuals and conceivably recognize individuals at risk for cancer among the vast majority of benign nodules.

Very little is known concerning the susceptibility factors for thyroid cancer. However, variations of thyroid cancer incidence in different geographic and ethnic groups suggest that exposition to distinct environmental factors may be very important in thyroid tumorigenesis process (Mack *et al.*, 2002; Memon *et al.*, 2002). For example, thyroid cancer is the second most common neoplasm among women in several countries in the Middle West, accounting for more than 8% of all cancers in Kuwait (Yu *et al.*, 2000). Polymorphisms of human drug-metabolizing enzymes influence individual susceptibility to chemical carcinogens and may help explain the variations of thyroid cancer incidence around the world (Strange and Fryer, 1999). The recent characterization of the *GSTO* class of genes allowed us to study their possible role in thyroid tumorigenesis. Since *GSTO1* variants present reduced enzyme activities and lower capacity to biotransform inorganic arsenic, genetic polymorphisms of the *GSTO* gene could have a role in thyroid tumorigenesis. Arsenic is

a notorious environmental carcinogen and contamination of drinking water with inorganic arsenic is a world-wide health problem. Methylation is considered as a principal pathway for detoxification of inorganic arsenic and a marked variation in methylation capacity, and, therefore, susceptibility to arsenic has been demonstrated among individuals (Hirakawa *et al.*, 2002). Unfortunately, our data do not support an important role for the *GSTO* gene or its variants in the risk for thyroid nodules or their pathologic and clinical characteristics.

We demonstrated that Brazilian population has a similar frequency of *GSTO1* variants ($f = 0.156$) as the one described in Australian ($f = 0.335$), Japanese ($f = 0.118$), Chinese ($f = 0.165$), Mexican ($f = 0.118$) and African ($f = 0.081$) populations (Cerda-Flores *et al.*, 2002; Whitbread *et al.*, 2003). Although we did not demonstrate the *GSTO1* genotype to have a role in thyroid tumours, it may influence susceptibility to other tumours. Data on the genotype distribution of *GST* genes in the Brazilian population may help to outline risk assessment and preventive actions in individuals exposed to environmental carcinogens.

References

Board PG, Coggan M, Chelvanayagam G, *et al.* (2000). Identification, characterization and crystal structure of the omega class of glutathione transferases. *J Biol Chem* **275**: 24798–24806.

Cerda-Flores RM, Budowle B, Jin L, *et al.* (2002). Maximum likelihood estimates of admixture in Northeastern Mexico using 13 short tandem repeat loci. *Am J Human Biol* **14**: 429–439.

Dulhunty A, Gage P, Curtis S, Chelvanayagam G, Board P (2001). The glutathione transferase structural family includes a nuclear chloride channel and a ryanodine receptor calcium release channel modulator. *J Biol Chem* **276**: 3319–3323.

Friedman GD, Ury HK (1983). Screening for possible drug carcinogenicity: second report of findings. *J Natl Cancer Inst* **71**: 1165–1175.

Granja F, Morari J, Morari EC, *et al.* (2004). GST profiling may be useful in the screening for thyroid nodule malignancy. *Cancer Letts* **209**: 129–137.

Haselkorn T, Stewart SL, Horn-Ross PL (2003). Why are thyroid cancer rates so high in Southeast Asian women living in the United States? The Bay Area thyroid cancer study. *Cancer Epidemiol Biomark Prev* **12**: 144–150.

Hayes JD, Strange RC (1995). Potential contribution of the glutathione S-transferase supergene family to resistance to oxidative stress. *Free Radic Res* **22**: 193–207.

Hirakawa M, Tanaka T, Hashimoto Y, *et al.* (2002). JSNP: a database of common gene variations in the Japanese population. *Nucleic Acids Res* **30**: 158–162.

Mack WJ, Preston-Martin S, Bernstein L, Qian D (2002). Lifestyle and other risk factors for thyroid cancer in Los Angeles County females. *Ann Epidemiol* **12**: 395–401.

Mannervik B (1985). The isozymes of glutathione S-transferase. *Adv Enzymol* **57**: 357–417.

McClain RM (1992). Thyroid gland neoplasia: non-genotoxic mechanisms. *Toxicol Lett* **64–**65: 397–408.

Memon A, Varghese A, Suresh A (2002). Benign thyroid disease and dietary factors in thyroid cancer: a case–control study in Kuwait. *Br J Cancer* **86**: 1745–1750.

Morari EC, Leite JLP, Granja F, Assumpção LVM, Ward LS (2002). The null genotype of glutathione S-transferase M1 and T1 locus increases the risk for thyroid cancer. *Cancer Epidemiol Biomarkers Prev* **11**: 1485–1488.

Ron E, Kleinerman RA, Boice Jr JD, *et al.* (1987). A population-based case–control study of thyroid cancer. *J Natl Cancer Inst* **79**: 1–12.

Schlumberger MJ (2000). Papillary and follicular thyroid carcinoma. *Baillière's Best Pract Res Clin Endo Metab* **14**: 601–613.

Strange RC, Fryer AA (1999). The glutathione S-transferases: influence of polymorphism on cancer susceptibility. In: Vineis P, Malatus N, Lang M (editors): *et al. Metabolic Polymorphisms and Susceptibility to Cancer.* Lyon: IARC Scientific Publication No. 148. IARC; pp. 231–249.

Tanaka-Kagawa T, Jinno H, Hasegawa T, *et al.* (2003). Functional characterization of two variant human GSTO1–1 s (Ala140Asp and Thr217Asn). *Biochem Biophys Res Commun* **301**: 516–520.

Whitbread AK, Tetlow N, Eyre HJ, Sutherland GR, Board PG (2003). Characterization of the human omega class glutathione transferase genes and associated polymorphisms. *Pharmacogenetics* **13**: 131–144.

Yin ZL, Dahlstrom JE, Le Couteur DG, Board PG (2001). Immunohistochemistry of omega class glutathione S-transferase in human tissues. *J Histochem Cytochem* **49**: 983–987.

Yu RC, Hsu KH, Chen CJ, Froines JR (2000). Arsenic methylation capacity and skin cancer. *Cancer Epidemiol Biomarkers Prev* **9**: 1259–1262.

AUTHOR QUERY FORM

LIPPINCOTT WILLIAMS AND WILKINS

.IOURNAL NAME **CE.I** **4/21/05**

ARTICLE NO: **2004-4073**

QUERIES AND / OR REMARKS

Query No	Details Required	Authors Response
AQ1	AQ1: “annealing temperature was 56<deg>C seconds” - deleter “seconds”?	

The Null Genotype of Glutathione S-Transferase *M1* and *T1* Locus Increases the Risk for Thyroid Cancer[1]

Elaine Cristina Morari, Janaína Luísa Pereira Leite, Fabiana Granja, Lígia Vera Montalli da Assumpção, and Laura Sterian Ward[2]

Laboratory of Cancer Molecular Genetics, Department of Medicine, State University of Campinas, 13085-857 Campinas, São Paulo, Brazil

Abstract

Susceptibility to chemical carcinogens plays an important role in the development of most cancers. Several polymorphisms of human drug-metabolizing enzymes influence this individual susceptibility. The genes that encode the isoenzymes of the glutathione s-transferase (GST) system present a polymorphic inheritance. The GST mu 1 (*GSTM1*) and GST theta 1 (*GSTT1*) genes have a null allele variant in which the entire gene is absent. The null genotype for both enzymes has been associated with many different types of tumors. To look for the influence of the inheritance pattern of these enzymes on thyroid cancer risk, we used a triplex PCR that included *β-globin* gene as a DNA quality control to compare 300 normal individuals of our population to 116 goiter patients. There were 49 cases of benign and 67 cases of malignant nodules: 50 papillary and 17 follicular carcinomas. Comparison between thyroid tumor specimens and normal corresponding samples of 35 cancer patients demonstrated identical patterns, suggesting that the GST system is not involved in the process of follicular dedifferentiation. There was no statistical difference between the prevalence of the deleted alleles in the normal individuals and in the goiter patients. However, papillary carcinoma patients (10%) and follicular carcinoma patients (17%) presented a higher prevalence of the null genotype than the normal population individuals (5%; $P < 0.05$). We found a 2.6 increased risk of thyroid cancer in individuals with the *GSTT1* and *GSTM1* combined null inheritance, suggesting that this genotype may be associated with an increased susceptibility to thyroid cancer.

Introduction

The majority of human tumors is considered to be a result of the interaction between environmental factors and personal genetic susceptibility (1, 2). However, people vary greatly in their likelihood of developing cancer in response to natural hazards. Individual differences in susceptibility to carcinogens play an essential role in the development of sporadic cancer. The biochemical basis for this susceptibility is related to genetic polymorphisms that normally occur in the general population regarding genes involved in predisposition to a specific cancer, in the metabolic activation or detoxification of environmental genotoxins, and in controlling DNA repair or cellular damage (3–5).

The etiology of thyroid cancer is markedly uncertain. Exposure to ionizing radiation, especially in childhood, remains the only factor clearly associated with benign and malignant thyroid tumors in humans (6). However, there is strong epidemiological evidence pointing toward the involvement of geographic, ethnic, and dietary factors in the risk of sporadic thyroid cancer (7). A variety of drugs, pesticides, goitrogenic xenobiotics, and chemicals have been shown to increase the incidence of thyroid tumors in rodents (8–10). However, chemicals have seldom been associated with human thyroid cancer, in contrast to lung, bladder, and many other cancers. No increase in the risk of human thyroid cancer has ever been consistently observed with any drug (6, 7, 11). On the contrary, a number of studies have reported a reduced risk of thyroid cancer in women who smoke cigarettes (12, 13).

The GST[3] system consists of a large multigenic group of detoxifying enzymes, the activity of which, catalyzing the conjugation of toxic and mutagenic compounds with glutathione, is essential for cell protection (14). Conversely, this important service may be disadvantageous during chemotherapy where GSTs have been associated with multidrug resistance of tumor cells (15, 16). At present, five classes of isoenzymes have been identified: alpha, mu, pi, sigma, and theta. The genes that encode the GST enzyme system are polymorphic in the general population (14–17). Both the *GSTM1* and *GSTT1* genes have a null variant allele in which the entire gene is absent. Persons with homozygous deletions of either the *GSTM1* or the *GSTT1* locus have no functional activity of the respective enzyme. Epidemiological studies suggest that individuals who are homozygous null have an increased risk of developing cancer at a number of sites like lung, bladder, colon, and breast (18).

The primary objective of this study was to test the hypothesis that individuals with an inherited homozygous deletion of the *GSTT1* and/or the *GSTM1* genes are at an increased risk of thyroid cancer. We conducted a prospective case control study in which we compared the proportion of *GSTT1* and *GSTM1* null genotypes between a group of patients with benign and malignant thyroid tumors and a control group. Heterogeneity of risk according to clinical and morphological subtypes of

Received 11/30/01; revised 6/3/02; accepted 6/14/02.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] Supported in part by Grant 99/03319-9 from Fundacão de Amparo à Pesquisa do Estado de São Paulo.
[2] To whom requests for reprints should be addressed, at Rua Olympia Pattaro 45, 13085-857 Campinas, São Paulo, Brazil. E-mail: Ward@unicamp.br.

[3] The abbreviations used are: GST, glutathione S-transferase; *GSTT1*, GST *T1* locus; *GSTM1*, GST *M1* locus; HC-FCM/UNICAMP, University Hospital–School of Medicine of the State University of Campinas; FC, follicular carcinoma; PC, papillary carcinoma.

Table 1 Distribution of thyroid carcinoma patients according to their histology, clinical features including age (X ± SD in years), gender (F, female; M, male), color (W, white; NW, non-white), the history of previous thyroid benign diseases, smoke habits, use of medicines, the presence of lymph node involvement and distant metastasis by the time of the diagnosis, and the diagnosis of recurrence and/or distant metastasis during the follow-up

Histology	Clinical characteristics								Diagnosis (presence of metastasis)		Follow-up
	Age	Sex		Color		Previous thyroid disease	Smokers	Use of medicines	Lymph node	Distant	Recurrence and/or distant metastasis
		M	F	W	NW						
PCs	42 ± 13	12	38	34	16	4	13	11	23	8	15
FCs	57 ± 17	7	10	10	7	1	3	4	3	9	9

thyroid tumors and their correspondent genotype was also explored.

Materials and Methods

Subjects. The study was approved by the Ethics Committee of the HC-FCM/UNICAMP, and informed written consent was obtained from a total of 416 individuals from our region, considered to have a normal iodine intake. Because of the highly heterogeneous ethnic composition of the Brazilian population, we included a large control group of 300 individuals (99 males and 201 females, 16–78 years old, 35 ± 23 years old) selected from the general population. To obtain a comparable control group with respect to gender proportion and the range of ages, we selected two to three women for every man who presented himself to donate blood, because thyroid cancer occurs more frequently in women than in men. Data on lifetime occupational history, smoking history, general health conditions, previous diseases, and other anamnestic data were obtained through interviews. Individuals with history of previous thyroid disease, exposure to radiation, and antecedents of malignancy were excluded. There were 252 healthy blood donors (78 males and 174 females, 18–60 years old, 31 ± 21 years old), who provided a representative group of the general population that seeks medical assistance in this region, and 48 volunteers (21 males and 27 females, 16–78 years old, 36 ± 25 years old) recruited among students and co-workers from the State University of Campinas. One hundred sixteen patients consecutively referred to the outpatient clinic of the University Hospital (HC-FCM/UNICAMP) for thyroid disease evaluation, who agreed to participate, were enrolled in the study. The study population included 49 cases of benign thyroid lesions [multinodular goiter (38 cases, 3 males and 35 females, 12–71 years old, 45 ± 15 years old) and follicular adenomas (11 cases, 2 males and 9 females, 22–77 years old, 43 ± 17 years old)] and 67 cases of thyroid tumors [50 papillary carcinomas (16–72 years old) and 17 follicular carcinomas (45–79 years old)]. Stage and grade of differentiation of the tumors were obtained from surgical and pathological records. Diagnoses were confirmed by experienced pathologists of the University Hospital (HC-FCM/UNICAMP). Patients were classified into whites and nonwhites. Clinical features of the patients with thyroid carcinomas are detailed in Table 1.

Data on general health conditions and medical history with emphasis on previous and/or current thyroid diseases were obtained through interviews. The use of drugs was also carefully assessed, in particular nutritional goitrogens, drugs that could interfere with thyroid function, as well as medicines for other concomitant diseases. Cigarette smoking habit was recorded but—because of the few 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 each pack) for 1 year in the last 5 years. None of the patients had a history of accidental or medical radiation exposure. All data, including pathological diagnoses, were confirmed in the patients' records. A peripheral blood sample was collected from all 116 patients. Also, thyroid tissue samples were obtained at surgery from 83 of these patients, snap frozen in liquid nitrogen immediately after surgery, and kept at –80°C until processed. We were able to obtain both cancer tissue samples and autologous blood specimens and/or normal thyroid tissue from the contralateral lobe in 35 cases of malignant tumors.

Patients were followed with periodic whole body scans, serum thyrotropin and thyroglobulin measurements according to a routine follow-up protocol that includes X-ray, ultrasonography, computer tomography scan, and other eventual procedures to detect distant metastasis for a period of 12–214 months (23 ± 58 months). Patients with high serum thyroglobulin levels (>2 mg/dl) and/or suspicious whole 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.

Methods. Genomic DNA was extracted from frozen specimens and from leukocytes separated from whole blood using a standard proteinase K-phenol-chloroform protocol. A tissue sample was collected from the central portion of the tumor to minimize the possibility of contamination of normal tissue.

A multiplex-PCR assay was used to simultaneously amplify the *GSTT1* and *GSTM1* genes. *β-Globin* gene coamplified as an internal positive control in a total volume of 50 μl containing 25 mM KCl; 10 mM Tris-HCl (pH 8.4); 1.5 mM $MgCl_2$; 0.1 mM each of dATP, dCTP, dGTP and dTTP; 500 ng of genomic DNA; and 2 units of Taq DNA polymerase recombinant (Life Technologies, Inc.). A fragment of 273 bp was obtained using 120 ng of each primer (sense and antisense) for *GSTM1* gene amplification, 150 ng of each primer for the *GSTT1* gene that amplified a fragment of 480 bp, and 95 ng of each primer for the *β-globin* gene that amplified a fragment of 630 bp (19). The reaction involved 35 cycles of incubation with denaturing at 94°C for 1 min, primer annealing at 62°C for 1 min, and extension at 72°C for 1 min. The PCR fragments were visualized into an ethidium bromide-stained 2% agarose gel as 273-bp and 480-bp products, corresponding to the normal presence of the allele of *GSTM1* and *GSTT1* genes, respectively. A 630-bp PCR fragment was obtained from the *β-globin* gene as shown in Fig. 1.

Statistical Analysis. The analysis was conducted using statistical software (Statistical Analysis System, version 8.1, 1999–2000; SAS Institute, Inc., Cary, NC). χ^2 or Fisher's exact tests were used to examine homogeneity between cases and controls

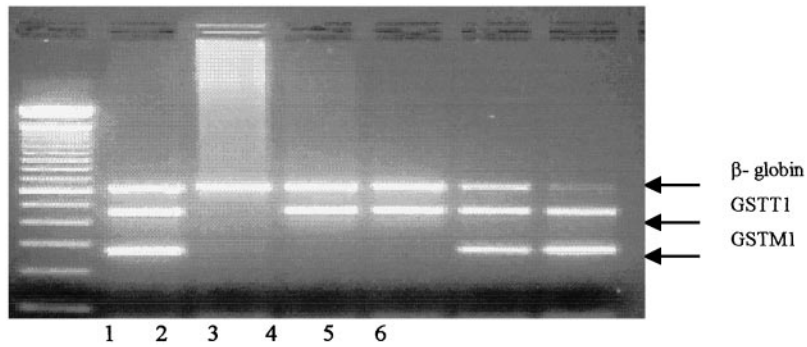


Fig. 1. Ethidium bromide-stained 2% agarose gel illustrating the multiplex PCR used for detection of null alleles of *GSTM1* and *GSTT1*. The products of 273 and 480 bp correspond to the normal presence of the allele for the *GSTM1* and *GSTT1* genes, respectively. The 630-bp PCR fragment corresponds to a *β-globin* gene fragment, including exon 3 and introns 2 and 3, that was used as a control for the DNA sample. *Lane 1*, DNA size marker ladder of 100 bp; *Lanes 2–4*, results from the amplification of DNA extracted from peripheral blood of PCs; *Lanes 5* and *6*, FCs.

Table 2 Comparison among the distribution of the different *GSTT1* and *GSTM1* genotypes in the normal population and the thyroid benign and malignant (papillary and follicular) patients

Genotype		Population		Benign goiter		Papillary		Follicular	
		n	%	*n*	%	*n*	%	*n*	%
GSTT1−	GSTM1−	15	5	4	8	5	10	3	18
GSTT1−	GSTM1+	52	17	6	12	5	10	4	24
GSTT1+	GSTM1−	111	37	20	41	20	40	5	29
GSTT1+	GSTM1+	122	41	19	39	20	40	5	29
Total		300		49		50		17	

regarding gender, color, previous thyroid disease, use of medicines, and cigarette smoking. Also, extent of disease was compared between PCs and FCs using Fisher's exact test. The Kruskal-Wallis test was used to compare age among groups. The Mann-Whitney or Wilcoxon tests were used to compare age among different genotype groups. The odds ratio and 95% confidence interval provide a measure of the strength of association (*e.g.,* indicating the increase in odds of a given benign or malignant thyroid tumor demonstrating a particular genotype compared with the control population). 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. Patients with FCs were older than the patients with PCs (Kruskal-Wallis, $P = 0.0012$). Nevertheless, there were no differences among the patients regarding color, gender distribution, smoking habit, the use of drugs, or preexisting benign thyroid diseases. The occurrence of lymph node involvement at the time of the diagnosis was more frequent among PC (39%) than FC (6%; χ^2, $P < 0.05$). However, these last tumors already presented long distance metastasis at the time of the diagnosis (53%) more frequently than PCs (16%; χ^2, $P < 0.02$). Follow-up data did not evidence differences between PC and FC concerning distant metastasis and/or recurrence of the tumor, although FC patients presented evidence of recurrence and/or metastasis in 50% of the cases against 30% of the PC cases (χ^2, $P = 0.13$). No patients died during the period of observation. Table 2 summarizes data of the overall proportions of the *GSTT1* and *GSTM1* genotypes in the control population and in the benign and malignant thyroid disease patients.

GSMT1 and *GSTT1* gene profiles proved to be exactly the same in the tumor and normal tissue samples, as well as in the corresponding peripheral blood samples in all tested samples.

The combined absence of both the *GSTT1* and *GSTM1* genes was more frequent in thyroid carcinomas (12%) than in the control population (5%; Fisher's exact test, $P < 0.05$) but did not distinguish benign from malignant tumors (Fisher's exact test, $P = 0.55$) or patients with benign thyroid tumors from the control population (Fisher's exact test, $P = 0.32$). There was no association between genotype and the patients' clinical features, tumor parameters of aggressiveness at diagnosis, or behavior during follow-up. Also, no relationship was found between the *GSTM1* and the *GSTT1* allele null genotypes and benign or malignant thyroid tumors. The combined *GSTT1* and *GSTM1* null genotype presented an odds ratio of 2.576 (95% confidence interval, 1.044–6.355) in thyroid cancer patients.

Discussion

The recognition of factors designed to identify people at risk of developing cancer is essential for good medical practice. Five to 10% of the population presents detectable nodules during their life span, mainly in iodine-deficient communities (6, 7, 20). However, most of these nodules will prove to be benign because thyroid cancer is responsible for only 0.6% to 1.6%, respectively, of all kinds of cancers that occur in men and women in the United States (6, 7). The environment has the principal role in causing sporadic cancer (2). Acting in concert with individual susceptibility, environmental factors such as smoking, diet, and pollutants play a role in most human cancers (1).

Variations of thyroid cancer incidence in different geographic and ethnic groups suggest that environmental factors may influence the thyroid tumorigenesis process, but available data regarding carcinogenic products are conflicting. Several chemicals produce thyroid neoplasia in rodents, generally acting through two basic mechanisms. The first involves a direct carcinogenic effect activating oncogenes, inactivating tumor suppressor genes, and producing specific alterations in the expression and function of genes involved in cell growth, differentiation, and life span. The second involves chemicals

that, through a variety of mechanisms, disrupt thyroid function and produce neoplasia secondary to hormone imbalance (21). However, there are important species-specific differences in thyroid gland physiology between humans and rodents. Broad screening of more than 200 drugs for carcinogenicity revealed that just two, griseofulvin and senna, were associated with increased risk of thyroid carcinoma in humans (22). Spironolactone and vitamin D, but not calcium supplements, were found to be significantly associated with thyroid cancer, mostly medullary carcinoma (23). Nutritional goitrogens intake, like vegetables containing cyanogenic glucosides (most forms of cabbage, cauliflower, broccoli, and other members of the cruciferous family), was not associated with increased risk of thyroid carcinoma in humans and may even exert a protective effect (23). There is no epidemiological evidence of increased risk of thyroid cancer in smokers. On the contrary, recent data suggest a protective effect of smoking, perhaps involving an effect on thyroid-stimulating hormone and estrogen metabolism (12, 13).

Individuals with a homozygous deletion of the *GSTT1* and *GSTM1* genes lack enzymatic conjugation of foreign compounds with glutathione. This results in diminished ability to detoxify many environmental carcinogens, including 1.3-butadiene, ethylene oxide, epoxybutanes, and monohalomethanes. Absence of GSTT1 and of GSTM1 activity in blood, corresponding to the *GSTT1* and *GSTM1* null genotypes, have been associated with carcinogen-induced and background chromosomal changes in some case-control studies in lung, bladder, and colon cancers, particularly mediating the risk of smoke-related cancers (24–26). We were not able to find any literature data regarding the role of detoxifying enzymes in thyroid tumors. Therefore, we carried out a study of benign and malignant thyroid lesions involving the *GSTT1* and *GSTM1* genes. We studied a large control group that presented a genotype profile similar to that previously described in our population, confirming its high heterogeneity (27, 28). Our data on thyroid nodules indicate a high prevalence of the combined null genotype in malignant lesions in FC (17%) and PC (10%), significantly higher than in the control population ($P < 0.05$). We were not able to find any association between clinical features, histology, parameters of aggressiveness at diagnosis or during follow-up, and the genotype. Moreover, genotypes of tumor and normal autologous cells were always identical, indicating that *GST* gene inheritance does not play any role in the follicular cell transformation process. However, an estimated 2.6-fold greater risk of malignant thyroid nodules was observed in individuals with combined null genotypes. These data suggest that individuals with *GSTT1* and *GSTM1* combined null inheritance may be genetically predisposed for an increased risk of developing thyroid cancer.

Acknowledgments

This study would not have been possible without the cooperation of Dr. Alfio José Tincani and colleagues from the Department of Surgery, HC-FCM/UNICAMP, in particular Drs. José Geraldo dos Santos, Carlos Frazatto, Jr., and Gustavo Magaldi. We also thank Luís Francisco Cintra Baccaro for valuable suggestions regarding statistical evaluation and clinical data.

References

1. Perera, F. P. Molecular epidemiology: insights into cancer susceptibility, risk assessment, and prevention. J. Natl. Cancer Inst., *88:* 496–509, 1996.

2. Lichtenstein, P., Holm, N. V., Verkasalo, P. K., Iliadou, A., Kaprio, J., Koskenvuo, M., Pukkala, E., Skytthe, A., and Hemminki, K. Environmental and heritable factors in the causation of cancer. N. Engl. J. Med., *343:* 78–85, 2000.

3. Palli, D., Vineis, P., Russo, A., Berrino, F., Krogh, V., Masala, G., Munnia, A., Pânico, S., Taioli, S., Taioli, E., Tumino, R., Garte, S., and Peluso, M. Diet, metabolic polymorphisms and DNA adducts: the EPIC-Italy cross-sectional study. Int. J. Cancer, *87:* 444–451, 2000.

4. Autrup, H. Genetic polymorphisms in human xenobiotic metabolizing enzymes as susceptibility factors in toxic response. Mutat. Res., *464:* 65–76, 2000.

5. Nebert, D. W. Drug-metabolizing enzymes, polymorphisms and interindividual response to environmental toxicants. Clin. Chem. Lab. Med., *38:* 857–861, 2000.

6. Schlumberger, M. J., and Torlantano, M. Papillary and follicular thyroid carcinoma. Baillieres Best Pract. Res. Clin. Endocrinol. Metab., *14:* 601–613, 2000.

7. Schlumberger, M., and Pacini, F. Nodular thyroid diseases. In: M. Schlumberger and F. Pacini (eds.), Thyroid Tumors, pp. 13–30. Paris: Editions Nucléon, 1999.

8. Capen, C. C. Mechanistic data and risk assessment of selected toxic end points of the thyroid gland. Toxicol. Pathol., *25:* 39–48, 1997.

9. Hard, G. C. Recent developments in the investigation of thyroid regulation and thyroid carcinogenesis. Environ. Health Perspect., *106:* 427–436, 1998.

10. Hurley, P. M. Mode of carcinogenic action of pesticides inducing thyroid follicular cell tumors in rodents. Environ. Health Perspect., *106:* 437–445, 1998.

11. Hallquist, A., Hardell, L., Degerman, A., and Boquist, L. Thyroid cancer: reproductive factors, previous diseases, drug intake, family history and diet. A case-control study. Eur. J. Cancer Prev., *3:* 481–488, 1994.

12. Kreiger, N., and Parkes, R. Cigarette smoking and the risk of thyroid cancer. Eur. J. Cancer, *36:* 1969–1973, 2000.

13. Galanti, M. R., Hansson, L., Lund, E., Bergstrom, R., Grimelius, L., Stalsberg, H., Carlsen, E., Baron, J. A., Persson, I., and Ekbom, A. Reproductive history and cigarette smoking as risk factors for thyroid cancer in women: a population-based case-control study. Cancer Epidemiol. Biomark. Prev., *5:* 425–431, 1996.

14. Mannervik, B. The isozymes of glutathione S-transferase. Adv. Enzymol., *57:* 357–417, 1985.

15. Chasseaud, L. F. The role of glutathione and glutathione S-transferases in the metabolism of chemical carcinogens and other electrophilic agents. Adv. Cancer Res., *29:* 175–274, 1979.

16. Hayes, J. D., and Pulford, D. J. The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. Crit. Rev. Biochem. Mol. Biol., *30:* 445–600, 1995.

17. Seidegard, J., Vorachek, W. R., Pero, R. W., and Pearson, W. R. Hereditary differences in the expression of human glutathione transferase active on trans-stilbene oxide are due to a gene deletion. Proc. Natl. Acad. Sci. USA, *85:* 7293–7297, 1988.

18. Vineis, P. Diet, genetic susceptibility and carcinogenesis. Public Health Nutr., *4:* 485–491, 2001.

19. Comstock, K. E., Sanderson, B. J., Claflin, G., and Henner, W. D. GST1 gene deletion determined by polymerase chain reaction. Nucleic Acids Res., *18:* 3670, 1990.

20. Aghini-Lombardi, F., Antonangeli, L., Martino, E., Vitti, P., Maccherini, D., Leoli, F., Rago, T., Grasso, L., Valeriano, R., Balestrieri, A., and Pinchera, A. The spectrum of thyroid disorders in an iodine-deficient community: the Pescopagano survey. J. Clin. Endocrinol. Metab., *84:* 561–566, 1999.

21. McClain, R. M. Thyroid gland neoplasia: non-genotoxic mechanisms. Toxicol. Lett., *64–65:* 397–408, 1992.

22. Friedman, G. D., and Ury, H. K. Screening for possible drug carcinogenicity: second report of findings. J. Natl. Cancer Inst., *71:* 1165–1175, 1983.

23. Ron, E., Kleinerman, R. A., Boice, J. D., Jr., LiVolsi, V. A., Flannery, J. T., and Fraumeni, J. F., Jr. A population-based case-control study of thyroid cancer. J. Natl. Cancer Inst., *79:* 1–12, 1987.

24. Ford, J. G., Li, Y., O'Sullivan, M. M., Demopoulos, R., Garte, S., Taioli, E., and Brandt-Rauf, P. W. Glutathione S-transferase M1 polymorphism and lung cancer risk in African-Americans. Carcinogenesis (Lond.), *21:* 1971–1975, 2000.

25. Setiawan, V. W., Zhang, Z. F., Yu, G. P., Li, Y. L., Lu, M. L., Tsai, C. J., Cordova, D., Wang, M. R., Guo, C. H., Yu, S. Z., and Kurtz, R. C. *GSTT1* and *GSTM1* null genotypes and the risk of gastric cancer: a case-control study in a Chinese population. Cancer Epidemiol. Biomark. Prev., *9:* 73–80, 2000.

26. Hong, Y. J., Lee, J. K., Lee, G. H., and Hong, S. I. Influence of glutathione S-transferase M1 and T1 genotypes on larynx cancer risk among Korean smokers. Clin. Chem. Lab. Med., *38:* 917–919, 2000.

27. Arruda, V. R., Grignolli, C. E., Gonçalves, M. S., Soares, M. C., Menezes, R., Saad, S. T., and Costa, F. F. Prevalence of homozygosity for the deleted alleles of glutathione S-transferase mu (GSTM1) and theta (GSTT1) among distinct ethnic groups from Brazil: relevance to environmental carcinogenesis? Clin. Genet., *54:* 210–214, 1998.

28. Gattas, G. J., and Soares-Vieira, J. A. Cytochrome P450–2E1 and glutathione S-transferase mu polymorphisms among Caucasians and mulattos from Brazil. Occup. Med. (Lond.), *50:* 508–511, 2000.

Lack of mutation in exon 10 of *p53* gene in thyroid tumors

Patricia Lia Santarosa[a], Fabiana Granja[a], Elaine Cristina Moraria, Janaína Luisa Leite[a], Ligia Vera Montalli da Assumpção, Laura S Ward.

Ausencia de mutaciones del exón 10 del gen p53 en tumores tiroideos

***Antecedentes**: p53 es una proteína nuclear que tiene un rol importante en la regulación de la proliferación celular y comanda cascadas de señalización para la reparación de ADN y apoptosis. En muchos tipos de cáncer, hay una alta frecuencia de mutaciones de p53. Estas mutaciones también son muy prevalentes en el cáncer indiferenciado de tiroides, pero no se encuentran en tumores benignos y son infrecuentes en el cáncer bien diferenciado. La mayor parte de las mutaciones se localizan en los exones 5 a 8 del gen. Recientemente se ha descrito una mutación de la línea germinal del exón 10 en el codón 337 del p53, en niños brasileños con tumores suprarrenales.* **Objetivo**: Buscar mutaciones del codón 337, del exón 10 de p53 en tumores tiroideos.* **Material y métodos**: *Se estudiaron 74 tumores tiroideos (5 carcinomas foliculares incluyendo 3 altamente invasivos, 22 carcinomas papilares incluyendo 6 variantes con células altas, 11 adenomas foliculares, 1 carcinoma medular y 35 bocios benignos). El ADN se extrajo de una sección central de los tumores y desde tejidos tiroideo normal contralateral o sangre en 38 pacientes. Los productos de PCR para el exón 10 de p53 fueron examinados por análisis de conformación de polimorfismos de hebra simple. Se secuenciaron 2 muestras en que se sospechó la presencia de bandas con migración aberrante y 3 productos de PCR adicionales provenientes de muestras de tumor con patrones normales de polimorfismo, pero no se detectaron mutaciones.* **Resultados**: *En todas las muestras estudiadas, no se detectaron mutaciones.* **Conclusiones**: *El exón de p53 no presenta mutaciones en los tumores tiroideos. Esto sugiere que esta mutación es específica para tumores suprarrenales. (Rev Méd Chile 2004; 132: 1513-6)*

Recibido el 11 de mayo, 2004. Aceptado en versión corregida el 25 de octubre, 2004.
Laboratory of Cancer Molecular Genetics, Department of Medicine, Faculty of Medical Sciences, State University of Campinas (FCM/UNICAMP), Campinas, São Paulo, Brazil.
[a]Biologist. Postgraduate doctoral student.

The tumor suppressor gene *p53* is a transcription factor that acts in cell cycle regulation, inducing cell cycle arrest or cell death in response to DNA-damaging agents, such as viral infection, radiation and chemotherapeutics[1]. The *p53* protein resides primarily in the nucleus, binds to specific DNA sequences, and functions at least in part as a transcriptional regulator[2]. Inactivated *p53* mutations have been described in some 50% of human cancers and are believed to be a major determinant of the phenotype of many forms of cancer[1-3].

Address for correspondence: Laura S Ward. Olympio Pattaro 45, 13085-045 Campinas, São Paulo, Brazil. F/Fax: 55-19-3788.7878 or 3289.4107. E-mail: ward@unicamp.br

Several studies, both with immunocytochemical and genetic analyses, have shown that *p53* mutations are highly prevalent in poorly differentiated and undifferentiated thyroid carcinomas, as well as thyroid cancer cell lines[4-6]. However, they 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[7]. These data suggest that mutational inactivation of the *p53* gene may be a key event in the progression from differentiated to anaplastic carcinoma[4,7]. There is also evidence that *p53* may interfere with thyroid cell differentiation. Introduction of a mutated *p53* markedly impairs the differen-

tiated gene expression of PCC13 thyroid cells[8]. By contrast, wild-type *p53* reintroduction into an undifferentiated thyroid carcinoma cell line leads to reexpression of thyroid peroxidase, a characteristic differentiated marker of the thyroid cell[9].

Typically, mutations in *p53* gene are located in exons 5-8, a highly conserved DNA binding domain of *p53*. Recently, a distinct nucleotide substitution in the exon 10 of *p53* was identified at a high frequency, 77 to 97% of children with benign and malignant adrenocortical sporadic tumors investigated by 2 distinct groups[10,11]. This germline mutation leading to an Arg337His mutation of exon 10 was also identified in asymptomatic relatives of the patients but in none of the unrelated controls, suggesting that the mutation is a risk factor associated with adrenocortical tumors rather than a benign polymorphism commonly found in southern Brazil[10,11].

Sporadic tumors often appear to have the same gene mutations as their familial counterparts. Many germline mutations have been demonstrated to be associated with sporadic tumors, including thyroid cancer[12-16]. We recently showed that a polymorphism at codon 72 of exon 4 of *p53* was associated with sporadic thyroid carcinomas[17].

Because of the high prevalence of the codon 337 of exon 10 of *p53* mutation in southern Brazilian population and the possibility that this polymorphism could be also associated to other cancers, we designed this study to screen a large amount of samples for this *p53* mutation in thyroid tumors.

Material and methods

Subjects. The Ethics Committee of the University Hospital - School of Medicine of the State University of Campinas (HC-FCM/UNICAMP) approved the study and informed written consent was obtained from a total of 74 subjects (55 females, 19 males, 16 to 81 years old, 49±21 years old) that were consecutively referred to thyroid surgery because of thyroid nodules that presented clinical or epidemiological suspicion of cancer. The diagnosis of thyroid carcinoma was established by fine-needle aspiration cytological study and confirmed by the histological analysis of thyroid tissues. There were 28 thyroid malignant tumors: 5 follicular carcinomas (3 widely invasive and 2 minimally invasive); 22 papillary carcinomas (14 of the classic variant, 2 follicular variants, 6 tall cell variants) and 1 medullary carcinoma. Other 46 cases (35 females, 11 males, 21 to 75 years old, 47±19 years old) of benign goitres included 19 follicular adenomas, 22 multinodular goitres and 5 Basedow-Graves disease. Thyroid tissue samples were obtained at the time of surgery at the University Hospital and immediately frozen in liquid N2. Besides collecting a central portion of all tumors, we obtained samples from the contra lateral normal thyroid lobe of 26 patients with thyroid cancer. In addition, peripheral blood samples were collected from 18 different patients with benign goitres. Tumor stage and degree of differentiation were obtained from surgical and pathological records. Experienced pathologists of the University Hospital of the Faculty of Medical Sciences of the State University of Campinas (UNICAMP) confirmed all diagnoses.

Methods. Genomic DNA was extracted from frozen tumors using a standard phenol-chloroform method. We used the same primers described by Latronico et al[10]. PCR was performed in 25 µl volumes of a mixture containing 100 ng DNA, 50 nM of each primer (5'-CTGAGGCACAAGAATCAC-3' and 5'-TCCTATGGCTTTCCAACC-3'), 10 mM Tris- HCl (pH 8.0), 1.5 mM MgCl2, 100 uM of each dinucleotide triphosphate and 0.5 U Taq DNA polymerase. Amplifications were carried out for 35 cycles of 94°C for 45 seconds, 62°C for 45 seconds 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 Perkin-Elmer 9600 GeneAMp PCR system. The amplified 447 bp DNA fragments were examined on a 2% agarose gel, containing ethidium bromide. After confirming amplification, the samples were mixed with 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol and 50 mM NaOH, denatured at 94°C for 10 min, and loaded on to 6% polyacrylamide gels. The electrophoresis was conducted at 2-5 W at room temperature overnight. The gel was then stained with silver nitrate. DNA samples homo and heterozygous for the Arg337His mutation, obtained from adrenocortical tumors, were used as positive controls of the gels.

Results

Figure 1 depicts an example of our results. All samples showed the same pattern of running, with no significant differences. Two samples suspected of presenting aberrant migrating bands were exci-

sed from the gel and purified using a commercial kit according to the manufacturer's instructions (Life Technologies, Paisley, UK). PCR products were sequenced with the ABI prism big dye sequencing kit (Perkin Elmer, Warrington, Cheshire, UK) using an ABI 377 Prism DNA Sequencer (Perkin Elmer). In all cases a wild-type sequence was found. In addition, we directly sequenced 3 additional PCR products from tumor samples with normal SSCP patterns, and all were wild type.

Discussion

The *p53* gene is one of the best studied tumor suppressor genes, located on chromosome 17p13.1. Its mutation has been reported mainly in aggressive forms of tumors, especially anaplastic carcinomas[18]. It has been found in up to 40% of dedifferentiated and undifferentiated thyroid carcinomas and in less than 10% of the differentiated thyroid tumors[19]. However, mutant *p53* protein has also been detected in follicular and papillary carcinomas[20]. More recently, *p53* mutant protein was also demonstrated in 11 out of 66 nodular hyperplasia cases (16.7%) and in 7 out of 50 (14%) cases of follicular adenomas[21].

Although somatic mutations of *p53* are the most common genetic changes observed to date, the frequency of germline *p53* mutations is found to be very low in sporadic malignant tumors[4]. It has been postulated that *de novo* germline *p53* mutations may occur in a substantial population of patients in the pediatric age group, who die of their disease and do not propagate the mutation[22,23]. On the other hand, recent reports suggest that germline *p53* splicing mutations have been described infrequently in the literature because the method of mutation detection, in many studies, does not include all splice junctions24. The low figures reported in the literature might also reflect the use of less-sensitive mutation detection methods and, certainly, the fact that most researches focused on exons 5-8, within the DNA-binding domain of *p53*, instead of screening all 11 exons of T*P53*[24]. Indeed, because 85% of *p53* mutations are expected to occur in exons 5 through 8, thyroid tumor screening efforts, in almost all reports, were restricted to these regions of the gene (http://www.iarc.fr/*p53*/; http://cancergenetics.org/*p53*.htm).

The spectrum and frequency of cancers associated with germline *p53* mutations are uncertain. Some cancers like breast carcinoma, soft tissue sarcomas, osteosarcoma, brain tumors, adrenocortical carcinoma, Wilms' tumor and phyllodes tumor are strongly associated with germline *p53* mutations while carcinoma of pancreas is moderately associated and leukaemia and neuroblastoma are weakly associated[25].

Screening exon 10 by PCR-SSCP and by direct sequencing, we did not find mutations in a large number of thyroid samples. These results support the concept that germline T*P53* mutations do not simply increase general cancer risk. Instead, they promote tissue-specific effects. Although our results are constrained by the fact that we did not screen poorly differentiated or undifferentiated tumors, they suggest that the Arg337His germline mutation described in Brazilian children is restricted to adrenocortical tumors.

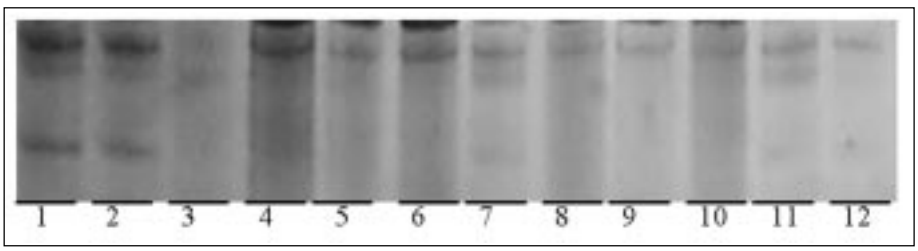


Figure 1. Gel of single-stranded conformation polymorphism analysis of PCR products (PCR-SSCP) representative of our results for exon 10 of p53 gene screening for mutations. Lanes 1 and 2 were loaded with the positive controls for the homo- and the heterozygous Arg337His mutation of exon 10 of the p53 gene, respectively. Lanes 3-7 and 8-12 were loaded with PCR products from follicular and papillary carcinomas, respectively.

References

1. Levine AJ. *p53*, the cellular gatekeeper for growth and division. *Cell* 1997; 88: 323-31.
2. Mendoza-Rodríguez CA, Cerbon MA. Tumor suppressor gene *p53*: mechanisms of action in cell proliferation and death. *Rev Invest Cl* 2001; 53: 266-73.
3. Goodsell DS. The molecular perspective: *p53* tumor suppressor. *Oncologist* 1999; 4: 138-9.
4. Farid NR. *p53* mutations in thyroid carcinoma: tidings from an old foe. *J Endocrinol Invest* 2001; 24: 536-45.
5. Shahedian B, Shi Y, Zou M, Farid NR. Thyroid carcinoma is characterized by genomic instability: evidence from *p53* mutations. *Mol Gen Metab* 2001; 72: 155-63.
6. Smida J, Zitzelsberger H, Kellerer AM, Lehmann L, Minkus G, Negele T et al. *p53* mutations in childhood thyroid tumors from Belarus and in thyroid tumors without radiation history. *Int J Cancer* 1997; 73: 802-7.
7. Ward LS, Fagin JA. Molecular genetics of thyroid cancer: evidence that inactivation of tumor suppressor genes occurs at late stages of tumor progression. *Molecular and Cellular Pediatric Endocrinology*. New Jersey: editor Handwerger S, 1998; 201-11.
8. Battista S, Martelli ML, Fedele M, Chiappetta G, Trapasso F, De Vita G et al. A mutated *p53* alters differentiation of thyroid cells. *Oncogene* 1995; 72: 2029-37.
9. Fagin JA, Tang S-H, Zeki K, Di Lauro R, Fusco A, Gonsky R. Reexpression of thyroid peroxidase in a derivative of an undifferentiated thyroid carcinoma cell line by introduction of wild-type *p53*. *Cancer Res* 1996; 56: 765-71.
10. Latronico AC, Pinto EM, Domenice S, Fragoso MC, Martin RM, Zerbini MC et al. An inherited mutation outside the highly conserved DNA-binding domain of the *p53* tumor suppressor protein in children and adults with sporadic adrenocortical tumors. *J Clin Endocr Metab* 2001; 86: 4970-3.
11. Ribeiro RC, Sandrini F, Figueiredo B, Zambetti GP, Michalkiewicz E, Lafferty AR et al. An inherited *p53* mutation that contributes in a tissue-specific manner to pediatric adrenal cortical carcinoma. *P Nat Acad Sci USA* 2001; 98: 9330-5.
12. Oliveira C, Ferreira P, Nabais S, Campos L, Ferreira A, Cirnes L et al. E-Cadherin (CDH1) and *p53* rather than SMAD4 and Caspase-10 germline mutations contribute to genetic predisposition in Portuguese gastric cancer patients. *Eur J Cancer* 2004; 40: 1897-903.
13. Lim W, Olschwang S, Keller JJ, Westerman AM, Menko FH, Boardman L et al. Relative frequency and morphology of cancers in STK11 mutation carriers. *Gastroenterology* 2004; 126: 1788-94.
14. Chang BL, Zheng SL, Isaacs SD, Wiley KE, Turner A, Li G et al. A polymorphism in the CDKN1B gene is associated with increased risk of hereditary prostate cancer. *Cancer Res* 2004; 64: 1997-9.
15. Harach HR. Familial adenomatous polyposis associated with sporadic MEN 1 and thyroid carcinoma related to APC mutation. *Am J Surg Pathol* 2003; 27: 412-3.
16. Eng C. The role of PTEN, a phosphatase gene, in inherited and sporadic nonmedullary thyroid tumors. *Recent Prog Horm Res* 1999; 54: 441-52.
17. Granja F, Morari J, Morari EC, Correia LA, Assumpção LVM, Ward LS. Proline homozygozity in codon 72 of *p53* is a factor of susceptibility for thyroid cancer. *Cancer Letters* 2004; 210: 151-7.
18. Fagin JA. Molecular pathogenesis of follicular ephitelium. In: Braverman LE, Utiger RD, editors. *Werner's and Ingbar's The Thyroid*. 8th ed. Boston: Lippincott Williams & Wilkins, 2000; 886-98.
19. Goretzki PE, Dotzenrath C, Simon D, Roher H-D. Studies of oncogenes and tumor suppressor genes in human thyroid carcinomas, and their clinical implications. *Langenbecks Arch Surg* 1999; 384: 1-8.
20. Hosal SA, Apel RL, Freeman JL, Azadian A, Rosen IB, Livolsi VA et al. Immunohistochemical localization of *p53* in human thyroid neoplasms: correlation with biologic behavior. *Endocr Pathol* 1997; 8: 21-8.
21. Omar E, Madhavan M, Othman NH. Immunohistochemical localization of RET and *p53* mutant protein of thyroid lesions in a North-Eastern Malaysian population and its prognostic implications. *Pathology* 2004; 36: 152-9.
22. Saed GM, Ladin D, Olson J, Han X, Hou Z, Fivenson D. Analysis of *p53* gene mutations in keloids using polymerase chain reaction-based single-strand conformational polymorphism and DNA sequencing. *Arch Dermatol* 1998; 134: 963-7.
23. Birch JM, Alston RD, McNally RJ, Evans DG, Kelsey AM, Harris M et al. Relative frequency and morphology of cancers in carriers of germline T*P53* mutations. *Oncogene* 2001; 20: 4621-8.
24. Varley JM, Attwooll C, White G, McGown G, Thorncroft M, Kelsey AM et al. Characterization of germline T*P53* splicing mutations and their genetic and functional analysis. *Oncogene* 2001; 20: 2647-54.
25. Frebourg T. Germline mutations of the *p53* gene. *Pathol Biol* (Paris) 1997; 45: 845-51.

Acknowledgment.
We thank Dr. Berenice B Mendonça from the Department of Medicine - Endocrinology, USP - São Paulo, for kindly providing us with the adrenal samples used as positive controls in this research.