Original Article

Lack of Association of *GSTT1, GSTM1, GSTO1, GSTP1* and *CYP1A1* Polymorphisms for Susceptibility and Outcome in Brazilian Prostate Cancer Patients

(benign prostatic hyperplasia / genetic inheritance / outcome / prostate cancer / risk factors)

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Abstract. The polymorphic inheritance of human drug-metabolizing enzymes, such as those encoded by the *GST* and *CYP* systems, has been implicated in both cancer risk and prognostic. In an effort to increase our understanding of the interaction between potential environmental exposure, lifestyle, and genetic factors in the predisposition and response to radiotherapy of prostate cancer patients, we examined *GSTT1, GSTM1, GSTO1, GSTP1* and *CYP1A1* genotypes in a Brazilian population. We studied 125 prostate cancer patients and 100 benign prostatic hyperplasia patients paired for ethnic and lifestyle characteristics. Lifetime occupational history, dietary patterns, cigarette-smoking, and other anamnestic data were obtained through interviews. Outcome was evaluated in 42 stage ≤ T2a patients presenting a Gleason score ≤ 6, PSA ≤ 10 ng/ml, treated with radiotherapy and followed up for 12 to 34 months (15 ± 8 months). None of the studied polymorphisms was found associated to prostate cancer risk either considered separately or in combination, in uni- or multivariate regression logistic analysis. Also, there was no association between genotypes and possible clinical factors of risk or any parameter of tumour aggressiveness at diagnosis or during follow-up. Patients' response to radiotherapy treatment was not associated to any genotype. In conclusion, our data suggest that *GST* and *CYP1A1* genotypes are not associated with the susceptibility to prostate cancer or its outcome in the Brazilian population.

Introduction

Similarly as observed worldwide, the incidence of prostate cancer varies substantially among different regions in Brazil, suggesting that other factors are involved in the pathogenesis of prostate cancer. Epidemiologic studies have pointed to a series of environmental and lifestyle factors, including pollutants, smoking and diet, as well as geographical and racial factors as possible contributors to the risk of prostate tumours (Instituto Nacional do Cancer, 2007; Fleshner et al., 2007).

Individual differences in the susceptibility to carcinogens play an essential role in the development of sporadic cancer. The biochemical basis for the genetic susceptibility to environmental hazards is related to genetic polymorphisms that normally occur in the general population, and involves a series of genes implicated in the metabolic activation or detoxification of environmental genotoxins. Several polymorphic genes encoding enzymes involved in the biotransformation of carcinogens have been studied as possible prostate cancer risk modifiers, including the phase II glutathione S-transferase (*GST*) system, and phase I cytochrome P450 (*CYP)* genes (Reszka et al., 2002; Keshava et al., 2004).

GSTM1 and *GSTT1* genes are polymorphic in humans, and the phenotypic absence of enzyme activity is due to the absence of a homozygous and inherited gene (Seidegard et al., 1998). GSTM1 and GSTT1 detoxify the reactive metabolites of cigarette smoke-derived

Received March 11, 2008. Accepted June 10, 2008.

The study was supported by Fundação de Apoio à Pesquisa do Estado de São Paulo- FAPESP, grant 06/01651-1, and Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq, grant 470317/2006-0.

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Abbreviations: CYP – cytochrome P450, GST – glutathione S transferase, PSA – prostate-specific antigen.

chemicals such as benzo[a]pyrene and other polycyclic aromatic hydrocarbons (Guengerich et al., 1995). GSTP1 is the most important detoxification enzyme, in a quantitative sense, in prostate tissues (Ali-Osman et al., 1997). A polymorphic site at nucleotide 313 causes a substitution of Ile by Val in the GSTP1 protein and modifies the enzyme's specific activity and affinity for electrophilic substrates (Ali-Osman et al., 1997). More recently, a newly characterized gene, *GSTO*, has been related to the ability of cells containing ryanodine receptors to resist apoptosis (Dulhunty et al., 2001). GSTO1 is abundant in the prostate and its activity is believed to be required for normal cellular processes (Board et al., 2000). A polymorphism involving a C-A transition at nucleotide position 419 in exon 4 of *GSTO1* that might be associated to a lower activity of the variant enzyme in a substrate-dependent manner may help explain the variation between individuals in their susceptibility to oxidative stress and inorganic arsenic (Tanaka-Kagawa et al., 2003). The role of *GSTO1* polymorphism in the risk of prostate cancer has not yet been investigated.

CYP phase I enzymes catalyse the activation process of chemical carcinogens to electrophilic reactive forms and are involved in the oxidative metabolism of oestrogens, which has been suggested to play a critical role in the aetiology of prostate cancer (Williams et al., 2000). *CYP1A1* polymorphisms and, also, their combination with the *GSTM1* genotype, have been reported associated with prostate cancer susceptibility (Williams et al., 2000; Murata et al., 2001; Chang et al., 2003; Aktas et al., 2004).

In addition, because GSTs and CYPs are important in the resistance to a number of drugs and are fundamental in the oxidative stress produced by therapeutic irradiation, they might influence the response to prostate cancer treatment. The primary objective of this prospective case control study was to investigate the combined effect of *GSTT1, GSTM1, GSTP1, GSTO1* and *CYP1A1* genes in prostate cancer risk, and their association to other clinical, epidemiological and pathological markers of susceptibility. We also aimed to evaluate a possible utility of polymorphism genotyping of these genes in the prediction of prostate cancer patient's response to radiotherapy and outcome.

Material and Methods

Subjects

The study was approved by the Ethics Committee of the Irmandade de Misericordia Hospital and the Medical Sciences School of the State University of Campinas (FCM/UNICAMP), and informed written consent was obtained from all individuals. The study population included 100 benign prostatic hyperplasia and 125 prostate cancer patients consecutively referred for prostate disease evaluation. Patients who had a first-degree relative (brother or father) with a confirmed diagnosis of prostate cancer were excluded in order to avoid familial prostate cancer cases. All 225 patients were submitted to standard 12-punctures at the parasagittal midline of the prostate (sextant punctures); right lobe: base, mid region, and apex; and left lobe: base, mid region, and apex; lateral punctures equidistant between the margin and the parasagittal midline. Each specimen was separately submitted to histological study. The clinical stage was obtained from the results of the digital rectal examination using the 2002 American Joint Committee on Cancer staging system. The Gleason score was determined by histological examination. Data on general health conditions and medical history with emphasis on previous and/or current prostate diseases were obtained through interviews, using a structured questionnaire administered by the same interviewer. This questionnaire included a lifestyle inquiry on occupational and professional history; dietary habits including red meat, fish, vegetables and fat intake; alcohol and drug consumption. 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 one year during the last five years. Skin colour was determined by the interviewer in accordance with the Brazilian Institute of Geography and Brazilian Institute of Geography and Statistics, but, because of the difficulty in classifying our highly heterogeneous population, we further grouped individuals into whites and non-whites.

Treatment and Follow-up

Forty-eight patients were primarily submitted to radical prostatectomy, 42 patients to radiotherapy, 18 to orchiectomy, six to hormonal therapy, one to cryosurgery, and 10 to watchful waiting. Cancer patients were followed up for three to 64 months (15 ± 12 months) with periodic serum PSA measurements according to a routine protocol that includes thoracic X-ray, bone scans, and computed tomography of the abdomen and pelvis as clinically indicated at three- to six-month intervals. Patients with two or more high serum PSA levels (> 0.2 ng/ml) after radical prostatectomy were submitted to a thorough image search.

In order to evaluate the response to therapy and survival, we focused on the more homogeneous group of 42 patients stage ≤ T2a presenting a Gleason score ≤ 6, PSA ≤ 10 ng/ml, primarily treated with radiotherapy and followed up for 12 to 34 months (15 ± 8 months). The outcome was defined as favourable if the patient did not present any clinical sign of disease or PSA elevation. Recurrence was defined as serum PSA levels ≥ 2 ng/ml or ≥ 4 ng/ml above the nadir and/or two consecutive increases in the serum PSA levels ≥ 0.5 ng/ml after radiotherapy and/or the evidence of disease at any image method. Six patients died because of their prostate cancer during the follow-up, including two patients primarily treated with radiotherapy.

Polymorphism analysis

Genomic DNA was extracted from leukocytes of all 225 patients using a standard proteinase K-phenol-chloroform protocol. A multiplex-polymerase chain reaction (PCR) assay was used to simultaneously amplify the *GSTT1*, *GSTM1* and *β-globin* gene as described previously (Morari et al., 2002). *GSTP1* was studied using a PCR-restriction fragment length polymorphism analysis 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 µM of each deoxyribonucleotide triphosphate, 2.0 mM $MgCl_2$ and 0.5 U Taq DNA polymerase. Amplifications were carried out for 35 cycles of 94° C for 45 s, annealing temperatures 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. Thirteen µl of each PCR product were subsequently submitted to Alw26I overnight digestion. The digests were electrophoresed in 3% agarose gels stained with ethidium bromide and photographed under ultraviolet light. *GSTO1* variants (Ala/Asp and Asp/Asp) were studied using a PCR-single strand conformation polymorphism analysis (SSCP)-sequencing approach previously described (Granja et al., 2005). Twenty-five samples suspected of presenting aberrant migrating bands were directly sequenced using the ABI prism big dye sequencing kit (Perkin Elmer, Warrington, Cheshire, UK) and the ABI 377 Prism DNA Sequencer (Perkin Elmer). The polymorphism of *CYP1A1 m1* (6235T>C) was demonstrated using the following primers: 5'-CAG TGA AGA GGT GTA GCC GCT-3' and 5'-TAG GAG TCT TGT CTC ATG CCT -3' under the same conditions described above for *GSTP1* amplification and *Msp*I enzyme digestion as described previously (Bufalo et al., 2006). Positive and negative control samples were included in all PCR, SSCP runs and products digested with the restriction enzymes 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 (SAS Institute Inc, Cary, NC, 2000). Power of calculation was performed using PS program version 2.1.31. Chi-square (χ^2) or Fisher's (F) exact tests were used to examine the homogeneity between benign prostatic hyperplasia and prostate cancer regarding colour, cigarette smoking, vegetables, red meat and fat intake, exercising and alcoholic habit. Mann-Whitney (MW) or Wilcoxon tests were used to compare age, Gleason score and PSA levels among different genotype groups. Deviations from Hardy-Weinberg equilibrium were considered using χ^2 statistics. Logistic regression was used to evaluate the effect of genotypes, after adjusting for other potential confounders such as age, colour, diet, tobacco, and alcohol consumption. Comparison between overall survival rates were calculated using the Kaplan-Meier method, and the Cox proportional hazard regression model for multivariate analysis was used to determine whether any of the genetic, clinical or treatment variables predicted for disease-free state or survival. 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 patients. There were no differences between the benign prostatic hyperplasia and the prostate cancer patients regarding age (64 ± 11 years versus 69 ± 8 years), colour (82% white and 18% non-white versus 80% white and 20% non-white individuals), and cigarette smoking habits (62% ever smokers and 38% never smokers versus 60% ever smokers and 40% never smokers). The demographic and lifestyle characteristics of the subjects from both groups, including alcohol consumption, red meat, vegetables and fat intake, education, and exercise were similar. However, prostate cancer patients (89%) were more frequently older than 60 years than benign prostatic hyperplasia patients (31%) (χ^2 = 14.84; P < 0 .001). As expected, prostate cancer patients presented PSA levels more elevated (30 ± 74) than benign prostatic hyperplasia patients (3 ± 3) (MW;

Table 1. Distribution of patients according to their histology, serum PSA levels, Gleason score, prostatectomy T stage, the diagnosis of locally advanced or distant metastasis by the time of the first staging evaluation, and the diagnosis of recurrence and/or distant metastasis and/or death during the follow-up

	Diagnosis									**Follow-up**
	PSA			**Gleason score**		**Prostatectomy T stage**		**Locally advanced**	**Distant**	
	≤4	**4-10**	**>10**	**2-6**	**7-10**	**T1-T2**	**T3-T4**			**Recurrence and/or distant metastasis**
Adenocarcinoma (7 deaths)	14	52	59	72	45	94	18	27	1	34
BPH	60	22	4							

Table 2. Distribution of the different studied genotypes in patients with prostate cancer (PCa = 125 individuals) and benign prostatic hyperplasia (BPH = 100 individuals)

		PCa		BBH		
Gene	**Genotype**	**N**	**%**	**N**	**%**	**p value**
GSTP1	Ile 105 Val					
	Ile/Ile	65	52.0	55	55.0	0.6880
	Ile/Val	38	30.4	33	33.0	
	Val/Val	22	17.6	12	12.0	
GSTO1	Ala140Asp					
	Ala/Ala	116	92.8	92	92.0	1.0000
	Ala/Asp	6	4.8	4	4.0	
	Asp/Asp	3	2.4	4	4.0	
GSTT1						
	Present	83	66.4	78	78.0	0.0739
	Null	42	33.6	22	22.0	
GSTM1						
	Present	56	44.8	47	47.0	0.7883
	Null	69	55.2	53	53.0	
GSTT1/GSTM1						
	Present/present	37	29.6	34	34.0	0.0993
	Present /null	47	37.6	40	43.0	
	Null /present	20	16.0	14	14.0	
	Null /null	21	16.8	9	9.0	
CYP1A1 M1	6235T>C					
	T/T	94	75.2	69	69.0	0.3678
	T/C	26	20.8	27	27.0	
	C/C	5	4.00	4	4.0	
GSTM1/CYP1A1 M1						
	Present/(T/T)	45	36.0	32	32.0	0.8954
	Present/(T/C)	12	9.6	14	14.0	
	Present/(C/C)	3	2. 4	2	2.0	
	Null/(T/T)	47	37.6	37	37.0	
	Null/(T/C)	15	12.0	14	14.0	
	Null/(C/C)	3	2.4	1	1.0	

$P < 0.001$). The majority of cases (83 %) were diagnosed with localized stage disease and had moderate grade tumours (Gleason score 2–6 = 62 % of the cases).

Table 2 shows the overall distribution of genotypes in both benign prostatic hyperplasia and prostate cancer patients and the major statistical comparisons between groups. The power of calculation was 13 %, 22 %, 60 %, 73 % and 86 % for *GSTO1, CYP1A1, GSTP1, GSTM1* and *GSTP1* polymorphisms, respectively. There was no association between *GSTT1* and *GSTM1* null genotypes, either considered individual ly or in combination, and the risk of prostate cancer. Also, there was no difference between *GSTP1*, *GSTO1* and *CYP1A1* variants (T/C or C/C) incidence in the benign prostatic hyperplasia and the prostate cancer patients. Univariate regression logistic analysis indicated that age over 60 years ($P < 0.001$) and total PSA levels ($P < 0.001$) were the only factors related to the susceptibility to prostate cancer.

Because both the GSTM1 and CYP1A1 enzymes act in the detoxification process of polycyclic aromatic hydrocarbons, such as benzo[a]pyrene found in cigarette smoke, we examined whether the risk of prostate cancer varied by *GSTM1* and *CYP1A1* genotypes and smoking. As demonstrated in Table 3, there was no significant interaction between *GSTM1* or *CYP1A1* genotypes and smoking. We were also unable to find any interaction between red meat, fat, and vegetable intake and the risk of cancer.

Also, we examined whether prostate cancer risk varied by the *GST* or *CYP* genotypes and age at diagnosis, measures of prostate cancer aggressiveness such as total PSA level, Gleason score and stage at diagnosis. We defined more aggressive prostate cancer cases as regional/distant stage T3 and T4 or Gleason score 7–10. We were unable to demonstrate any relationship between genotypes and parameters of aggressiveness at diagnosis or during the follow-up. Also, there was no relationship between the response to radiotherapy or the outcome of this group of patients and any genotype.

There were no interactions between any of the *GST* genotype combinations or *GST* and *CYP* combinations.

Discussion

A series of polymorphisms in germ-line DNA have been investigated in an effort to delineate polygenic

Table 3. Comparison among the distribution of the different studied genotypes in patients with prostate cancer who consumed at least 20 packages of cigarettes for one year during the last five years (ever smokers) and patients who never smoked (never smokers). One patient was excluded because of the lack of reliable information on his smoking habits

Gene	Genotype	Ever smokers N	Ever smokers %	Never smokers N	Never smokers %		P value
GSTP1	Ile/Ile	36	46.7	25	53.1	$\chi^2 = 0.2607$	0.609
	Ile/Val or Val/Val	41	53.2	22	46.8		
GSTO1	Ala/Ala	70	90.9	43	91.4	$\chi^2 = 5.658$	0.9940
	Ala/Asp or Asp/Asp	8	10.3	4	8.5		
GSTT1	Present	46	59.7	35	74.4	$\chi^2 = 2.182$	0.1396
	Null	31	40.2	12	25.5		
GSTM1	Present	39	50.6	22	46.8	$\chi^2 = 0.05286$	0.8182
	Null	38	49.3	25	53.1		
CYP 1A1 M1	TT	55	71.4	37	78.7	$\chi^2 = 0.4749$	0.4907
	T/C or C/C	22	28.5	10	21.2		
GSTT1/ GSTM1	Present/present	25	32.4	17	36.1	$\chi^2 = 2.062$	0.1510
	Present/null	25	32.4	20	42.5		
	Null/present	10	12.9	5	10.6		
	Null/null	17	22.0	5	10.6		
CYP 1A1 M1+ GSTM1	Null+ Allelic Variants	12	15.5	7	14.8	$\chi^2 = 0.01073$	0.9175
	Others	65	84.4	40	85.1		

models of cancer susceptibility and prognosis. Such models are particularly interesting since they may help select men for specific chemopreventive interventions and determine which men with localized prostate cancer are most likely to benefit from specific therapies. Unfortunately, these studies have produced inconsistent results mostly derived from small samples, but also from population stratification secondary to ethnic diversity, lack of exposure conditions reports, heterogeneity of the therapeutic measures employed, and consideration limited to only one rather than combinations of polymorphisms. In addition, an important limitation in case-control studies is the fact that some population controls may have undetected prostate cancer that could mislead the interpretation of the observed associations.

A recently published case-control study performed in a large cohort of prostate cancer patients (N = 752 cases) showed a modest increase in the risk of prostate cancer in subjects with homozygous deletion of the *GSTM* gene (Agalliu et al., 2006). Unfortunately, controls in this study were selected only on the basis of self-reported history of prostate cancer and were not screened either by digital rectal examination or by PSA level (Agalliu et al., 2006). This is an important issue: the selection of an adequate control group is essential in this type of study given the high prevalence of prostate cancer. The use of 12-punctures biopsy in all patients is the strength of our study since this is an effective screening method for low-risk prostate cancer patients (Slongo et al., 2003). Concurring with our results, a recently published meta-analysis of *GSTM1, GSTT1* and *GSTP1* genotyping concluded that these polymorphisms are unlikely to be major determinants of susceptibility to prostate cancer (Ntais et al., 2005).

There is very limited and controversial information on the role of the *CYP1A1* gene in prostate cancer risk despite the multiple functions of this gene (Williams et al., 2000; Murata et al., 2001; Chang et al., 2003; Aktas et al., 2004). An increase in the risk for prostate cancer was reported in a study of 115 prostate cancer patients and 200 benign prostatic hyperplasia Japanese controls (Murata et al., 2001). In addition, the authors found the combination of *CYP1A1* polymorphisms with the *GSTM1* null type to increase the risk for prostate cancer (Murata et al., 2001). Both GSTs and CYPs are implicated in the metabolism of polycyclic aromatic hydrocarbons derived from cigarette smoking and also from the dietary consumption of red meat cooked at high temperatures. However, similarly as ours, most studies indicate that smoking does not exert an important role in prostate cancer occurrence or outcome (Merrick et al., 2004).

We were unable to demonstrate any significant association between any of the genetic polymorphisms studied, either considered separately or in combination, in uni- or multivariate regression logistic analysis, and tumour parameters of aggressiveness at diagnosis or behaviour during the follow-up. Also, an interesting gene that has not been previously investigated, *GSTO1,* was not found associated to prostate cancer risk or behaviour.

The strong points of the present report are the detailed epidemiological information regarding the studied populations, the uniform mechanism of prostate cancer di-

Table 4. Comparison between the genetic profiles of 125 prostate cancer patients classified as Gleason score 2 to 6 (N = 77 patients) and Gleason score 7 to 10 (N = 48 patients)

		GSTM1		**Test; P value**
		Null	**Present**	
Gleason 2–6	(%)	58	41.9	
Gleason 7–10	(%)	38.8	61.1	χ^2; 0.134
		GSTT1		
		Null	**Present**	
Gleason 2–6	(%)	31.5	68.4	
Gleason 7–10	(%)	33.3	66.7	χ^2; 0.880
		GSTP1		
		Ile/Ile	**Ile/Val or Val/Val**	
Gleason 2–6	(%)	53.3	46.6	
Gleason 7–10	(%)	61.1	38.8	χ^2; 0.545
		GSTO1		
		Ala/Ala	**Ala/Asp or Asp/Asp**	
Gleason 2–6	(%)	94.5	5.4	
Gleason 7–10	(%)	77.7	22.2	F; 0.038
		CYP1A1		
		T/T	**T/C or C/C**	
Gleason 2–6	(%)	73.9	26	
Gleason 7–10	(%)	77.7	22.2	F; 0.999
		GSTM1 + CYP1A1		
		***GSTM1* Null/ *CYP1A1* T/C or C/C**	**Other combinations**	
Gleason 2–6	(%)	84.7	15.2	
Gleason 7–10	(%)	88.8	11.1	F; 0.999
		GSTT1 + GSTM1		

		***GSTT1* Null/ *GSTM1* Null**	***GSTT1* Present/ *GSTM* Null**	***GSTT1* Null/ *GSTM1* Present**	***GSTT1* Present/ *GSTM1* Present**	
Gleason 2–6	(%)	16.3	41.3	15.2	27.1	
Gleason 7–10	(%)	11.1	27.7	22.2	38.8	F; 0.553

χ^2 = χ^2 test
F = Fisher's exact test

agnosis for all cases, the combinational laboratory approach (i.e. five genes) and the thorough experimental and statistical analysis. Also, the highly heterogeneous composition of the Brazilian population might help dilute the ethnic bias. Unfortunately, very little is known about the exposure to the most important carcinogenic compounds, and genetic susceptibility is certainly affected by the exposure level. Moreover, most of the effects of polymorphisms of low-penetrance genes require several hundreds or thousands patients to identify. Hence, further studies with more subjects and meta-analysis of the published data are needed to confirm our results.

Acknowledgements

We thank the statistical team of the School of Medical Sciences FCM/UNICAMP for useful discussion and important suggestions.

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