

Short Communication

GSTM1, GSTT1, and GSTP1 Polymorphisms and Risk of Advanced Colorectal Adenoma

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Abstract

Cigarette smoking is a risk factor for colon adenoma. The glutathione S-transferase enzymes are involved in the detoxification of carcinogenic compounds including those found in tobacco smoke, and thus, may be important modifiers of individual risk of developing this disease. We examined the prevalence of *GSTM1* and *GSTT1* gene deletions, and two *GSTP1* polymorphisms in 772 cases with advanced colorectal adenomas (>1 cm, villous elements or high-grade dysplasia) of the distal colon (descending or sigmoid colon or rectum) and 777 sigmoidoscopy negative controls enrolled in the screening arm of the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial. Epidemiologic data on smoking was collected by self-administered questionnaire and DNA was extracted from whole blood or buffy coat. For *GSTM1* and *GSTT1*, we used a newly developed TaqMan-based assay capable of discriminating heterozygous (+/-) individuals from those with two active alleles (++) and homozygous deletions (--). For *GSTP1*, the I105V and the A114V substitutions were identified using end point 5' nuclease assays (TaqMan). Adjusted odds ratios

(OR) and 95% confidence intervals (95% CI) were determined using unconditional logistic regression, controlling for age, race, and gender. Advanced adenoma risk was increased in current/former smokers (OR, 1.4; 95% CI, 1.2-1.8). Risks were decreased in subjects with ≥ 1 inactive *GSTM1* alleles (OR, 0.6; 95% CI, 0.4-0.9); and the association was independent of smoking status (P interaction = 0.59). Having ≥ 1 inactive *GSTT1* allele was associated with increased risk among smokers (OR, 1.4; 95% CI, 1.1-1.9; $P_{\text{trend}} = 0.02$) but not among never smokers (OR, 0.9; 95% CI, 0.6-1.3) and a significant interaction between smoking and genotype was observed (P interaction = 0.05). In summary, this is the first study to report associations between colorectal adenomas and *GSTM1* wild-type and *GSTT1* null allele among smokers. These findings only became apparent using a newly developed assay able to distinguish heterozygous from wild-type individuals. Our data provide evidence that phenotypic differences between these two groups exist. (Cancer Epidemiol Biomarkers Prev 2005; 14(7):1823-7)

Introduction

Colorectal adenoma is a recognized precursor of colorectal cancer based on epidemiologic, histologic, and genetic studies demonstrating shared genetic alterations (1, 2). Tobacco smoking is an established risk factor for colorectal adenoma and tobacco smoking. Tobacco smoke is a rich source of polycyclic aromatic hydrocarbons (PAH), aromatic amines, *N*-nitroso compounds and other carcinogens (3, 4). The colon is also exposed to PAHs and heterocyclic amines through consumption of foods cooked at high temperatures, another potential risk factor for colorectal adenoma (5, 6). Activated PAHs and *N*-nitroso compounds are substrates for both *GSTM1* and *GSTT1* enzymes and are able to induce their expression (7). Expression is also induced by dietary consumption of isothiocyanates found in cruciferous vegetables (8).

The *GSTM1* gene detoxifies hydrophobic electrophiles such as PAH-derived epoxides found in cigarette smoke (9), whereas *GSTT1* catalyzes the conjugation of glutathione with substrates such as halogenated alkanes and epoxides (10, 11). The *GSTP1*

enzyme selectively detoxifies the carcinogenic epoxide of benzo(*a*)pyrene, a highly carcinogenic metabolite of PAHs (10).

Polymorphic variants of both genes were previously identified by the presence or absence of a PCR fragment using gel electrophoresis, a method which groups individuals having two functional alleles (++) and heterozygotes with one active and one inactive allele (+/-) as one group. In this study, a novel quantitative PCR-based method able to discriminate subjects carrying 0, 1, or 2 active alleles was used. Two single nucleotide polymorphisms in the *GSTP1* gene were also analyzed, one at codon 105 (Ile→Val) and the other at codon 114 (Ala→Val).

In this study, we investigated advanced colorectal adenoma risk in relation to polymorphic variants in the *GSTM1*, *GSTT1*, and *GSTP1* genes among participants randomized to the screening arm of the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial. We hypothesized that variation in risk associated with glutathione S-transferase polymorphisms would primarily be observed among smokers.

Materials and Methods

The Prostate, Lung, Colorectal, and Ovarian Cancer Trial. This trial, which is being carried out by the National Cancer Institute, has randomized 77,465 screening arm participants (38,350 men and 39,115 women) and an equal number of nonscreened controls, ages 55 to 74, at 10 screening centers throughout the U.S. (12, 13).

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Study Population. Cases and controls for this study were drawn from the screening arm participants at the 10 screening centers of the Prostate, Lung, Colorectal, and Ovarian Cancer Trial who filled out a risk factor questionnaire, had a successful sigmoidoscopy (insertion to at least 50 cm with >90% of mucosa visible or a suspect lesion identified), and provided a blood sample for use in etiologic studies (September 1993-September 1999, applied conditions described; $n = 42,037$; ref 14). Of these participants, we excluded 4,834 with a self-reported history of ulcerative colitis, Crohn's disease, familial polyposis, colorectal polyps, Gardner's syndrome, or cancer (except basal cell and squamous cell skin cancer). We randomly selected 772 of 1,234 cases with at least one advanced colorectal adenoma (adenoma ≥ 1 cm or containing high-grade dysplasia or villous, including tubulovillous elements) in the distal colon (descending colon and sigmoid or rectum), and 777 or 26,651 control participants with a negative sigmoidoscopy screening (i.e., no polyp or other suspect lesion), frequency-matched to the cases by gender and ethnicity (non-Hispanic White, non-Hispanic Black, Hispanic, and others). Study subjects were predominantly non-Hispanic Whites (94%). Among the 772 cases, 572 (74%) had a lesion ≥ 1 cm, 489 (63%) showed advanced histologic features, and 245 (32%) had multiple adenoma. Also, 631 (82%) cases had an advanced adenoma of the descending colon or sigmoid and 232 (30%) had an advanced adenoma of the rectum, including subjects having lesions at both sites. Questionnaire data and blood collection were previously reported (15). For risk evaluation related to recent smoking, smokers were categorized as long-term quitters (quit ≥ 10 years before enrollment) and current or recent smokers (quit < 10 years before enrollment). Because risks associated with polymorphic variants were similar for both former and current and recent smokers, they were combined as ever smokers to increase power in this group. Dietary nutrient intake was calculated by multiplying the reported frequency of consumption for relevant food items by gender- and nutrient-specific portion sizes (16), using the nutrient database from the U.S. Department of Agriculture (17). Dietary intake including intake of benzo(a)pyrene, heterocyclic amines [PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine), and MeIQx (2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline)] were estimated using data from the CHARRED database (<http://charred.cancer.gov>).

GSTM1, GSTT1, and GSTP1 Genotype Assays. A new method was developed at the National Cancer Institutes Core Genotyping Facility to distinguish heterozygotes from subjects with two active *GSTM1* and *T1* alleles. The method used TaqMan real-time PCR technology with two 5' nuclease probes and four PCR primers (<http://snp500cancer.nci.nih.gov/assays>). Because *GSTT1* and *GSTM1* are members of a multigene family, we chose to compare the members of the same class to ensure a gene-specific assay design. All sequences used in assay design were obtained from Genbank: *GSTM1*, NM_000561; *GSTM2*, NM_000848; *GSTM3*, NM_000849; *GSTM5*, NM_000851, and *GSTT1*, NM_000853; *GSTT2*, NM_000854f. Gene-specific primers and probes (<http://snp500cancer.nci.nih.gov/assays>) were selected for specificity to one gene target and subsequently checked using Primer3 software (18).

Assays were independently done using 10 μ g of lyophilized genomic DNA in a 5 μ L TaqMan reaction. Reactions were done in a 384-well plate (96 \times 4) format. Three controls with known gene copy numbers (Coriell DNA controls) as well as no-template controls were analyzed alongside samples. We designed two assay sets for the *GSTM1* genotype assay and two assay sets for the *GSTT1* genotype assay. Each assay set contains two PCR primers and one dual labeled probe. The control assay sets used a human-specific genomic region in IVS 10 of the *BRCA1* gene as a two-copy gene control. The test assay sets were designed to hybridize within a commonly deleted area of the inactive genetic variant making it possible to quantify the active allele copy number by the rate of ampli-

fication. The *GSTM1* and *GSTT1* real-time assays were conducted using 2.5 μ L of the 2 \times Universal Master Mix (ABI, Foster City, CA). Specific primers and probes used in the reaction are presented on the National Cancer Institute's Core Genome Facility web site (<http://snp500cancer.nci.nih.gov/assays>). Thermocycling conditions were: 50°C for 2 minutes, 95°C for 10 minutes, followed by 49 cycles 95°C for 30 seconds, and 60°C for 1 minute. Real-time fluorescence was monitored during PCR amplification and results analyzed using methods similar to Covault et al. (19). Assay validation, optimization, and genotype prevalence was conducted using Coriell samples from 102 individuals of four ethnicities. This information is available on the National Cancer Institute's SNP500 database web page at <http://snp500cancer.nci.nih.gov/> (20).

Two single nucleotide polymorphisms at the *GSTP1* locus were genotyped using the end point 5' nuclease TaqMan assay. Specific primers, probes, and methods used to detect polymorphisms at codons 105 and 114 are also publicly available at <http://snp500cancer.nci.nih.gov/> (20). All TaqMan output was processed electronically for downloading into analytical programs.

Coriell DNA samples containing the homozygous major and minor allele and heterozygotes served as internal laboratory quality controls for each polymorphism studied. Four of each control type and four no-template controls were included per plate. External blinded samples from 40 individuals were interspersed throughout analyses. The blinded samples showed 100% interassay concordance for all assays.

Genotype data were successfully obtained for 89% to 91% of study subjects, excluding those with insufficient DNA (~6%), genotyping failures (~3-5%), and those whose fingerprint profiles showed subject-specific ambiguities (<1%).

Statistical Analysis. Adenoma risk was estimated by calculating odds ratios (OR) and 95% confidence intervals (CI) using unconditional logistic regression (Stata 8.1, College Station, TX). Analyses were adjusted for gender, race (non-Hispanic White, non-Hispanic Black, others), age at entry (55-59, 60-64, 65-69, 70-74), and when not used as a stratifying factor, smoking status (never, ever smoker). Adjustments for other suspected confounders, including education, history of colorectal cancer in a first-degree relative, body mass index, and dietary red meat intake were not included in the model because they did not alter the OR by 10% and/or did not have a *P* value ≤ 0.1 in the multivariate model. Associations between broccoli consumption was analyzed by dividing cases and controls above and below the median intake of broccoli (g/d), and also by quartiles (Q1, 0-3.59; Q2, >3.59-9.06; Q3, >9.06-15.60; Q4, >15.60). Subjects who reported never eating broccoli were also used as a comparison group. Dietary analyses included adjustment for exercise and fiber intake in addition to factors listed above (age, sex category, and race). Tests for interaction were conducted using a likelihood ratio test.

Based on a novel extension of polytomous logistic regression for multivariate outcome analysis (21), we studied whether the prevalence of glutathione S-transferase alleles varied for three characteristics of adenomas: size (≥ 1 versus < 1 cm), multiplicity (multiple versus single), and advanced histologic features (high-grade dysplasia or villous structure versus absence of these features), estimating case-case ORs for each characteristic after controlling for the other two characteristics. All *P* values were two-sided. Individuals with missing values were excluded from specific analyses.

Results

Distributions between cases and controls were essentially the same for matching factors, gender, and race. However, cases tended to be older, more likely to report a first-degree family history of colorectal cancer, have less education, and a higher body mass index at study entry (Table 1).

Table 1. Description of subjects enrolled in the nested case-control study of colorectal adenoma within the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial

Characteristics		Controls (n = 777)		P χ^2
		n (%)	Cases (n = 772) n (%)	
Sex	male	536 (69.0)	535 (69.3)	0.9
	female	241 (31.0)	237 (30.7)	
Age at interview	55-59	363 (46.7)	257 (33.3)	<0.0001
	60-64	200 (25.7)	244 (31.6)	
	65-69	140 (18.0)	172 (22.3)	
	70-74	74 (9.5)	99 (12.8)	
Race	White	729 (93.8)	725 (93.9)	1.0
	Black	23 (3.0)	22 (2.9)	
	others*	25 (3.2)	25 (3.2)	
First-degree family member with history of colorectal cancer	no	707 (91.0)	675 (87.4)	0.02
	yes	70 (9.0)	97 (12.6)	
Level of education (y)	≤11	50 (6.4)	72 (9.3)	0.001
	12 or high school	176 (22.7)	191 (24.8)	
	some college [†]	247 (31.8)	276 (35.8)	
	college and above	303 (39.1)	232 (30.1)	
Body mass index at interview	<18.5	2 (0.3)	5 (0.7)	0.3
	≥18.5 to <25	219 (28.2)	200 (25.9)	
	≥25 to <30	357 (46.0)	349 (45.2)	
	≥30	188 (24.2)	215 (27.9)	
Pathologic characteristics of adenoma(s) Size (cm)	<1 [‡]	—	200 (25.9)	—
	≥1 [‡]	—	572 (74.1)	
Number	1	—	527 (68.3)	—
	>1	—	245 (31.7)	
Histology	nonadvanced	—	283 (36.7)	—
	advanced	—	489 (63.3)	

*Hispanic (0.9%), Asian (1.7%), Pacific Islander (0.4%), and American Indian native (0.3%).

[†]Post-high school and some college education.

[‡]Adenoma size <1 cm, with high-grade dysplasia or villous elements.

[§]Two hundred and eighty-nine subjects (50.5%) with adenoma size ≥1 cm, with high-grade dysplasia or villous elements.

^{||}Adenomas with high-grade dysplasia or villous elements.

Cigarette smoking was associated with advanced adenoma, with greater risks for current smokers and recent smokers who quit <10 years ago (OR, 2.4; 95% CI, 1.8-3.1) than for former smokers who quit >10 years ago (OR, 1.1; 95% CI, 0.9-1.4; $P_{\text{trend}} < 0.001$; Table 2).

Also in Table 2, genotype distributions are presented for cases and controls. A lower risk of colorectal adenoma was observed among those carrying ≥1 null *GSTM1* allele (−/− and +/−) when compared with individuals carrying two active alleles (+/+) (OR, 0.6; 95% CI, 0.4-0.9). Lower relative risks were not observed when heterozygotes were grouped with those carrying two active alleles as would have been observed by PCR fragment analysis (OR, 0.9; 95% CI, 0.8-1.2, data not shown). Neither the *GSTT1* nor *GSTP1* variants were associated with adenoma risk. By multivariate disease characteristic analysis (21), only the *GSTP1* GG genotype at codon 105 was associated with large adenomas (>1 cm) compared with small adenomas ($P_{\text{trend}} < 0.006$); no differentials were noted for any other genotype with tumor multiplicity or histologic characteristics (data not shown).

Having ≥1 *GSTM1* null allele was protective regardless of smoking status (Table 3). Ever smokers having ≥1 null *GSTT1* allele had an increased risk of adenoma ($P_{\text{trend}} = 0.02$); however, tests for gene-environment interactions were only significant when +/− and −/− individuals were combined ($P_{\text{interaction}} = 0.05$).

We also examined adenoma risk by *GSTM1* and *GSTT1* genotype and broccoli intake (<, ≥median) but the findings were difficult to interpret because a consistent association between broccoli intake and adenoma risk was not observed (data not shown). A protective effect of high consumption of broccoli was only observed among *GSTM1* (+/+) individuals (OR, 0.5; 95% CI, 0.2-1.2) compared with *GSTM1* (+/+) individuals with low consumption of broccoli (<9.1g/d). For

the *GSTT1* gene, individuals with ≥1 null allele continued to be at lower risk regardless of broccoli intake. Increased risks were only associated with the *GSTT1* (−/−) polymorphism in the high broccoli consumption group (OR, 1.7; 95% CI, 1.1-2.7) compared with *GSTT1* (+/+) in the low broccoli consumption group. The same results were obtained when individuals who never consumed broccoli were used as a comparison group. Risks associated with genetic variants were not modified by dietary red meat or estimated dietary benzo(a)pyrene, PhIP, or MeIQx intake when all subjects or only subjects who had never smoked were considered (data not shown).

Discussion

This investigation of glutathione S-transferase polymorphisms and advanced adenoma risk revealed reduced risks in carriers of ≥1 *GSTM1* null allele, however, this association was not related to tobacco use or other factors examined. If this result were seen only among subjects who consumed higher amounts of broccoli, this finding could have been explained by isothiocyanate-induced protection, however, this was not what we observed. These findings remain puzzling, as they could have been caused by small numbers in the homozygous active (+/+) group, and should be confirmed. Excess risks associated with carriage of ≥1 *GSTT1* null allele were only observed among smokers. Both of these findings would not have been observed by the method of PCR fragment analysis on gel electrophoresis. The two single nucleotide polymorphisms associated with amino acid substitutions at codons 105 and 114 of the *GSTP1* gene did not contribute to overall adenoma risk in this study, however, the GG genotype at codon 105 was significantly associated with adenoma size.

GSTM1 and *GSTT1* have been widely studied in relation to colorectal cancer because of their high expression in the

Table 2. Risk of left-sided advanced colorectal adenoma, by smoking history, broccoli intake, and genotype

	Controls (n = 777)		Cases (n = 772)		OR (95% CI)*
	n (%)	n (%)	n (%)	n (%)	
Smoking status					
Never	315 (40.8)		260 (33.8)		1.0
Former, quit ≥10 y	302 (39.2)		272 (35.4)		1.1 (0.9-1.4)
Current and recent quitters (<10 y)	111 (14.4)		198 (25.8)		2.4 (1.8-3.1)
<i>P</i> _{trend} *†					<0.001
Ever	419 (57.1)		473 (64.5)		1.4 (1.2-1.8)
Cigar/pipe	43 (5.6)		39 (5.1)		1.2 (0.7-1.9)
<i>GSTM1</i>					
+/+	37 (5.4)		62 (9.4)		1.0
+/-	276 (40.1)		249 (37.6)		0.6 (0.4-0.9)
-/-	376 (54.6)		352 (53.1)		0.6 (0.4-0.9)
+/- and -/- combined	652 (94.7)		601 (90.7)		0.6 (0.4-0.9)
<i>GSTT1</i>					
+/+	241 (34.3)		221 (32.0)		1.0
+/-	343 (48.9)		340 (49.3)		1.1 (0.9-1.4)
-/-	118 (16.8)		129 (18.7)		1.2 (0.9-1.7)
+/- and -/-	461 (65.7)		469 (68.0)		1.1 (0.9-1.4)
<i>GSTP1-I105V</i>					
AA	317 (45.4)		282 (41.4)		1.0
AG	293 (42.0)		314 (46.1)		1.2 (1.0-1.5)
GG	88 (12.6)		85 (12.5)		1.1 (0.8-1.6)
<i>P</i> _{trend}					0.2
<i>GSTP1-A114V</i>					
CC	596 (83.5)		591 (84.4)		1.0
CT	114 (16.0)		103 (14.7)		0.9 (0.7-1.2)
TT	4 (0.6)		6 (0.9)		1.6 (0.4-5.7)
<i>P</i> _{trend}					0.8

*Adjusted for age, race, and gender.

†Smoking pipe and cigar and former smokers combined were not included in the trends estimation.

gastrointestinal tract and their role in detoxification of food- and tobacco-derived carcinogens. Studies have not generally found significant associations (22-25). We observed associations for *GSTM1* and *GSTT1* with adenoma risk only when +/- and +/+ individuals were analyzed separately. Our data provide supportive evidence that phenotypic differences between +/+ and +/- individuals exist. As in previous studies, our findings would have been negative had subjects with ≥1 active alleles been combined.

Although genotype-phenotype concordances have been shown with the PCR fragment analysis approach for *GSTM1* and *GSTT1*, it is unclear whether a gene dosage effect exists

(26-29). Experimental studies suggested a bimodal distribution of *ex vivo* *GSTM1* overall (26); however, the authors also reported that ~20% of the subjects were considered "very highly active." Similar studies of *GSTT1* (27-29) enzymatic activity were clearly trimodal, however, additional experimental studies employing genotyping approaches that categorize allele count are needed to more precisely specify these relationships.

The results of this study do not support a relationship between *GSTP1* polymorphisms and adenoma risk. We also found no evidence for a modifying effect of glutathione S-transferase genotype on the association between adenoma

Table 3. Risk of left-sided advanced colorectal adenoma associated with cigarette smoking, and specific *GSTM1*, *GSTT1*, and *GSTP1* polymorphisms

	Never smokers			Ever smokers			<i>P</i> interaction
	Controls n (%)	Cases n (%)	OR (95% CI)*	Controls n (%)	Cases n (%)	OR (95% CI)*	
<i>GSTM1</i>							
+/+	12 (4.4)	18 (8.1)	1.0 (reference)	23 (6.1)	40 (9.8)	1.0 (reference)	
+/-	110 (40.4)	87 (39.6)	0.5 (0.2-1.2)	150 (39.7)	150 (36.9)	0.6 (0.3-1.0)	
-/-	150 (55.2)	115 (52.3)	0.5 (0.2-1.1)	205 (54.2)	217 (53.3)	0.6 (0.3-1.0)	0.59
+/- and -/-	260 (95.6)	202 (91.9)	0.5 (0.2-1.1)	355 (93.9)	367 (90.2)	0.6 (0.3-1.0)	0.79
<i>GSTT1</i>							
+/+	83 (29.6)	75 (33.2)	1.0 (reference)	149 (39.1)	133 (31.2)	1.0 (reference)	
+/-	147 (52.5)	107 (47.3)	0.8 (0.5-1.2)	176 (46.2)	217 (50.8)	1.4 (1.0-1.9)	
-/-	50 (17.9)	44 (19.5)	1.0 (0.6-1.7)	56 (14.7)	77 (18.0)	1.6 (1.0-2.4)	0.12
+/- and -/-	197 (70.4)	151 (66.8)	0.9 (0.6-1.3)	232 (60.9)	294 (58.8)	1.4 (1.1-1.9)	0.05
<i>GSTP1-I105V</i>							
AA	132 (48.5)	97 (44.1)	1.0 (reference)	171 (44.1)	173 (40.8)	1.0 (reference)	
AG + GG	140 (51.5)	123 (55.9)	1.2 (0.9-1.8)	251 (56.9)	251 (59.2)	1.2 (0.9-1.6)	0.79
<i>GSTP1-A114V</i>							
CC	238 (84.4)	189 (81.1)	1.0 (reference)	324 (83.3)	375 (87.2)	1.0 (reference)	
CT + TT	44 (15.6)	44 (18.9)	1.2 (0.8-2.0)	65 (16.7)	55 (12.8)	0.7 (0.5-1.1)	0.18

*Adjusted for age, race, and gender.

and dietary sources of PAHs or hydrogenated amorphous carbons. Our analysis of dietary isothiocyanates from broccoli was difficult to interpret, possibly due to small numbers per subgroup and misclassification with respect to dietary exposure.

In conclusion, a lower risk of colorectal adenoma was observed among individuals carrying ≥ 1 inactive *GSTM1* allele. Having ≥ 1 inactive *GSTT1* allele was associated with a moderate increased risk among smokers. A significant interaction between ever smoking and genotype was observed when +/– and –/– individuals were combined. *GSTP1* variants were unrelated to risk. In summary, this is the first study to report associations between colorectal adenomas and *GSTM1* wild-type and *GSTT1* null alleles among smokers. These findings only became apparent using a newly developed assay to distinguish heterozygous and wild-type individuals. Our data provide evidence that phenotypic differences between these two groups exist.

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BLOOD CANCER DISCOVERY

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