CYP2A6 Activity Determined by Caffeine Phenotyping: Association with Colorectal Cancer Risk[1]

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Abstract

Cytochrome P450 2A6 (CYP2A6) catalyzes the metabolic activation of several procarcinogens including dietary and environmental nitrosamines, and the involvement of CYP2A6 in cancer development has been postulated. CYP2A6 phenotype was determined using caffeine as a probe drug in individuals participating in a case-control study of colorectal cancer (127 cases and 333 controls matched on age, gender, race, and geographic region). Conversion of the caffeine metabolite 1,7-dimethylxanthine (17X) to 1,7-dimethyl uric acid (17U) is catalyzed primarily by CYP2A6, and this activity can be assayed by comparison of urinary molar ratios of metabolites. Caffeine (200 mg) was administered to each participant, and a 4–5 h postadministration urine sample was collected. Urinary metabolites of caffeine were separated by high-performance liquid chromatography and quantified by comparison to authentic standards. We examined the distributions of the ratio, 17U:17X, according to subject characteristics among controls. In case-control comparisons, subjects in the medium and high tertiles of CYP2A6 activity had an increased risk of colorectal cancer compared with subjects with low activity. Odds ratios from a conditional logistic regression model for medium and high 17U:17X ratio were 2.0 (95% confidence interval, 1.1–3.7) and 2.6 (95% confidence interval, 1.5–4.5), respectively (*P* for trend = 0.001). CYP2A6 phenotype has not been compared previously between cancer cases and controls. We found a strong relationship between CYP2A6 activity, measured by urinary caffeine metabolite ratio, and colorectal cancer risk.

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Introduction

Caffeine is widely used as a probe drug and can be used to simultaneously assess the phenotypes of various drug-metabolizing enzymes including *N*-acetyltransferase 2 (1), cytochrome P450 1A2 (2, 3), flavin-containing monooxygenases (4), xanthine oxidase (4, 5) and CYP2A6 (6–10).[3] CYP2A6 catalyzes the hydroxylation of the caffeine metabolite 17X to yield 17U (6, 11). The urinary caffeine metabolite ratio of 17U:17X has been used to reflect CYP2A6 activity, with an increase in this ratio indicating higher CYP2A6 activity (8–10).

CYP2A6 is the primary P450 responsible for the biotransformation of nicotine to cotinine (12). Additionally, CYP2A6 catalyzes the metabolic activation of several promutagens and procarcinogens including aflatoxin B_1 (13, 14) and 3-methylindole (15). This isoform is also involved in the metabolism of dietary and tobacco-specific nitrosamines, such as *N*-nitrosodiethylamine and 4-methylnitrosoamino-1-(3-pyridyl)-1-butanone (16, 17). Elevated expression of CYP2A6 has been implicated in increased risk of liver cancer in populations where aflatoxin exposure is common (18–20). Additional studies have linked genetic polymorphisms in CYP2A6 to impaired capacity to metabolize nicotine, resulting in a decreased risk of lung cancer (21–23). Polymorphisms in CYP2A6 have been suggested to affect tobacco-dependent behavior and number of cigarettes smoked (22, 23). However, another study did not support this conclusion (24).

Several polymorphisms have been identified in the *CYP2A6* gene (reviewed in Ref. 25). There is substantial ethnic variation in CYP2A6 genotype, but the allelic variants identified thus far have a low frequency in Caucasians (26). Nevertheless, the literature shows that there is wide interindividual variability in the metabolism of coumarin, a CYP2A6-specific probe drug, in Caucasian populations (27–29). Therefore, phenotype analysis provides information concerning individual CYP2A6 metabolic variation in Caucasians that is not explained by the polymorphisms identified thus far.

To date, studies of CYP2A6 in relation to colorectal cancer have not been performed. However, a recent report on a cohort of adult Finns found a significant association between dietary intake of the nitrosamine *N*-nitrosodimethylamine and risk of colorectal cancer (30). Although no other epidemiological studies have assessed exposure to nitrosamines in relation to colorectal cancer, several studies (reviewed in Refs. 31, 32) have reported increased colorectal cancer risk associated with consumption of processed meats, which are important sources of a variety of nitrosamines. CYP2A6 catalyzes the metabolic activation of dietary nitrosamines other than *N*-nitrosodimethylamine; consequently, a role for CYP2A6 in the etiology of

[3] The abbreviations used are: CYP2A6, cytochrome P450 2A6; HPLC, high-performance liquid chromatography; 17X, 1,7-dimethylxanthine; 17U, 1,7-dimethyluric acid; CI, confidence interval; OR, odds ratio.

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colorectal cancer can be hypothesized. In this study, we sought to determine whether CYP2A6 activity, measured by analysis of caffeine metabolites in urine, was positively associated with colorectal cancer incidence.

Materials and Methods

***In Vitro* Studies.** Recombinant CYP1A1, CYP1A2, CYP2A6, CYP2E1, CYP2C9, and CYP3A4 were purchased from Gentest Corporation (Woburn, MA). Activity assays were performed as described by Gu *et al.* (33), except that for incubations using CYP2A6, 0.1 M Tris (pH 7.5) was substituted for phosphate buffer and the concentration of 17X in all of the assays was 0.1 mM. HPLC analysis of the product was carried out as described below for the analysis of urinary caffeine metabolites.

Study Population. Participants in this study included those diagnosed with histologically confirmed cancer of the colon or rectum, diagnosed in 1993–1999, and community controls. This study population has been described previously (34). Case subjects were recruited from the University of Arkansas for Medical Sciences University Hospital and the Central Arkansas Veteran's Health Care System in Little Rock, AK. Control participants were selected using the Arkansas state driver's license/identity card records and were matched to cases on race, age (within 10 years), sex, and county of residence. The University of Arkansas for Medical Sciences Institutional Review Board approved the study protocol. Exclusion criteria for the case-control study included a history of cancer (other than nonmelanoma skin cancer), uncontrolled cardiovascular disease, hepatic dysfunction as determined by bilirubin >1.5 mg/dl, aspartate aminotransferase >40 units/liter, alkaline phosphatase >140 units/liter, and abnormal renal function as determined by blood urea nitrogen (BUN) >20 mg/dl and serum creatinine >1.8 mg/dl. Twenty-six potential case subjects were excluded based on hepatic dysfunction. During 1994–1999, we conducted in-person interviews with each subject and asked participants to complete a caffeine phenotyping assay (see below). The interview addressed risk factors for colorectal cancer including cigarette smoking history, occupational history, diet, and medical history. Meat consumption was assessed using an instrument developed by Sinha and Rothman (35), which included detailed questions about meat, fish, and eggs. Information for each item included how often the food was consumed and usual portion size; food models were used as an aid for estimation of portion size. The reference period for the diet questionnaire was the last year before the interview. There were 156 cases and 366 controls interviewed; 24 case subjects and 16 controls did not complete the phenotyping assay. Omitting subjects for whom no case/control match was available, we report on caffeine phenotyping results for 127 cases and 333 controls (most cases had two, three, or four matched controls). The range of ages for case subjects was 32–85 years (mean 61.1, median 63); for control subjects, the range was 32–88 years (mean 62.1, median 65); 30% of cases and 34% of controls were females. The majority of subjects were Caucasian; 14% of cases and 11% of controls were African American, and none reported other racial background.

Caffeine phenotype data were also examined from patients participating in a separate study of breast cancer, recruited from the same institutions. The exclusion criteria were the same for the breast cancer study as outlined for the colorectal cancer study.

Caffeine Phenotyping Assay. Subjects were instructed to abstain from methylxanthine-containing foods and beverages (*i.e.*, coffee, tea, chocolate, and cola drinks) from midnight before phenotyping until 5 h after dosing. A 200-mg tablet of No-Doz (Bristol-Myers) was administered to each participant. Four hours after administration, the subject emptied his or her bladder, and a urine sample representing the 4–5 h urine was collected 1 h later. If a patient was receiving chemotherapy or radiation therapy, the caffeine phenotyping procedure was postponed until at least 6 weeks after completion of therapy. The majority of case subjects completed the caffeine phenotyping procedure within 1 year of diagnosis; the time from diagnosis to phenotyping was <6 months for 47 (37%) case subjects, 7–12 months for 39 subjects (31%), and >1 year for 40 subjects (32%).

Laboratory Analysis of Urinary Caffeine Metabolites. Urinary caffeine metabolites were extracted and prepared for HPLC analysis as described by Butler *et al.* (3). The HPLC system consisted of a Waters 996 Photodiode Array Detector, a 717 Autosampler, and a 600E Solvent Delivery System with column heater set at 32°C. The column and guard columns were Beckman Ultrasphere ODS 5 μm (4.6 mm $\times$ 25 cm and 4.6 mm $\times$ 4.5 cm). The mobile phase was 0.05% acetic acid (A), methanol (B), and acetonitrile (C) with a flow rate of 0.8–1.1 ml/min. The methanol concentration was 8% for the first 15 min of the HPLC run, increasing to 20% by 20 min. Between 20 and 23 min the 0.05% acetic acid remained at 80%, whereas the methanol concentration dropped to 12% and 8% acetonitrile was substituted followed by a column wash and reequilibration (from 30 to 48 min). Caffeine metabolites were identified and quantified by comparison to the spectral characteristics and retention time of authentic standards. Caffeine (1,3,7-trimethylxanthine), 17U, 1-methylxanthine, and 1-methyluric acid were purchased from Sigma Chemical Co.-Aldrich (St. Louis, MO). 17X was purchased from Fluka (Milwaukee, WI) and 5-acetylamino-6-formylamino-3-methyluracil was provided by R. Fumeaux, Nestec Lte. Research Center (Lausanne, Switzerland).

Data Analysis. We calculated caffeine metabolite ratios from molar concentrations of caffeine metabolites in the 4–5 h urine samples. Enzyme activity of CYP2A6 was estimated by the ratio of 17U:17X (8).

Others have reported that distributions of urinary caffeine metabolite ratios are typically skewed (3). The distribution of the 17U:17X ratio among controls was tested for normality using Wilk-Shapiro test. We constructed kernel-smoothed probability density functions of the values of each ratio or of the natural logs of the ratio values. We evaluated relationships between metabolite ratios and subject characteristics (*i.e.,* smoking status, gender, age, and race) among control subjects using Wilcoxon rank-sum test and nonparametric test for trend, and using multivariate linear regression analysis. We established categories for each caffeine metabolite ratio based on tertiles of activity among controls.

We evaluated CYP2A6 activity as a risk factor for colorectal cancer using conditional logistic regression analysis of matched case-control pairs, calculating ORs for the middle and highest tertile of enzyme activity, with low activity as the reference category. A likelihood ratio test for a variable representing the ordered tertiles of activity was used to test for trend. Current smoking and educational background, potential confounders of dietary exposures, were included in the model as covariates. Age categories for matching cases and controls were broad, so we assessed potential confounding by age by comparing model results for several coding schemes including years of age, categories of decade of age, and linear spline terms. We calculated the amount of each food consumed per week in

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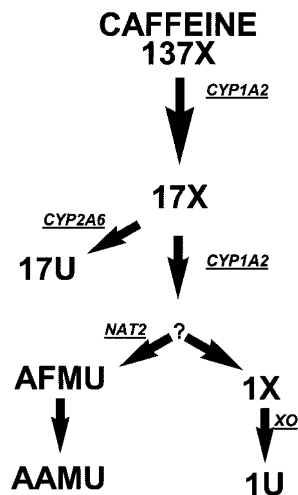

Fig. 1. Major pathways of caffeine metabolism. The metabolite pathway was described by Tucker *et al.* (41). The metabolites of caffeine are in **bold,** and the major enzymes catalyzing the reaction are *underlined* and *italicized*.

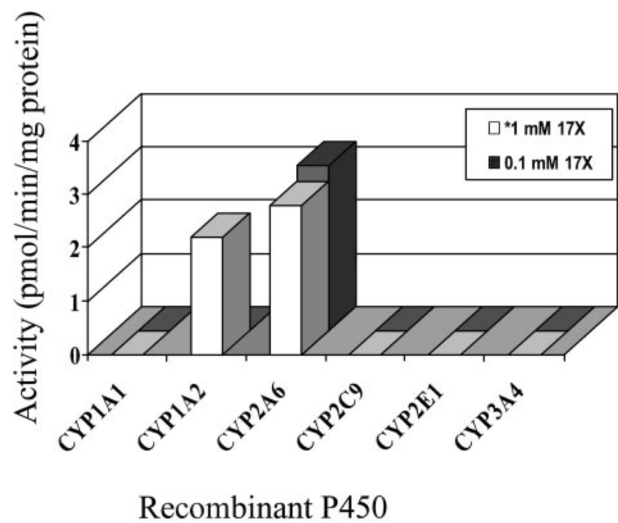

Fig. 2. Conversion of 17X to 17U by recombinant cytochrome P450s. Enzymatic activity was determined as described in "Materials and Methods." Incubations were carried out for 1 h at 37°C with 1 mg/ml cell protein and 0.1 mM 17X. *Black bars* represent our data and are the average of duplicate determination, and *white bars* represent data at 1 mM substrate concentration from Gu *et al.* (26).

ounces as the product of number of servings per week and serving size. The total amount of meat consumed was estimated by summing the following meat items: hamburger, beef steak, pork chops, ham steak, bacon, sausage, hot dogs, fried chicken, other chicken, turkey, roast beef, beef stew, ground beef, ham, bologna, salami, tomato sauces containing meat, and meat soups. The amount of preserved meat consumed per week was estimated by the sum of bacon, sausage, hot dogs, ham, bologna, and salami. Categories of meat consumption were established according to the median consumption and tertiles among controls. ORs for CYP2A6 activity within categories of meat consumption were calculated using interaction terms entered into the conditional logistic model. Stata software (Stata Corp., College Station, TX) was used for statistical analysis.

Results

Cytochrome P450-catalyzed Conversion of 17X to 17U. The major pathways of caffeine metabolism are illustrated in Fig. 1. To determine the isoform specificity of the conversion of 17X to 17U, we examined this reaction using six different cDNA expressed human cytochrome P450s and plotted the results together with the data of Gu, *et al.* (33). When this assay was performed using a substrate concentration of 0.1 mM, in contrast to the 1 mM concentration used in (33), CYP2A6 was the only P450 capable of catalyzing this conversion (Fig. 2).

Metabolite Ratio. For the 17U:17X ratio, the range was 0.25–16.52, with a median of 1.63 and mean (SD) of 2.03 (1.6); normality was rejected ($P < 0.001$), but a normal distribution was not rejected for log-transformed 17U:17X values ($P = 0.42$). The shape of the probability density functions did not suggest a bimodal distribution. Intraindividual variability of the caffeine metabolite analysis was assessed by phenotyping five healthy individuals weekly for 4 weeks. The intraindividual coefficient of variation in the 17U:17X ratio ranged from 12 to 24%. None of the variation observed resulted in a change in tertile assignment for the individuals. Repeat analyses of the same sample over 4 weeks gave essentially the same values (data not shown).

CYP2A6 Activity and Smoking Status, Age, Gender, and Race. Table 1 shows the mean and median of CYP2A6 activity ratios among control subjects by subject characteristics. When

Table 1 CYP2A6 activity, measured by urinary caffeine metabolite ratio by subject characteristics, among controls

	17U/17X				
	n	Mean	Median	P^a	P^b
All control subjects	333	2.03	1.63		
Sex					
Males	217	1.98	1.57		
Females	116	2.12	1.76	0.41	0.11
Race					
Caucasian	297	1.96	1.66		
African-American	36	2.60	1.53	0.70	0.76
Age					
<40	21	1.73	1.42		
40–49	24	1.73	1.38		
50–59	77	2.04	1.56		
60–69	125	2.03	1.59		
≥70	86	2.16	1.91	0.05	0.09
Current smoker					
No	289	2.08	1.66		
Yes	44	1.69	1.47	0.36	0.36
Cigarettes per day[c]					
1–19	53	1.97	1.42		
20	62	2.07	1.55		
21–80	65	1.99	1.62	0.55	0.69

[a] For sex, race, and current smoking, *P* is from Wilcoxon rank sum test for comparison of two groups; for age and number of cigarettes per day, *P* is from nonparametric trend test for differences across groups.
[b] *P* from linear regression of natural log of 17U:17X with all covariates shown.
[c] Among 271 current or former smokers reporting number of cigarettes smoked per day.

groups were compared using nonparametric tests, age was an important predictor of CYP2A6 activity, with older subjects having higher activity. The distribution of values for 17U:17X is very similar for smokers and nonsmokers (Fig. 3). Whereas age remained the most important predictor of 17U:17X ratio, there was limited evidence ($P = 0.11$) of higher activity in females compared with males. There was no evidence that this

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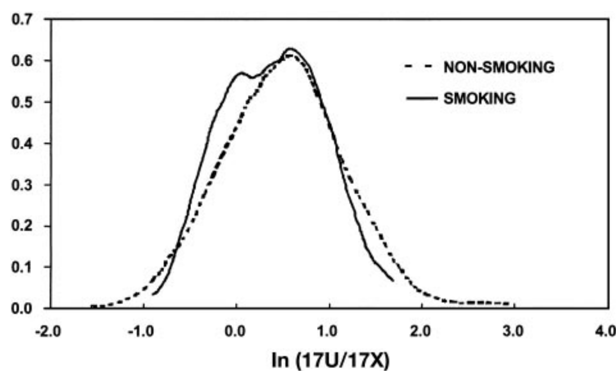

Fig. 3. CYP2A6 Activity among control subjects by smoking status, probability density functions. Activity estimated by urinary 17U:17X ratio, among nonsmokers, n = 289, and current smokers, n = 44.

ratio varied by race or number of cigarettes smoked per day. Barbiturates are the only known inducers of CYP2A6 activity. Subjects in this study were asked about current use of medications, and none reported taking barbiturates. As shown in Table 1, the median CYP2A6 activity for African-American subjects was slightly but nonsignificantly lower than the median in Caucasians. The proportion of African-Americans with CYP2A6 activity falling in the lowest tertile was 39% *versus* 33% for Caucasians, but this difference was also nonsignificant.

CYP2A6 Activity and Colorectal Cancer Risk. Table 2 details the characteristics of the colorectal cancer patients compared with the control individuals. We compared the CYP2A6 phenotype in case subjects to that of controls, based on tertiles established in control participants. However, when the conditional logistic model was constructed using a continuous variable for CYP2A6 activity instead of tertiles of activity, higher CYP2A6 activity remained positively associated with colorectal cancer risk ($P < 0.001$). A high proportion of case subjects (49%) had values in the highest tertile of activity (Table 3). Elevated ORs for colorectal cancer were observed for the second and third tertiles, with a trend of increased risk associated with increased CYP2A6 activity (P for trend < 0.001). ORs for categories of 17U:17X were very similar to those shown in Table 3 when the analysis was limited to men or women, or to cases with cancer of the colon or rectum. When alternate categorizations of age, smoking status, and amount smoked were considered, the ORs were very similar from all of the models, indicating no important confounding by age or smoking history. Median 17U:17X ratios among cases by decade of age were: 1.24 for age younger than 40, 2.70 for ages 40–49, 2.22 for 50–59, 1.96 for 60–69, and 2.14 for 70 and older. The median value from the case group was higher than the control group median in all of the age categories except the youngest. When age, sex, and race were taken into account in a multivariate regression model, 17U:17X ratio remained unrelated to current smoking status. We considered whether the 17U:17X ratio varied among cases according to time between the diagnosis of a case subject and the collection of the urine sample. Median 17U:17X ratio was 2.25 for samples collected within 6 months of diagnosis, 2.00 for samples collected at 7–12 months after diagnosis, and 1.93 for samples collected >1 year, indicating little difference by time to sample collection (in a nonparametric test for trend, $P = 0.21$). We also examined the 17U:17X ratio in relation to stage of disease at diagnosis. The proportion of case subjects with 17U:17X falling in the highest tertile of control values by stage of disease were: stage I, 45%; stage II, 57%; stage III, 29%; and stage IV, 50%.

Table 2 Characteristics of colorectal cancer cases and controls

	Cases (%)	Controls (%)
[a]Sex		
Males	89 (70.1)	217 (65.2)
Females	38 (29.9)	116 (34.8)
[a]Race		
Caucasian	110 (86.6)	297 (89.2)
African-American	17 (13.4)	36 (10.8)
[a]Age		
<50	25 (19.7)	45 (13.5)
50–59	29 (22.8)	77 (23.1)
60–69	35 (27.6)	125 (37.5)
≥70	38 (29.9)	86 (25.8)
Education		
<High school grad	33 (26.2)	46 (13.8)
High school grad	45 (35.7)	79 (23.7)
Some college or vocational	26 (20.6)	103 (30.9)
Bachelor's degree or higher	22 (17.5)	105 (31.5)
[b]Cigarette smoking		
Never	52 (41.3)	152 (45.7)
Current	26 (20.6)	44 (13.2)
Former	48 (38.1)	137 (41.1)
[b]Total meat (ounces/week)		
<28.8	26 (20.6)	103 (31.1)
28.8–47.7	42 (33.3)	120 (36.3)
>47.7	58 (46.0)	108 (32.6)
Preserved meat (ounces/week)		
	21 (16.7)	114 (34.2)
	40 (31.8)	110 (33.0)
	65 (51.6)	109 (32.7)

[a] Proportions by age, sex and race are unequal because the number of controls matched to each case varied between 1 and 4.
[b] One case was missing information on smoking and meat consumption. Two controls were missing information on some meat variables.

Table 3 CYP2A6 phenotype among colorectal cancer cases and controls

Urinary 17U:17X ratio, tertiles	Cases (%)	Controls (%)	OR[a]	95% CI
Low	23 (18.1)	111 (33.3)	1.0	—
Medium	41 (32.2)	111 (33.3)	2.0	1.0–3.7
High	63 (48.9)	111 (33.3)	2.9	1.6–5.0

[a] OR for matched cases and controls (one or more controls per case, matched on age, sex, race, and geographic region) calculated from conditional logistic regression model, adjusted for age and smoking status.

CYP2A6 Phenotype, Colorectal Cancer, and Preserved Meat. The relationship between CYP2A6 phenotype and colorectal cancer in context of consumption of preserved meats, an important source of nitrosamine exposure, was examined. There was a significant trend of increased colorectal cancer risk with higher categories of reported meat consumption (P = 0.02) and preserved meat consumption (P = 0.01). For meat consumption above, *versus* below, the median, the OR was 1.3 (95% CI, 0.8–2.1), adjusted for smoking and education. For preserved meat, the OR for consumption above the median was 2.0 (95% CI, 1.2–3.5), adjusted for smoking, education, and total meat consumption. Elevated CYP2A6 activity was associated with increased risk of colorectal cancer in both the low- and high-preserved meat consumption groups (Table 4). Among subjects with low consumption of preserved meat, the ORs indicate elevated risk only for the high tertile of CYP2A6 activity. Among subjects with high consumption of preserved

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Table 4 CYP2A6 phenotypes by consumption of preserved meat and by smoking history among colorectal cancer cases and controls

	Tertiles of CYP2A6 Activity								
	Low			Medium			High		
	Cases Controls	OR[a]	95% CI	Cases Controls	OR[a]	95% CI	Cases Controls	OR[a]	95% CI
Preserved meat[b]									
Low	9			7			21		
	57	1.0	—	60	0.8	0.3–2.3	47	3.7	1.4–9.6
High	14			34			41		
	54	1.4	0.5–4.0	51	4.0	1.6–10.4	64	3.2	1.3–7.9
Cigarette smoking									
No or <40 pack years	16			32			42		
	93	1.0	—	89	2.3	1.1–4.8	86	2.9	1.4–5.8
≥40 pack years	6			9			19		
	18	1.8	0.5–6.0	21	1.8	0.6–5.4	24	4.5	1.7–12.0

[a] OR from conditional logistic regression model, matched on age group, race, sex, and geographic region, with additional adjustment for smoking, exact age, total meat consumption, and education.
[b] Preserved meat items in the questionnaire were bacon, hot dogs, and bologna or other lunch meats; "low" and "high" were divided at the median consumption, 5 ounces per week.

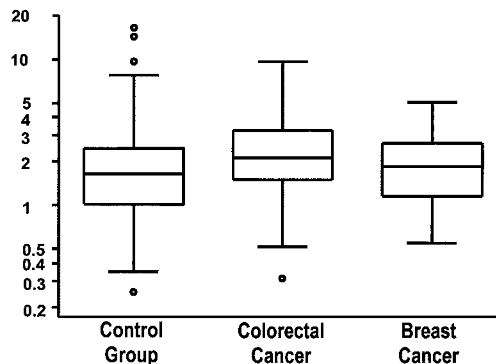

Fig. 4. CYP2A6 activity among three groups of subjects: colorectal cancer controls (n = 333), colorectal cancer cases (n = 127), and breast cancer cases (n = 36). Box plot of urinary 17U:17X ratio, on log scale, with the median of each group represented as a *horizontal line in the box* and interquartile range (75th and 25th percentile) as the *top and bottom of the box.*

meats, there was increase in risk for both the middle and high tertiles. These differences in the dose-response pattern were reflected in a statistical interaction in the multiplicative model (P = 0.02).

CYP2A6 Phenotype in a Breast Cancer Study. To assess whether elevated CYP2A6 activity in the colorectal cancer patients was because of the presence of neoplastic disease, we analyzed caffeine metabolite ratios in a group of women with breast cancer (n = 36). The distributions of CYP2A6 activities in the colorectal cancer control subjects, colorectal cancer case subjects, and breast cancer case subjects are compared in Fig. 4. The median 17U:17X ratios among female subjects were: 1.84 for the breast cancer case subjects, 1.76 for colorectal cancer control subjects, and 1.91 for colorectal cancer case subjects. When CYP2A6 activities in the breast cancer case subjects were compared with female colorectal cancer control subjects in a logistic regression model, with adjustment for age and race, ORs for breast cancer risk of 0.9 (95% CI, 0.3–2.5) and 1.4 (95% CI, 0.5–3.8) were calculated for the second and third tertiles of the 17U:17X ratio. Thus, higher CYP2A6 activity, as measured by 17U:17X ratio, was associated with increased risk of colorectal cancer, but not of breast cancer.

Discussion

Colorectal cancer is the fourth most common cancer worldwide and the United States, along with other developed countries, is considered to be a high-risk area for its development (36). Studies have shown that diet, in particular meat consumption, is the factor most consistently associated with the development of this disease. Recent results from large prospective studies have indicated that both exposure to nitrosamines in the diet and smoking cigarettes are positively associated with the risk of developing colorectal cancer (30, 37), but these findings have not been consistent across all studies. Because CYP2A6 is involved in the metabolic activation of nitrosamines from both of these sources, we examined CYP2A6 phenotype in a case-control study of colorectal cancer.

Coumarin has been used as a probe drug to assess CYP2A6 activity in humans because coumarin 7-hydroxylase activity is specific to CYP2A6 (38, 39). Others have used caffeine as an *in vivo* probe for several enzymes, including CYP2A6 (6–8), based on their preferential catalysis of different steps in the metabolism of caffeine, as shown in Fig. 2. However, before this study, there was little experimental evidence to support the use of 17U:17X as a marker of CYP2A6 activity. Earlier studies had demonstrated that both CYP2A6 and CYP1A2 catalyzed the conversion of 17X to 17U at substrate concentrations of 1 mM (33). We investigated the isoform specificity of this reaction at a substrate concentration of 0.1 mM (a concentration that may more closely reflect *in vivo* conditions) and found that at this lower concentration, CYP2A6 selectively catalyzes the conversion of 17X to 17U. Additional support for the ratio reflecting CYP2A6 activity comes from the observation that this ratio is not influenced by smoking status, a condition that has a profound effect on the activity of CYP1A2.

We have performed caffeine phenotyping on many colorectal cancer patients and control individuals. Although the initial goal of the caffeine phenotyping study was to examine CYP1A2 activity,[4] we were also able to assess the metabolic activity of CYP2A6 using metabolites from the caffeine-phenotyping procedure. The present study, with 333 control subjects available for analysis, reports on the largest healthy

[4] Unpublished data.

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study population described to date in regard to CYP2A6 activity measured by caffeine phenotyping. This number of control participants allowed us to consider in detail the relationships between CYP2A6 activity and gender, age, race, and smoking. The 17U:17X ratio was significantly influenced only by age. Data presented by Iscan *et al.* (39) suggests a possible increase in CYP2A6 activity measured by coumarin phenotyping, although these authors did not find the correlation with age to be statistically significant among 100 subjects.

When CYP2A6 phenotype was compared between colorectal cancer case subjects and control individuals, high CYP2A6 activity was associated with increased risk of cancer (Table 2), with a strong trend of increased risk with higher activity. ORs were essentially unchanged when alternate modeling of age and smoking status were examined, so the association is unlikely to be the result of confounding by these characteristics.

Because CYP2A6 activates putative carcinogens found in preserved meat and cigarette smoke, we examined CYP2A6 activity in the context of these exposures. When smoking was examined in a multivariate regression model with adjustments for age, sex, and race, CYP2A6 activity remained unrelated to current smoking status. The proportion of control subjects who reported being current smokers is lower than expected. The prevalence of current smoking is less in individuals above the age of 65, the median age of our control group, so age may partly explain the low prevalence. Male Caucasians between the ages of 45 and 64 in our control population reported a smoking prevalence of 14%, which is low compared with 27% for this age group in the 1998 National Health Interview Survey (40). This suggests that our control group was influenced by selection bias or information bias and that it is not appropriate to calculate an OR for cigarette smoking based on this data. However, when these ORs are examined (Table 4), smoking history of forty-pack years or more and the highest tertile of CYP2A6 activity give an OR of 4.5 (95% CI, 1.7–12.0).

When the ORs for CYP2A6 among high and low consumers of preserved meats are compared (Table 4), there is a suggestion of different shapes of the dose-response depending on exposure. However, the 127 case subjects phenotyped in the present study gave us limited power to consider subgroups of exposures, and the differences in dose-response may be because of chance. Rigorous evaluation of potential interaction between CYP2A6 activity and environmental exposures will require larger study populations.

When metabolic profiles are compared between diseased and nondiseased study subjects, the possible impact of disease on phenotype is a concern. An assumption of case-control studies is that exposures measured among case subjects represent exposures present before disease. In the present study, the higher CYP2A6 activity among cases implies it is a risk factor for disease only if the higher activity was characteristic of the individual before the development of disease. It was not possible to compare phenotypes for the same individuals before and after development of cancer. However, we were able to take advantage of the availability of caffeine phenotype data from another group of cancer patients to consider whether elevation in CYP2A6 results from the presence of cancer or from cancer treatment. CYP2A6 activities of patients recruited for a pilot study of breast cancer were compared to the colorectal study control group. Taking into account age and gender, the distribution of CYP2A6 activity among the breast cancer patients was similar to the control group (Fig. 4). The high activity tertile showed a nonsignificant, slightly elevated OR, but the OR was much less than the OR for the high activity tertile among colorectal cancer subjects. Although the number of breast cancer subjects was small, the similarity of CYP2A6 activities between this group and the control subjects indicates that the presence of cancer alone is unlikely to be the explanation for the high CYP2A6 activity observed among colorectal cancer cases.

Limitations of the present case-control study population include use of a hospital-based rather than population-based, case group, and lack of information about nonparticipants so that we are unable to compare characteristics of participating subjects *versus* nonparticipants. However, the variable of interest, CYP2A6 activity, is unlikely to be unduly influenced by selection bias. Evaluation of predictors of CYP2A6 activity among controls showed that only age, and possibly gender, influenced phenotype; both of these characteristics were accounted for in case-control matching.

Disease states may affect activity of hepatic enzymes. Studies have demonstrated increased expression of CYP2A6 in liver cells immediately adjacent to areas of fibrosis or inflammation because of the presence of hepatitis B or cirrhosis (19). We are unable to completely rule out the co-occurrence of hepatitis or other liver pathology with colorectal cancer as an explanation for the elevated activity among colorectal cancer study subjects. However, the exclusion criteria for study subjects include blood chemistry analysis to detect abnormal liver function, and 26 potential subjects were excluded based on elevated liver enzymes.

The proportion of case subjects with 17U:17X ratio falling in the highest tertile of control values, by stage of disease at diagnosis, are: stage I, 45%; stage II, 57%; stage III, 29%; and stage IV, 50%. Thus, elevated 17U:17X ratios were apparent for patients diagnosed with stage I and II disease, so it seems unlikely that the elevated 17U:17X ratios that we observed among cases are attributable to change in liver function because of liver metastasis.

In conclusion, CYP2A6 phenotype has not been compared previously between colorectal cancer patients and control individuals. We found a strong relationship between CYP2A6 activity, measured by urinary caffeine metabolite ratios, and colorectal cancer risk. The mechanistic basis of this observation remains to be elucidated. Whereas this finding is not a definitive demonstration of the role of nitrosamines in colorectal carcinogenesis, the data are suggestive of this because of the role of CYP2A6 in the activation of procarcinogens found in cigarette smoke and in foods, particularly preserved meats. Nevertheless, the present finding is novel, and the suggested association between CYP2A6 activity and colorectal cancer should be investigated in independent study populations.

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