Combined Effects of Well-done Red Meat, Smoking, and Rapid *N*-Acetyltransferase 2 and CYP1A2 Phenotypes in Increasing Colorectal Cancer Risk[1]

Loïc Le Marchand,[2] Jean H. Hankin, Lynne R. Wilkens, Lisa M. Pierce, Adrian Franke, Laurence N. Kolonel, Ann Seifried, Laurie J. Custer, Wendy Chang, Annette Lum-Jones, and Timothy Donlon

Etiology Program, Cancer Research Center of Hawaii, University of Hawaii, Honolulu, Hawaii 96813

Abstract

Heterocyclic amines (HAAs) are suspected carcinogens that are formed in meat when it is cooked at high temperature for long durations. These compounds require metabolic activation by CYP1A2 and *N*-acetyltransferase (NAT) 2 or NAT1 before they can bind to DNA. It has been hypothesized that well-done meat increases the risk of colorectal cancer (CRC), especially in individuals with the rapid phenotype for CYP1A2 and NAT2. This association may be particularly strong in smokers because smoking is known to induce CYP1A2. We conducted a population-based case-control study on Oahu, Hawaii to specifically test this hypothesis. An in-person interview assessed the diet and preference for well-done red meat of 349 patients with CRC and 467 population controls. A urine collection after caffeine challenge and a blood collection were used to assess phenotype for CYP1A2 and NAT2 and genotype for *NAT2* and *NAT1*, respectively. No statistically significant main effect association with CRC was found for red meat intake, preference for well-done red meat, the *NAT2* rapid genotype, the CYP1A2 rapid phenotype or the *NAT1*10* allele. However, in ever-smokers, preference for well-done red meat was associated with an 8.8-fold increased risk of CRC (95% confidence interval, 1.7–44.9) among subjects with the NAT2 and CYP1A2 rapid phenotypes, compared with smokers with low NAT2 and CYP1A2 activities who preferred their red meat rare or medium. No similar association was found in never-smokers, and there was no increased risk for well-done meat among smokers with a rapid phenotype for only one of these enzymes or for smokers with both rapid phenotypes who did not prefer their red meat well-done. These data provide additional support to the hypothesis that exposure to carcinogens (presumably HAAs) through consumption of well-done meat increases the risk of CRC, particularly in individuals who are genetically susceptible (as determined by a rapid phenotype for both NAT2 and CYP1A2) and suggest that smoking, by inducing CYP1A2, facilitates this effect.

Introduction

CRC[3] remains the third most common malignancy in the United States, with 130,200 new cases expected in 2000 (1). Few causative factors are known with certainty for this disease. Hereditary syndromes, such as familial adenomatous polyposis and hereditary nonpolyposis CRC, account for <10% of all cases. First-degree family history of large bowel cancer is associated with a 2-fold increased risk for the disease, after exclusion of the known familial syndromes, suggesting the existence of unidentified genetic susceptibility factors. In addition, it is suspected that overnutrition, physical inactivity, a high red meat diet, alcohol use, smoking, and a low intake of vegetables may increase CRC risk, but the data are not conclusive (2). Possibly explaining the association with red meat is the observation that potent chemical carcinogens (*e.g.*, heterocyclic aromatic amines and polycyclic aromatic hydrocarbons) are formed when meat is cooked at high temperature for a prolonged period of time (3).

HAAs require *N*-oxidation by CYP1A2, followed by *O*-esterification by NAT1 or NAT2, before they can exert their genotoxicity (4, 5). At least 10 single-base substitution variants have been identified in the *NAT2* gene, seven of which result in amino acid changes. Combinations of these substitutions result in alleles coding for decreased enzyme activity, and five these alleles (**5A*, **5B*, **5C*, **6A*, and **7B*) occur with significant frequency (>1%; Ref. 6). CYP1A2 is also thought to be polymorphically expressed, although no genetic variant has been clearly demonstrated to explain the interindividual variation in enzyme activity. CYP1A2 is also known to be inducible by various environmental exposures including smoking (7). It has been hypothesized that the rapid NAT2 phenotype confers an increased CRC risk, especially when combined with the rapid CYP1A2 phenotype and consumption of well-done meat (7, 8). Recent data have only been weakly supportive of this hypothesis (9). This may be due to the fact that these studies did not consider CYP1A2 and grouped together rapid and intermediate acetylators, in effect "diluting" the high-risk group (10). They

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[2] To whom requests for reprints should addressed, at Etiology Program, Cancer Research Center of Hawaii, University of Hawaii, 1236 Lauhala Street, Suite 407, Honolulu, HI 96813.

[3] The abbreviations used are: CRC, colorectal cancer; HAA, heterocyclic amine; NAT, *N*-acetyltransferase; AFMU, 5-acetylamino-6-formylamino-3-methyluracil; 17U, 1,7-dimethyluric acid; 17X, 1,7-dimethylxanthine; 1X, 1-methylxanthine; 137X, caffeine; OR, odds ratio; CI, confidence interval.

also may not have considered the possible modifying effect of smoking. A variant allele for *NAT1* (**10*) associated with higher acetylation activity in colon tissue has also been suggested to increase CRC (11).

Intrigued by the high frequency of the rapid acetylator phenotype in Japanese and the rapid increase in CRC incidence and the high red meat intake observed among Japanese migrants to Hawaii (12), we conducted a population-based case-control study to test the relationships of *NAT2* and *NAT1* genotypes, CYP1A2 and NAT2 phenotypes (as assessed by caffeine challenge), and doneness of meat with CRC in Hawaii.

Materials and Methods

Cases were identified through the rapid reporting system of the Hawaii Tumor Registry, a member of the United States National Cancer Institute's Surveillance, Epidemiology, and End Results program. Eligible cases consisted of all Japanese, Caucasian, or native Hawaiian Oahu residents diagnosed before the age of 85 years with a primary adenocarcinoma of the colon or rectum in the main medical centers of Oahu between January 1994 and August 1998. Controls were selected from participants in an ongoing health survey conducted by the Hawaii State Department of Health among an annual 2% random sample of the state households following a design modeled after that of the National Health Survey (13). This source was supplemented with controls from Health Care Financing Administration participants on Oahu. One control was matched to each case on sex, ethnicity, and age (±2 years). Personal interviews were obtained from 768 matched pairs of cases and controls, resulting in a participation rate of 58.2% and 53.2% for cases and controls, respectively. The reasons for nonparticipation in cases were as follows: (*a*) refusal (22.5%); (*b*) death before contact (10.9%); (*c*) severe disease (6.7%); and (*d*) inability to locate (1.7%). The reasons for nonparticipation in controls were as follows: (*a*) refusal (34.8%); (*b*) inability to locate (9.2%); and (*c*) major illness or death (2.8%). Compared with noninterviewed cases, interviewed cases had the same ethnic and sex distribution but were less likely to have a regional or distant metastasis (46% *versus* 55%) and were younger by an average of 1.8 years. A blood sample was obtained for 548 cases (71.3% of interviewed cases) and 656 controls (85.4% of interviewed controls). A caffeine test was successfully completed on 349 of these cases (45.4% of interviewed cases) and 467 of these controls (60.8% of interviewed controls).

In-person interviews were conducted at the subjects' homes by trained interviewers. The median interval between diagnosis and interview for cases was 4.5 months (25th–75th percentiles, 3.3–8.4 months). The questionnaire included detailed information on demographics, including the race of each grandparent; a quantitative food frequency questionnaire; a lifetime history of tobacco, alcohol, and aspirin use; a history of recreational sports activities since age 18; a personal history of various relevant medical conditions; a family history of CRC in parents and siblings; information on height and weight at different ages; and for women, a history of reproductive events and hormone use. The food frequency questionnaire has been described previously (14) and validated (15) in this population. Frequencies and amounts consumed during the reference period (the year prior to onset of symptoms for cases or interview for controls) were obtained for 268 food items or categories. Daily intake in grams for each food item was computed for each individual. A food composition nutrient database that was based primarily on the United States Department of Agriculture's nutrient database and supplemented from other sources for ethnic-specific foods and recipes was applied to the food items to compute nutrient intakes. Food groupings were computed by summing intake from appropriate food items and relevant proportions from mixed dishes. Red meat was defined as including all beef and pork items, but not processed meat. Participants also reported their intakes and dosages of vitamin and mineral supplements during the reference period. In a separate question referring to 5 years prior to the interview, subjects were also asked whether they ate red meat and, if so, how they liked it cooked (rare, medium-rare, medium, well done, or very well done).

In preparation for the caffeine test (16), subjects were asked to refrain from consuming foods that may induce or inhibit CYP1A2 (well-done meat, cruciferous vegetables, and grapefruit) during the 2 days prior to the test, and they were asked to refrain from consuming caffeine-containing beverages and caffeine- and acetaminophen-containing drugs during the day preceding the test (17). Subjects were also asked to fast from 10 p.m. to the time of the blood draw the following morning. After venipuncture, the subjects drank a cup of coffee made of two packets of Maxwell House instant coffee (57 mg caffeine/packet), maintained fasting for another 2 h, voided 4 h after coffee consumption, and provided a 1 h urine specimen at the end of the fifth hour after dosing. No other source of caffeine was consumed during the 5-h period. The urine samples were acidified with ascorbate, kept on ice, and processed within 4 h of collection. The volume of urine was recorded, and the samples were stored at −70°C until analysis.

All laboratory analyses were performed blind to the case-control status of the samples. Urinary ratios of AFMU:1X and of (17U + 17X):137X were measured as described previously (10, 16, 17) to assess NAT and CYP1A2 activity, respectively. Each analytical batch included the same number of samples from cases and controls. The intra-assay coefficients of variation assessed by analyzing 10% blind duplicates were 4.3% for (17U+17X):137X and 10.6% for AFMU:1X. Past data from our group on intraindividual variability for both ratios suggested that one measurement performed reasonably well in characterizing long-term activity for both NAT2 and CYP1A2 (10, 17).

DNA was purified from blood lymphocytes, and the subjects were genotyped for *NAT2* and *NAT1* according to published protocols (10, 11). This *NAT2* genotyping method identifies nucleotide substitutions C481T, G590A, and G857A, which are diagnostic for the common slow activity alleles **5A,B*, **6A,B*, and **7A,B*. In addition, we genotyped our samples for T341C to identify the slow activity allele **5C* using primers 5′-CACCTTCTCCTGCAGGTGACCG-3′ and 5′-TGTCAAGCAGAAAATGCAAGGC-3′. The PCR conditions consisted of an initial denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 10 min. Twenty-five μl of the reaction product were digested with *Aci*I and subjected to electrophoresis on a 3.5% MetaPhor agarose gel. The T341C substitution creates an *Aci*I recognition site that results in the digestion of the 141-bp fragment into 121 and 20 bp (18).

Individuals were classified as "slow" acetylators when they had two low acetylation activity alleles (any two of *NAT2 *5A,B*, **5C*, **6A,B*, or **7A,B*) or "intermediate" acetylators when they had one such allele. The remaining subjects were assumed to be wild type or to carry the rare C282T variant, both of which are associated with high acetylation activity, and were classified as "rapid" acetylators. Similarly, individuals who carried two or one *NAT1*10* allele(s) were classified as "rapid" or "intermediate" acetylators for NAT1, respectively.

Table 1 Characteristics[a] of all interviewed subjects and subjects phenotyped by the caffeine test

	All interviewed subjects		Phenotyped subjects	
	Cases (N = 727)	Controls (N = 727)	Cases (N = 349)	Controls (N = 467)
Male (%)	58.1	58.1	63.0	59.3
Female (%)	41.9	41.9	37.0	40.7
Japanese (%)	60.2	60.2	58.2	60.8
Caucasian (%)	25.9	25.9	27.8	28.1
Hawaiian (%)	13.9	13.9	14.0	11.1
Family history[b] (%)	15.4	9.5	16.0	9.2
Ever used aspirin regularly (%)	17.6	28.7	20.2	27.4
Age (yrs)	67 (58–74)	68 (59–74)	65 (56–72)	67 (57–73)
Education (yrs)	12 (12–15)	14 (12–16)	13 (12–15)	14 (12–16)
Pack-years	8 (0–34)	1 (0–24)	8 (0–34)	1 (0–25)
Body mass index 5 yrs ago	25 (22–28)	24 (22–27)	25 (22–28)	24 (22–26)
Lifetime recreational physical activity (h)	2824 (180–9048)	4624 (960–11684)	3264 (336–9000)	4560 (960–11568)
Total calories (kcal)	2005 (1532–2600)	1894 (1461–2454)	2052 (1615–2743)	1918 (1415–2411)
NSP from vegetables[c] (g/day)	2.4 (1.4–3.7)	2.8 (1.8–4.4)	2.3 (1.2–2.6)	2.7 (1.8–4.4)
Total calcium[d] (mg/day)	819 (528–1895)	955 (561–2610)	790 (520–1743)	958 (556–2642)
Red meat (g/day)	41 (21–70)	37 (19–68)	45 (24–76)	40 (20–70)
Processed meat (g/day)	20 (9–39)	15 (6–31)	21 (12–41)	15 (6–31)

[a] Median (25th–75th percentile), except where indicated.
[b] Percent with family history of CRC among parents and siblings.
[c] Nonstarch polysaccharides (NSP) from vegetable sources.
[d] Calcium from food and supplements. NSP and calcium are adjusted for total caloric intake.

Forty-one case-control pairs were excluded because of a missing covariate on the case or control. The statistical analysis used conditional logistic regression for analyses including all of the matched interviewed case-control pairs (19). The models were adjusted for variables found to be associated with CRC in this study, namely, pack-years of cigarette smoking, lifetime recreational physical activity, body mass index 5 years ago, lifetime use of aspirin (in months), years of schooling, and intakes of non-starch polysaccharides from vegetables and calcium from foods and supplements. Because the relationships observed were dose dependent, all of these covariates were entered as continuous variables. Models with CYP1A2 phenotype were further adjusted for number of cigarettes, cigars, or pipes smoked by the subject during the 2 weeks preceding the caffeine test because smoking is known to induce CYP1A2. Nonsmokers were given the value 0 for amount smoked. Nutrient intakes were adjusted for total caloric intake by the method of residuals (20). The urinary metabolic ratios were log-transformed and categorized into tertiles or halves to maximize cell counts. Other continuous exposure variables were categorized according to the quantile distributions among the controls. Dose-response relations were evaluated by inclusion in the model of a trend variable assigned the median value of the appropriate quantile for quantitative exposures and the value 1, 2, or 3 for increasing number of variant alleles for genotypes. The case-control matching was broken for the analysis of the genotyping and phenotyping data because not all subjects provided biospecimens. Unconditional logistic regression was then used with further adjustment for age, sex, and ethnicity. The likelihood ratio test was used to determine interaction among certain variables with respect to CRC. The test compares a main effects, no interaction model with a fully parameterized model containing all possible interaction terms for the variables of interest.

Results

Sixty percent of the subjects were Japanese, 26% were Caucasian, and 14% were Hawaiian. Table 1 compares characteristics of subjects who agreed to the caffeine test with those of all interviewed subjects by case-control status. The controls who were phenotyped were similar to all interviewed controls for virtually all of the variables examined, whereas the phenotyped cases were slightly younger, more physically active, and more likely to be male and have used aspirin regularly than all interviewed cases. Both phenotyped cases and controls ate slightly more red meat than all interviewed cases and controls. Importantly, case-control differences were conserved in the phenotyped group. Compared with controls, cases were less educated, more likely to have a first-degree relative family history of CRC, and less likely to have used aspirin regularly. Cases had smoked more cigarettes and exercised less during their lifetimes. They were also heavier and consumed more calories, less calcium, less non-starch polysaccharides from vegetables, and more processed meats than controls.

The adjusted ORs for red meat intake as assessed with the food frequency questionnaire and the adjusted ORs for the red meat preference question are shown in Table 2 for all interviewed subjects and for phenotyped subjects. Results were similar in the two sexes and are thus presented for both sexes combined. The age-, sex-, and ethnicity-adjusted ORs and 95% CIs for CRC by increasing quartile of red meat were 1.0, 1.0 (95% CI, 0.7–1.3), 1.3 (95% CI, 1.0–17), and 1.4 (95% CI, 1.0–1.8; P for trend = 0.01). However, after adjustment for other covariates (Table 2), the relationship was no longer present, indicating that this association was confounded by known CRC risk factors. The proportions of all interviewed cases and controls who reported eating their meat well-done or very well-done (29.0% and 24.3%, respectively) were similar to those for phenotyped cases and controls (28.4% and 23.3%, respectively). CRC was not statistically significantly associated with preferring well-done or very well-done red meat in either group, although a weak association was suggested in the entire group of interviewed subjects.

Table 3 shows the adjusted ORs for *NAT2* and *NAT1* genotypes for all genotyped and all phenotyped subjects and the ORs for CYP1A2 and NAT activities for all subjects pheno-

Table 2 CRC ORs[a] and 95% CIs for red meat and red meat preference among all interviewed and phenotyped subjects

	All interviewed		Phenotyped	
	N[b]	OR (95% CI)	*N*	OR (95% CI)
Red meat intake (g/day)				
≤18.9	162/184	1.0	68/113	1.0
19.0–37.4	170/180	1.0 (0.7–1.4)	74/111	1.0 (0.7–1.6)
37.5–68.5	209/183	1.1 (0.8–1.5)	108/121	1.2 (0.8–1.9)
>68.6	186/180	1.0 (0.7–1.4)	99/122	1.0 (0.6–1.5)
		$P = 0.98$[c]		$P = 0.86$
Red meat preference				
Did not eat/rare/medium-rare	328/337	1.0	158/214	1.0
Medium	188/213	1.0 (0.7–1.9)	92/144	0.8 (0.6–1.1)
Well done/very well done	211/177	1.2 (0.9–1.5)	99/109	1.1 (0.8–1.6)
		$P = 0.29$[d]		$P = 0.73$

[a] ORs for all interviewed subjects adjusted by conditional logistic regression for pack-years of cigarette smoking, lifetime recreational physical activity, lifetime aspirin use, body mass index 5 years ago, years of schooling, and intakes of nonstarch polysaccharides from vegetables and calcium from foods and supplements. A total of 727 sex-, age-, and ethnicity-matched case-control pairs were interviewed. ORs for phenotyped subjects (349 cases and 467 controls) were adjusted by unconditional logistic regression for the same variables and age, sex, and ethnicity.
[b] Number of cases/number of controls.
[c] *P* for trend based on a trend variable assigned the median value for the appropriate red meat quartile.
[d] *P* for trend based on a trend variable respectively assigned 1, 2, and 3 for the three preference categories.

Table 3 CRC ORs[a] and 95% CIs for *NAT2* and *NAT1* genotypes and CYP1A2 and NAT2 phenotypes

	All genotyped subjects		Phenotyped subjects	
	N[b]	OR (95% CI)	*N*	OR (95% CI)
NAT2 genotype				
Slow	124/157	1.0	76/120	1.0
Intermediate	223/270	1.1 (0.8–1.5)	148/189	1.3 (0.8–1.9)
Rapid	196/227	1.1 (0.7–1.5)	124/157	1.2 (0.8–1.9)
		$P = 0.76$[c]		$P = 0.51$
NAT1 genotype				
Slow	249/268	1.0	158/193	1.0
Intermediate	189/269	0.8 (0.6–1.0)	127/187	0.9 (0.6–1.2)
Rapid	101/112	1.0 (0.7–1.4)	60/83	0.8 (0.5–1.3)
		$P = 0.58$		$P = 0.28$
CYP1A2 phenotype[d]				
≤3.48			104/156	1.0
3.49–7.25			112/155	1.1 (0.7–1.6)
>7.26			133/156	1.3 (0.9–1.8)
				$P = 0.22$
NAT2 phenotype[e]				
≤0.62			104/156	1.0
0.63–1.56			135/155	1.4 (0.9–2.0)
>1.57			110/156	1.0 (0.6–1.5)
				$P = 0.81$

[a] Adjusted by unconditional logistic regression for age, sex, ethnicity, pack-years of cigarette smoking, lifetime recreational physical activity, lifetime aspirin use, body mass index 5 years ago, years of schooling, and intake of nonstarch polysaccharides from vegetables and calcium from foods and supplements. The model for CYP1A2 phenotype was also adjusted for the number of cigarettes, cigars, and pipes smoked during the 2 weeks preceding the caffeine test.
[b] Number of cases/number controls.
[c] *P* for trend based on a trend variable assigned 1, 2, or 3 for the slow, intermediate, and rapid genotype, respectively, or assigned the median for the appropriate phenotype category.
[d] As determined by the urinary ratio (17U + 17X):137X.
[e] As determined by the urinary ratio AFMU:1X.

typed with the caffeine test. The proportion of controls with a rapid NAT2 genotype was 48.5% for Japanese, 4.7% for Caucasians, and 31.0% for Hawaiians. The frequencies of the specific *NAT2* genotypes are shown for each ethnic group in the "Appendix." Among controls, the frequency of the *NAT1*10* allele was 28.5% in Japanese, 17.1% in Caucasians, and 55.2% in Hawaiians. There was no association between CRC and the rapid *NAT2* genotype in all genotyped subjects or in the subset of phenotyped subjects (Table 3). The mean, median, and interquartile range for AFMU/1X were 0.4, 0.2, and 0.1–0.4 for the slow *NAT2* genotype; 1.3, 1.1, and 0.7–1.5 for the intermediate *NAT2* genotype, and 2.3, 1.8, and 1.3–2.5 for the rapid *NAT2* genotype. No association with CRC was found for the NAT2 phenotype or the *NAT1* genotype (Table 3). Among the controls, the mean, median, and interquartile range values for (17U+17X):137X were 12.4, 7.6, and 4.2–13.6 for current smokers and 8.8, 4.9, and 2.7–8.6 for nonsmokers. No association was observed between CYP1A2 phenotype and CRC (Table 3); however, there was the suggestion of an association for rectal cancer with ORs of 1.0, 1.6 (95% CI, 0.9–3.0), and 1.8 (95% CI, 1.0–2.3; *P* for trend = 0.07) for increasing tertiles of (17U+17X):137X. The corresponding values for colon cancer were 1.0, 0.9 (95% CI, 0.6–1.4), and 1.0 (95% CI, 0.7–1.6; $P = 0.83$).

Two-way interactions between *NAT2* genotype and red meat preference, CYP1A2 activity and red meat preference, *NAT2* genotype and CYP1A2 phenotype, and *NAT2* and *NAT1* genotype were explored. No suggestion of interactions was detected (data not shown). Table 4 presents the ORs for the three-way interaction among *NAT2* genotype, CYP1A2 activity, and red meat preference. For this analysis, subjects were cross-classified on their urinary CYP1A2 metabolic ratio (>median *versus* ≤median), red meat preference (well done/very well done *versus* did not eat/rare/medium-rare), and *NAT2* genotype (rapid *versus* slow/intermediate). The reference group included subjects with a slow/intermediate *NAT2* genotype and a CYP1A2 activity ≤ median who did not eat red meat or preferred it cooked rare, medium-rare, or medium. Compared with this group, individuals with the rapid *NAT2* genotype and a CYP1A2 activity > median who preferred their red meat well done or very well done had a 3.3-fold increased risk of CRC (95% CI, 1.3–8.1). The CRC ORs for the other categories of subjects were very close to 1.0, indicating that the increased risk with red meat was limited to those with the rapid phenotype for both NAT2 and CYP1A2. The test for interaction ($P = 0.12$) approached statistical significance. This three-way interaction analysis was repeated, successively, in Japanese, men, and women and for colon and rectal cancers. The same pattern

Table 4 Three-way interaction for *NAT2* genotype, CYP1A2 phenotype, and red meat preference

CYP1A2 Phenotype[a]							
≤Median				>Median			
NAT2	Doneness[b]	*N*[c]	OR[d] (95% CI)	*NAT2*	Doneness	*N*	OR (95% CI)
Slow/intermediate	Rare/medium	72/112	1.0	Slow/intermediate	Rare/medium	93/123	1.1 (0.7–1.8)
Slow/intermediate	Well-done	31/34	1.2 (0.7–2.3)	Slow/intermediate	Well-done	28/40	1.0 (0.6–1.9)
Rapid	Rare/medium	43/61	1.0 (0.6–1.8)	Rapid	Rare/medium	41/61	0.9 (0.5–1.5)
Rapid	Well-done	19/27	1.0 (0.5–1.9)	Rapid	Well-done	21/8	3.3 (1.3–8.1)
P for interaction[e] = 0.12							

[a] As determined by the urinary ratio (17U + 17X):137X; median = 5.1.
[b] Rare/medium includes did not eat, rare, medium-rare, and medium. Well-done includes well-done and very well-done.
[c] Number of cases/number of controls.
[d] ORs (95% CIs) adjusted by unconditional logistic regression for age, sex, ethnicity, pack-years of cigarette smoking, number of cigarettes, cigars, and pipes smoked during the 2 weeks preceding the caffeine test, lifetime recreational physical activity, lifetime aspirin use, body mass index 5 years ago, years of schooling, and intakes of nonstarch polysaccharides from vegetables and calcium from foods and supplements.
[e] Based on the likelihood ratio test comparing the model with interactions with one containing only main effects for these three variables.

Table 5 Three-way interaction for NAT2 phenotype, CYP1A2 phenotype, and red meat preference

CYP1A2 Phenotype[a]							
≤Median				>Median			
NAT2[b]	Doneness[c]	*N*[d]	OR[e] (95% CI)	NAT2	Doneness	*N*	OR (95% CI)
≤Median	Rare/medium	59/93	1.0	≤Median	Rare/medium	65/91	1.0 (0.6–1.7)
≤Median	Well-done	28/27	1.6 (0.8–3.1)	≤Median	Well-done	16/24	0.9 (0.4–1.8)
>Median	Rare/medium	56/80	1.0 (0.6–1.8)	>Median	Rare/medium	70/94	1.1 (0.7–1.8)
>Median	Well-done	22/34	0.8 (0.4–1.6)	>Median	Well-done	33/24	2.1 (1.0–4.2)
P for interaction[f] = 0.13							

[a] As determined by the urinary ratio (17U + 17X):137X; median = 5.1.
[b] NAT2 phenotype as determined by the urinary ratio (AFMU:1X); median = 1.1.
[c] Rare/medium includes did not eat, rare, medium-rare, and medium. Well-done includes well-done and very well-done.
[d] Number of cases/number of controls.
[e] ORs (95% CIs) adjusted by unconditional logistic regression for age, sex, ethnicity, pack-years of cigarette smoking, number of cigarettes, cigars, and pipes smoked during the 2 weeks preceding the caffeine test, lifetime recreational physical activity, lifetime aspirin use, body mass index 5 years ago, years of schooling, and intakes of nonstarch polysaccharides from vegetables and calcium from foods and supplements.
[f] Based on the likelihood ratio test comparing the model with interactions with one containing only main effects for these three variables.

of increased risk limited to the well-done/rapid/rapid category was observed in each group, with ORs of 2.7 (95% CI, 0.9–8.1), 4.1 (95% CI, 1.2- 13.6), 3.0 (95% CI, 0.6–13.9), 2.3 (95% CI, 0.9–6.4), and 2.8 (95% CI, 0.9–9.2), respectively.

Table 5 shows the ORs for the three-way interaction with all subjects combined when acetylation status was assessed by the urinary ratio AFMU:1X instead of imputed from the *NAT2* genotype. A similar interaction was observed with a somewhat weaker association [OR = 2.1 (95% CI, 1.0–4.2)] for individuals with NAT2 and CYP1A2 activities > median who preferred their red meat well done or very well done. The weaker risk estimate may be due to the increased misclassification resulting from the greater intraindividual and laboratory variability associated with the phenotyping compared with genotyping.

To explore the modifying effect of smoking, we reran the three-way interaction model separately for ever-smokers and never-smokers (Table 6). Although it was based on small numbers, this stratified analysis clearly suggested that the interaction was observed only in ever-smokers and not in never-smokers. This is consistent with the well-known inducible effect of smoking on CYP1A2.

No interaction was found among red meat intake, *NAT2* genotype, and CYP1A2 phenotype. All analyses using urinary metabolic ratios were repeated, further adjusting for urine volume. The results were not materially changed.

Discussion

In this case-control study, we found a 9-fold increase in CRC risk for ever-smokers who preferred their red meat well-done and had a rapid metabolic phenotype for both NAT2 and CYP1A2. Well-done red meat was not associated with risk among never-smokers or smokers with the slow or intermediate phenotype for one or both of these enzymes. The association was not explained by other known risk factors. These data suggest that exposure to carcinogens through consumption of well-done meat increases the risk of CRC only in genetically susceptible individuals, as determined by these two polymorphically expressed genes, and only in smokers. The *NAT1*10* allele was not associated with risk.

A recent review of the epidemiological data on red meat intake and CRC concluded that red meat probably increases the risk of this malignancy, although it was unclear whether this association was due to animal fat, processing or cooking methods, or other factors (2). A recent study in Hawaii also found that the association between beef intake and CRC was much stronger in subjects with a family history of the disease, suggesting a genetic component (21). The few past studies that have specifically explored the role of cooking have found a higher risk of CRC or adenoma for well-done or heavily browned meat (22–25), although some studies have been negative (26, 27). Cooking of meat produces mutagens and car-

Table 6 Three-way interaction for *NAT2* genotype, CYP1A2 phenotype, and red meat preference by smoking status

CYP1A2 Phenotype[a]							
≤Median				>Median			
NAT2	Doneness[b]	*N*[c]	OR[d] (95% CI)	*NAT2*	Doneness	*N*	OR (95% CI)
Never smokers							
Slow/intermediate	Rare/medium	32/57	1.0	Slow/intermediate	Rare/medium	32/53	0.9 (0.5–1.8)
Slow/intermediate	Well-done	18/12	1.9 (0.8–4.8)	Slow/intermediate	Well-done	17/19	1.3 (0.6–2.9)
Rapid	Rare/medium	16/26	0.9 (0.4–2.1)	Rapid	Rare/medium	16/28	0.8 (0.3–1.9)
Rapid	Well-done	9/15	0.8 (0.3–2.2)	Rapid	Well-done	9/6	1.5 (0.4–5.0)
			P for interaction[e] = 0.81				
Ever smokers							
Slow/intermediate	Rare/medium	40/55	1.0	Slow/intermediate	Rare/medium	61/70	1.3 (0.7–2.4)
Slow/intermediate	Well-done	13/22	0.9 (0.4–2.2)	Slow/intermediate	Well-done	25/33	0.6 (0.2–1.5)
Rapid	Rare/medium	27/35	1.2 (0.6–2.6)	Rapid	Rare/medium	11/21	0.9 (0.4–1.9)
Rapid	Well-done	10/12	1.3 (0.5–3.5)	Rapid	Well-done	12/2	8.8 (1.7–44.9)
			P for interaction = 0.01				

[a] As determined by the urinary ratio (17U + 17X):137X; median = 5.1.
[b] Rare/medium includes did not eat, rare, medium-rare, and medium. Well-done includes well-done and very well-done.
[c] Number of cases/number of controls.
[d] ORs (95% CIs) adjusted by unconditional logistic regression for age, sex, ethnicity, lifetime recreational physical activity, lifetime aspirin use, body mass index 5 years ago, years of schooling, intakes of nonstarch polysaccharides from vegetables and calcium from foods and supplements, and pack-years of cigarette smoking, and number of cigarettes, cigars, and pipes smoked during the 2 weeks preceding the caffeine test (smokers model only).
[e] Based on the likelihood ratio test comparing the model with interactions with one containing only main effects for the three variables.

cinogens, such as HAAs, polycyclic aromatic amines, and possibly other agents (3). The amount of carcinogens formed is clearly related to the temperature and duration of cooking (3, 28). A recent Swedish study using a detailed questionnaire to estimate intake of HAAs found no association with colon or rectal cancers, although an increased risk was suggested at the highest level of intake (29).

The metabolic activation of HAAs is thought to occur via *N*-oxidation in the liver by CYP1A2, followed by *O*-acetylation by NAT2 or NAT1 in the liver or colon to form *N*-acetoxy arylamine that binds to DNA to give carcinogen-DNA adducts (4, 5). An association between *NAT1* and CRC has been found in only one (11) of five past studies (9). Most previous studies, however, focused on the relationship between NAT2 phenotype or genotype and CRC risk. Three of the four studies that used a pharmacological probe to assess the acetylation phenotype found a higher CRC risk among individuals with the rapid phenotype (8, 30–32). A recent review of past studies of *NAT2* genotype and CRC or adenoma concluded that no consistent association has been found, with 10 of 11 studies showing no association with the rapid/intermediate acetylator genotype (9). However, most studies that examined the joint effect of dietary exposure and the NAT2 phenotype or genotype found a stronger effect on CRC or adenoma risk for meat (32), fried meat (33), red meat (34), or a meat mutagen index (35) in rapid/intermediate acetylators than in slow acetylators, providing some evidence for an interaction.

Lang *et al.* (7) further hypothesized that the individuals at greatest risk for CRC would be those who are not only exposed to HAA carcinogens and possess a rapid *N*-acetylation phenotype but also have a rapid *N*-oxidation phenotype. In their hospital-based case-control study of 75 CRC or polyp cases and 205 controls in Arkansas, they found that preference for well-done meat was associated with a 2.1-fold increased risk of CRC (95% CI, 1.0–4.1) and that the rapid-rapid NAT2/CYP1A2 phenotype was associated with a 2.9-fold increased risk of CRC (95% CI, 1.4–6.1). Consistent with their hypothesis, they also found a (nonsignificant) 6-fold increased risk of CRC for individuals with the well-done preference and the rapid-rapid NAT2/CYP1A2 phenotype compared with those with a rare/medium preference and the slow/slow phenotype (7). The present study specifically attempted to reproduce these findings with a population-based design, larger sample size, and careful adjustment for confounders. The results that we obtained are also in agreement with the hypothesis. However, they differ somewhat from those of past studies in that they suggest that risk of CRC is increased by consuming red meat only when it is cooked well-done and only among smokers with both the rapid CYP1A2 and NAT2 phenotypes. No increased risk was observed in genetically susceptible individuals who were less likely to be exposed or preferred their meat well-done but never smoked.

As discussed elsewhere (12), this finding is consistent with the descriptive and analytical epidemiology of CRC among Japanese migrants who came to the United States at the turn of the last century. The rates for this disease increased rapidly among this group to surpass the level of the host population, and Hawaii Japanese now have the highest reported CRC rates in the world (36, 37). More recently, CRC rates have been increasing in Japan (36, 37), presumably also as the result of the Westernization of the diet. Beef intake has been identified as an important CRC risk factor in Hawaii Japanese in two previous case-control studies (21, 38). Japanese were found to be more likely to prefer their red meat well done or very well done in the present study (27.5% *versus* 17.1% for Caucasians and 23.4% for Hawaiians). Although CYP1A2 activity does not show significant difference between Japanese and Caucasians (10), the rapid acetylation phenotype is much more common in Japanese, possibly conferring a distinct genetic susceptibility to CRC on this population. Thus, it is tempting to speculate that this genetic susceptibility manifested itself when Japanese migrated to the United States (and in more recent years in Japan) due to an increased exposure to well-done meat carcinogens, presumably HAAs. Nevertheless, additional factors, such as low vegetable and fiber intake and overnutrition, are also likely to have contributed to the increased CRC risk of Hawaii Japanese (10).

The strengths and limitations of the data need to be considered. The study was specifically designed to test the hypothesis, making it less likely that the association found in this study

Table 7 Frequencies for *NAT2* alleles and imputed phenotypes by case-control status and ethnicity

Imputed phenotype	Genotype	Japanese		Caucasian		Hawaiian	
		Case	Control	Case	Control	Case	Control
Slow	*NAT2*						
	*5/*5	2	0	27	27	3	3
	*5/*6	0	2	30	41	3	4
	*5/*7	0	1	2	3	2	4
	*6/*6	14	21	15	12	4	8
	*6/*7	11	17	4	3	0	4
	*7/*7	5	7	2	0	0	0
Intermediate	*NAT2*						
	*4/*5	6	6	30	49	8	7
	*4/*6	90	89	15	27	22	27
	*4/*7	42	61	6	1	4	3
Rapid	*NAT2*						
	*4/*4	150	192	16	8	30	27
Total		320	396	147	171	76	87

was due to chance. Also, strengthening the likelihood of a true association is the fact that the association was consistently observed across sex-, ethnic-, and subsite-specific groups and with NAT2 phenotype assessed by both genotyping and caffeine metabolism. Although it is still unclear which of the proposed ratios of caffeine metabolites assesses the CYP1A2 phenotype with the least error, the (17X + 17U):137X ratio is being used in a variety of epidemiology and biomarker studies (39). A differential bias due to case-control differences in caffeine absorption or urine flow resulting from the disease or its treatment appears unlikely because it would have to operate more strongly among individuals with the rapid/rapid phenotype. The simple cooking preference question used to assess exposure to HAAs in this study is likely to have resulted in misclassification and attenuation of the risk estimates. Thus, the association between well-done meat and CRC may in reality be significantly stronger and may not be limited to subjects with the rapid phenotypes.

As in other recent molecular epidemiology studies, the response rates were less than optimal due to the extensive participation requested from subjects. However, we found very little indication that nonparticipants were different in any major way from the interviewed subjects or that refusal to provide a biospecimen was associated with lifestyle or genotype. It seems unlikely that a selection bias could explain the observed interaction, but this cannot be excluded. Similarly, recall bias does not seem likely to explain our findings because it would have had to occur only in rapid metabolizers. However, replication of these results is desirable, preferably with a prospective design. Additional polymorphic genes are admittedly involved in the activation and detoxification of well-done meat carcinogens. However, our failure to consider them in the present study, as well as additional rare *NAT2* alleles, is more likely to have attenuated our risk estimates than to have created a spurious association.

In summary, these data provide support to the hypothesis that exposure to carcinogens (presumably HAAs) through consumption of well-done meat increases the risk of CRC in smokers with the rapid phenotype for both NAT2 and CYP1A2. Means for reducing the amount of HAAs formed in meat during cooking have been described and include marinating, precooking by microwaving, and frequently turning the meat over during cooking (40, 41).

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Appendix

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Combined Effects of Well-done Red Meat, Smoking, and Rapid *N*-Acetyltransferase 2 and CYP1A2 Phenotypes in Increasing Colorectal Cancer Risk

Loïc Le Marchand, Jean H. Hankin, Lynne R. Wilkens, et al.

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