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Age-associated Risk of Cancer among Individuals with *N*-Acetyltransferase 2 (*NAT2*) Mutations and Mutations in DNA Mismatch Repair Genes[1]

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Abstract

Mutations in *N*-acetyltransferase 2 (*NAT2*), a highly polymorphic enzyme involved in the metabolism of xenobiotics and carcinogens, may affect risk for colorectal cancer (CRC), especially among individuals with germ-line mutations in DNA mismatch repair genes. We determined the *NAT2* genotypes and allele frequencies for 86 individuals with CRC who had mutations in *hMLH1*, *hMSH2*, or *hPMS1*. No significant difference in time to onset was observed between rapid (NAT2*4) and slow (NAT2*5, NAT2*6, and NAT2*7) acetylators. However, when individuals were stratified separately by *NAT2* polymorphism (NAT2*5, NAT2*6, and NAT2*7), those who were heterozygous at the mutant locus NAT2*7 after adjustment for the *NAT2* mutant loci NAT2*5 and NAT2*6 had a significantly higher risk of CRC (hazard ratio, 2.96; $P = 0.012$) and all of the cancers (hazard ratio, 3.37; $P = 0.00004$) than individuals homozygous for wild type at the *NAT2*7* allele. These findings suggest that *NAT2* genotype may be an important factor in tumorigenesis of CRC and cancers related to hereditary nonpolyposis CRC among individuals with mismatch repair defects.

Introduction

NAT2[3] is a polymorphic isozyme of *N*-acetyltransferase and is found in a variety of tissues including the colorectal mucosa (1, 2). It catalyzes the metabolism of xenobiotics and carcinogens by transferring an acetyl group to these agents. Phenotypic variation in the rate at which the acetylation of these agents occurs is attributable in part to the polymorphic nature of the *NAT2* gene (3).

We assessed the role of *NAT2* as a modifier gene in HNPCC, an autosomal dominant disorder accounting for 3–14% of all of the cases of CRC (4–6). HNPCC has been associated with germ-line mutations in DNA MMR genes, specifically *hMSH2*, *hMLH1*, *hMSH6*, *hPMS1*, and *hPMS2*; *hMLH1* and *hMSH2* are the most commonly mutated (7, 8).

Testing for mutations in the *NAT2* gene by PCR assays has made it possible to predict the rate at which drugs and chemicals containing primary aromatic amine or hydrazine groups will be metabolized (9). Humans can be classified as either slow or rapid acetylators, based on a DNA amplification assay developed by Bell *et al.* (10) that was 100% concordant with rapid acetylator phenotype (as measured by caffeine metabolite excretion) and 90% concordant overall.

Results from previous studies of the association between *NAT2* genotype and CRC have been mixed; some groups (11, 12) have reported a positive association between fast acetylator phenotype and risk of CRC, whereas another report (13) did not. The aim of our study was to determine the extent to which polymorphisms in the *NAT2* gene affect risk for CRC and other cancers among individuals with DNA MMR mutations. We hypothesized that some of the observed variation in time to onset of CRC among carriers of mutations in DNA mismatch repair genes may be because of genetic variation in the *NAT2* gene at these polymorphic loci. Using Kaplan-Meier product limit estimates and Cox proportional hazards modeling, we compared time to onset of CRC and HNPCC-related cancers between carriers and noncarriers of mutations in *NAT2*, all of whom were predisposed to colon cancer as a result of inheriting mutations in the DNA MMR genes *hMLH1*, *hMSH2*, or *hPMS1*.

Materials and Methods

Subjects. The patients selected for DNA MMR mutation testing came from either the M. D. Anderson Hereditary Colorectal Cancer Registry or a consecutive series of CRC patients evaluated at the M. D. Anderson Cancer Center. The patients in the registry came from HNPCC or HNPCC-like families or were very young (<45 years of age) at CRC diagnosis. The consecutive series of CRC patients included all of the newly registered patients with adenocarcinoma of the colon or rectum evaluated at the M. D. Anderson Cancer Center during an 11-month period beginning in September 1994. From these two sources, we identified 86 individuals with germ-line mutations in *hMLH1* or *hMSH2*. Of these, 43 were index cases, 37 were first-degree relatives of those cases, and the other 6 were more distant relatives. Of the 86 carriers, 49 were affected with CRC. There also was one case each of breast, cervical, uterine, and oral cancer, one unspecified malignant neoplasm of the female genitalia, and one malignant neoplasm of unspecified origin.

DNA Extraction from Peripheral Blood Leukocytes. Blood was drawn from each study subject in Vacutainer tubes containing EDTA (Becton Dickinson Vacutainer System; Becton Dickinson, Rutherford, NJ). DNA was isolated from the blood with a 341 Nucleic Acid Purification System (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

Testing for Mutations in *hMLH1* and *hMSH2* Using Heteroduplex and SSCP Analysis. The DNA was subjected to PCR with the primers for each exon of the *hMLH1* and *hMSH2* genes used by Wijnen *et al.* (14, 15), except that the GC clamp and M13 sequences were not included. PCR was performed on 500 ng of DNA in a 20-μl reaction mixture of 50 mM KCl; 10 mM Tris-HCl (pH 8.3); 1.5 mM $MgCl_2$; 0.2 mM dATP, dGTP, and dTTP; 0.1 mM dCTP; 20 pmol of each primer; 1 μCi of [^{32}P]dCTP (3000 Ci/mmol); and 1.0 unit of Taq polymerase (Perkin-Perkin-Elmer Corp., Norwalk, CT). Each PCR mixture was incubated at 94°C for 3 min and then subjected to 25 cycles of 94°C for 30 s, 58°C for 30 s, and 72° for 1 min. A 3-min final extension was performed at 72°C. For heteroduplex analysis, 4 μl of each PCR product was mixed with equal volumes of loading buffer containing 95% formamide, 20 mM EDTA, 0.05% xylene cyanol, and 0.05% bromphenol blue. This was heated at 94° for 10 min, slowly cooled to room temperature overnight, and subjected to electrophoresis on Mutation Detection Enhancement gels (J. T. Baker, Phillipsburg, NJ) as described previously by Jeon *et al.* (16). For SSCP analysis, the same procedure was followed except that after heating at 94°C, the DNA was promptly chilled on ice and then loaded onto the gel. The gels were then vacuum dried and subjected to autoradiography overnight at −80°C. Nucleo-

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[3] The abbreviations used are: *NAT2*, *N*-acetyltransferase 2; HNPCC, hereditary nonpolyposis colorectal cancer; CRC, colorectal cancer; MMR, mismatch repair; SSCP, single-stranded conformational polymorphism; HR, hazard ratio; CI, confidence interval.

tide sequence analysis was performed using one primer that was used to generate the PCR product.

Once an altered banding pattern was detected, exon-specific nucleotide sequence analysis was performed to determine the sequence. To accomplish this, the exon was subjected to PCR as described above except that the [^{32}P]dCTP was omitted. Before nucleotide sequencing, the PCR products were cleaned up by mixing them with 20 units of exonuclease 1 and four units of shrimp alkaline phosphatase followed by incubation at 37°C for 15 min and then at 85°C for 15 min to remove the unused primers and residual deoxynucleotide triphosphates. The products were then subjected to electrophoresis in 1.5% agarose gels in 1 × Tris-borate EDTA. Ethidium bromide was used to visualize the PCR products, verify the fragment length, and determine the concentration. The DNA sequences of PCR products were then determined using an Applied Biosystems model 377 sequencer.

***NAT2* Genotype Analysis.** The subjects were genotyped by PCR followed by SSCP analysis. Briefly, the PCR reaction conditions are the same as described above with the exception of the primers. The PCR primers used were: 2590F (5′-GGACCAAATCAGGAGAGAGCAG 3′) and 2590R (5′-GTTGGAGACGTCTGCAGGTATG-3′) for NAT2*5; 2857F (5′-GAAGAG-GTTGAAGAAGTG-CTG-3′) and 2857R (5′-GTTGGGTGATACATACA-CAAGG-3′) for NAT2*6; and 2481F (5′-AAGGATCAGCCTCAGGT-GCCTT-3′) and 2481R (5′-CTGCTCTCTCCTGATTTGGTCC-3′) for NAT2*7. The PCR was performed for 10 min at 94°C followed by 28 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 10 min. SSCP analysis was performed as described above. The identities of the *NAT2* alleles were confirmed by automated sequencing of several samples with an Applied Biosystems Model 377 sequencer as described above.

Statistical Analysis. To assess differences in time to onset of cancer for individuals of different *NAT2* genotypes, we used survival-analysis procedures (17). We calculated the median time to onset for cancer from the Product-Limit Kaplan-Meier estimator. To test for differences in the time to onset in individuals of different *NAT2* genotypes, we used Cox proportional hazard regression analysis (18). The estimates from Cox modeling were exponentiated to give an estimate of the increased risk conferred by having mutant genotypes at each of the three *NAT2* loci. Alleles *NAT2*5*, *NAT2*6*, and *NAT2*7* were assigned as indicator variables, and the wild-type genotype was used as reference. The time to onset to cancer for individuals in the same family may be correlated because of unmeasured covariables such as shared household environment and shared dietary exposures or because they share the same mutation. To allow for this possible correlation, we used the cluster function of S+ in the Cox proportional hazard model that we fitted. This action corrected the SEs of the robust estimates for familial correlation using a sandwich estimator of the variance. We analyzed the fast and slow acetylators of the NAT2 phenotypes by the same approach (19).

Results

***NAT2* Genotype, Age of Onset, and Cancer Risk.** Twenty-eight of the subjects had mutations in *hMLH1*, 57 had mutations in *hMSH2*, and 1 patient had a mutation in *hPMS1*. These 86 individuals were analyzed by *NAT2* genotype and age of onset for CRC and all of the cancers (Table 1 and Table 2). No subjects were homozygous for the *NAT2*7* mutant allele. In multivariate analysis of NAT2*5, NAT2*6, and NAT2*7, we found significantly increased risks for CRC among NAT2*5 ($P = 0.0034$) and NAT2*7 ($P = 0.012$) heterozygotes with HRs of 2.38 and 2.96, respectively. When the outcome was all of the cancers, only NAT2*7 heterozygotes had a significantly elevated HR of 3.37 ($P = 0.00004$). The median times to onset for NAT2*7 mutants for both CRC and all of the cancers were decreased compared with wild-type *NAT2*, but consistent effects were not seen for NAT2*5 and NAT2*6. Subjects heterozygous for the *NAT2*7* mutant allele had increased HRs for both CRC (HR, 2.1; $P = 0.053$) and for all of the cancers (HR, 2.8; $P = 0.00001$) compared with wild-type homozygotes (Table 3).

Table 1 *Genotype analysis of 86 patients with mutations in MMR genes at each of three NAT2 loci*

NAT2 locus	WT/WT[a]	WT/M[b]	M/M	Total *N*[c]
*NAT2*5* q[d] = 0.34 (56/168)	39	34	11	84
*NAT2*6* q = 0.30 (51/170)	44	31	10	85
*NAT2*7* q = 0.07 (11/168)	73	11	0	84

[a] WT, wild type at *NAT2* locus indicated in first column.
[b] M, mutant at *NAT2* locus indicated in first column.
[c] Not equal to total number of subjects because we were unable to obtain genotypes for some. All of the subjects that were genotyped for each locus are presented in this table including both affected and unaffected subjects.
[d] q, allele frequency.

Table 2 *Median time to diagnosis (years) for CRC and all of the cancers for all of the subjects stratified by NAT2 genotype and number of affected individuals*[a]

Cancer	*NAT2* locus	(WT/WT[b])	(WT/M[c])	(M/M)
CRC	*NAT2*5*	50.0 ($n = 21$)	48.0 ($n = 22$)	36.0 ($n = 5$)
	*NAT2*6*	47 ($n = 24$)	57.1 ($n = 17$)	46.7 ($n = 8$)
	*NAT2*7*	53.6 ($n = 42$)	46.7 ($n = 6$)	($n = 0$)
All cancers	*NAT2*5*	48.0 ($n = 25$)	48.0 ($n = 22$)	43.0 ($n = 6$)
	*NAT2*6*	42.0 ($n = 27$)	57.0 ($n = 19$)	42.0 ($n = 8$)
	*NAT2*7*	48.0 ($n = 46$)	39.5 ($n = 7$)	($n = 0$)

[a] *n*, number of affected individuals.
[b] WT, wild type at *NAT2* locus indicated in second column.
[c] M, mutant at *NAT2* locus indicated in second column.

Acetylator Phenotype. Of the 86 individuals tested, 38 (47%) had two of any of the three mutant *NAT2* alleles and, therefore, were classified as slow acetylators. The remaining 44 (53%) had only one or no mutant alleles and were classified as rapid acetylators. We used Cox proportional hazard regression of risk of CRC and cancer stratified by acetylator phenotype. When the rapid acetylator genotype was used as the reference genotype, the risk for CRC in slow acetylators *versus* rapid acetylators was not significantly different (HR, 1.53; 95% CI, 0.087–2.69; $P = 0.14$). Similarly, the risk for all of the cancers in slow acetylators *versus* fast acetylators was not significantly different either (HR, 1.14; CI, 0.69–1.91; $P = 0.61$).

Discussion

Some studies have indicated that slow acetylators have a lower risk of CRC (11, 12). In contrast, a recent study by Heinimann *et al.* (20) suggested an increased risk of cancer among slow acetylators with HNPCC, who had CRC. The *NAT2* genotype was determined for 26 unaffected *hMLH1/hMSH2* mutation carriers and 52 with cancer in 21 Swiss HNPCC families. Slow acetylators were significantly more prevalent among affected MMR mutation carriers than among unaffected mutation carriers. In our study on HNPCC, we did not observe an increase in prevalence of slow acetylators or fast acetylators among the mutation carriers with cancer. However, we did find that the *NAT2*7* mutant allele of *NAT2*, which confers the slow acetylator phenotype, increased the risk for CRC and all of the cancers in HNPCC carriers. We also found that the *NAT2*5* mutant allele of *NAT2* increased the risk for CRC in heterozygotes, although the level of significance was not so great as for NAT2*7.

The different conclusions regarding the association of slow acetylation phenotypes in different populations might be because of different genetic and environmental influences. Patients with HNPCC may be more sensitive to certain environmental influences than subjects without MMR gene defects. The detrimental effects of such environmental factors might be enhanced by the *NAT2*7* mutant allele in HNPCC while having little or no effect in other subjects.

Our findings also suggest that there may be differences in the slow acetylation phenotypes produced by different *NAT2* genotypes, perhaps because of variation in the enzyme activity depending on the substrate. A recent review by Hein *et al.* (21) discusses the complexities of assessing phenotype with the different *NAT2* genotypes. They point out that multiple mechanisms for reduction in *N*-acetyltransferase activity are associated with various nucleotide substitutions present on *NAT2* alleles and that the ability to distinguish acetylator

Table 3 *Cox proportional hazard regression of risk of CRC and cancer stratified by NAT2 genotype*

	Univariate results				Multivariate results (adjusted by NAT2*5, NAT2*6, and NAT2*7)			
	HR	95% CI-L[a]	95% CI-U[b]	*P*	HR	95% CI-L	95% CI-U	*P*
CRC								
NAT2*5 (wt[c]/wt)	1				1			
NAT2*5 (wt/m[d])	1.70	0.969	2.985	0.0640	2.38	1.332	4.251	0.0034
NAT2*5 (m/m)	1.20	0.388	3.691	0.7600	1.6	0.495	5.181	0.4300
NAT2*6 (wt/wt)	1				1			
NAT2*6 (wt/m)	0.62	0.351	1.086	0.0940	0.75	0.415	1.350	0.3300
NAT2*6 (m/m)	0.94	0.368	2.370	0.8900	1.82	0.586	5.674	0.3000
NAT2*7 (wt/wt)	1				1			
NAT2*7 (wt/m)	2.10	0.989	4.475	0.0530	2.96	1.280	6.854	0.0120
NAT2*7 (m/m)								
Cancer								
NAT2*5 (wt/wt)	1				1			
NAT2*5 (wt/m)	1.22	0.686	2.163	0.5000	1.34	0.730	2.460	0.3500
NAT2*5 (m/m)	1.1	0.435	2.776	0.8400	1.13	0.409	3.106	0.8200
NAT2*6 (wt/wt)	1				1			
NAT2*6 (wt/m)	0.59	0.340	1.031	0.0640	0.63	0.327	1.216	0.1700
NAT2*6 (m/m)	0.7	0.266	1.823	0.4600	0.94	0.300	2.948	0.9200
NAT2*7 (wt/wt)	1				1			
NAT2*7 (wt/m)	2.82	1.761	4.546	0.00001	3.37	1.918	5.909	0.00004
NAT2*7 (m/m)								

[a] CI-L, lower boundary of 95% confidence interval.
[b] CI-U, upper boundary of 95% confidence interval.
[c] WT, wild type at this *NAT2* locus.
[d] M, mutant at this *NAT2* locus.

phenotypes is complex and is a function of sensitivity and specificity of the phenotyping method.

The different results could also be because of different *NAT2*5*, *NAT2*6*, and *NAT2*7* mutant allele frequencies in different populations. In the group of subjects studied by Heinimann *et al.* (20), the frequency of the *NAT2*6* mutant allele is much lower than in our population. Our study suggested that although the *NAT2*5*, *NAT2*6*, and *NAT2*7* mutant alleles may all confer the slow acetylation phenotype, only *NAT2*5* and *NAT2*7* were associated with increased risk for cancer. Therefore, the higher frequency of the *NAT2*6* mutant alleles (which were not associated with increased risk) in our study may have reduced the actual effects of slow acetylation on cancer risk.

How the *NAT2* mutant alleles modify MMR defects to increase age-associated risk for cancer is not clear. A more complete understanding is needed of the tissue-specific action of *NAT2* and the drugs and compounds it metabolizes in the colon. Additional studies that include environmental exposures in *NAT2* mutation analysis might increase our knowledge of how variation in the *NAT2* gene affects time to onset and risk of CRC in carriers of mutations in DNA MMR.

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