DNMT3b Polymorphism and Hereditary Nonpolyposis Colorectal Cancer Age of Onset

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Abstract

Hereditary nonpolyposis colorectal cancer (HNPCC) is an autosomal dominant syndrome of familial malignancies resulting from germ-line mutations in DNA mismatch repair genes. Colorectal and endometrial cancers are most frequently observed. A polymorphic C-to-T change in the promoter region of the *DNMT3b* gene, −149 bp from the transcription start site, is reported to greatly increase promoter activity and is associated with increased risk for lung cancer and decreased postsurgical survival in patients with small cell carcinoma of the head and neck. We studied the influence of this *DNMT3b* polymorphism on HNPCC age of onset. We determined the *DNMT3b* genotype of 146 mismatch repair mutation carriers from 72 families. Of these, 74 participants had colorectal cancer. The participants were genotyped by single-strand conformational polymorphism analysis and DNA sequencing. We tested the association between age of onset and *DNMT3b* genotypes by comparing Kaplan-Meier survival curves, evaluating the homogeneity of the curves using the log-rank test, Wilcoxon's test, and Fleming-Harrington test and estimating the strength and direction of the association using the Cox proportional hazards regression model adjusting for potential demographic and genetic confounding factors. HNPCC patients carrying one or two copies of the *DNMT3b* variant T allele developed their colorectal cancer significantly earlier than HNPCC patients who were homozygous for the wild-type *DNMT3b* allele. Combining knowledge of an individual's *DNMT3b* genotype with information on other genetic and environmental risk factors may improve risk estimates and help to identify individuals who are genetically susceptible to developing HNPCC at an earlier age. (Cancer Epidemiol Biomarkers Prev 2006;15(5):886–91)

Introduction

Hereditary nonpolyposis colorectal cancer (HNPCC) is an autosomal dominant syndrome characterized by an 80% lifetime risk of developing cancer, predominately colorectal and endometrial cancer. Other extracolonic malignancies include gastric, ureter, renal pelvis, and ovarian cancers (1-4). The syndrome results from germ-line mutations in DNA mismatch repair (MMR) genes. The MMR genes most often mutated are *hMLH1* and *hMSH2* (5-9).

Although all of these MMR gene mutation carriers are predisposed to developing the cancers that are characteristic of HNPCC, there is considerable variability in their age of onset. The variability is probably due to a combination of genetic and environmental factors. In this study, we investigated the influence of a polymorphism in the promoter region of the *DNMT3b* gene on HNPCC age of onset in a population of MMR gene mutation carriers.

DNMT3a and DNMT3b are required for the establishment and maintenance of genomic methylation patterns and proper murine development (10). Both genes are up-regulated to differing degrees in some malignancies, including colon cancers (11). They directly repress transcription, independent of their methylating capacities, by way of different NH_2-terminal transcriptional repression domains (10).

The *DNMT3b* gene contains a C-to-T single nucleotide polymorphism (SNP) −149 bp from the transcriptional start site that may result in increased promoter activity of the gene (12). Heterozygous carriers of the polymorphism have been shown to have decreased survival following surgical resection in patients with small cell carcinoma of the head and neck (13). In addition, the presence of the variant allele (heterozygous or homozygous) has been associated with a 2-fold increase in risk for lung cancer (12). Although the mechanism of this association is unknown, it is suggested that up-regulated gene expression caused by the presence of the SNP may increase lung cancer risk by way of increased aberrant *de novo* methylation of CpG islands and thus transcriptional repression of some tumor suppressor genes (11, 12, 14, 15).

By this mechanism, if MMR mutation carriers are also carriers of the *DNMT3b* variant allele, the resulting influence on promoter hypermethylation of genes in other interacting pathways, such as cell cycle, apoptosis, or other DNA repair pathways, may enhance the level of DNA damage that accumulates as a result of the MMR deficiency. Previous studies suggest that adverse polymorphic genotypes of the *cyclin D1*, N-*acetyltransferase 2*, and *p53* genes influence age-associated risk for HNPCC (16-18). We hypothesize that the polymorphic genotype of *DNMT3b* may similarly affect HNPCC age of onset, accounting, in part, for the variation in age of onset seen in our HNPCC patients. Thus, in this study of MMR mutation carriers, we examined the HNPCC age of onset by *DNMT3b* genotype.

Materials and Methods

Subjects. We studied 146 confirmed MMR mutation carriers from 72 families in our HNPCC registry, who were identified

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and recruited as described previously (16). Demographic data are listed in Table 1. Most of the probands were patients seen at The University of Texas M. D. Anderson Cancer Center. The ethnic/racial distribution of the study participants was 122 Caucasian (83.5%), 13 African American (8.9%), 10 Hispanic (6.9%), and 1 Asian (0.7%). Of the 72 families, 26 families were represented in the study by more than one family member. Of the 146 study participants, 62 (42.5%) were probands and the remaining 84 (57.5%) were recruited relatives of probands. Of the 74 individuals who had colorectal cancer, 53 (71.6%) were probands, thus ascertained because of their cancer history. Of the 72 unaffected MMR mutation carriers, 9 (12.5%) were probands and 63 (87.5%) were recruited relatives. The mean age of individuals with and without colorectal cancer was 43.5 and 44.4 years, respectively.

All of the participants gave informed consent. Age of onset for colorectal cancer was defined as the patient's age at diagnosis. For the unaffected carriers, the age of onset was the age at which blood was drawn, the age at last contact, or the age at death. The MMR mutation status of all participants was confirmed by a Clinical Laboratory Improvement Act–certified laboratory. Each study subject contributed blood and DNA was extracted using an AUTOPURE LS Automated DNA Purification Instrument (Gentra Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions.

PCR and Single-Strand Conformational Polymorphism Analysis. PCR and single-strand conformational polymorphism (SSCP) analyses were used to genotype the C-to-T *DNMT3b* polymorphism. Briefly, PCR fragments were generated from 100 ng genomic DNA in a 20 μL reaction mixture containing 1× GeneAmp PCR buffer [500 mmol/L KCl, 100 mmol/L Tris-HCl (pH 8.3), 15 mmol/L $MgCl_2$; Applied Biosystems, Foster City, CA]; 0.2 mmol/L each of dATP, dGTP, dTTP, and dCTP (Invitrogen Corp., Carlsbad, CA); 20 pmol/L of each primer (Sigma/Genosys, The Woodlands, TX); 0.1 μL [^{2}P]dCTP (3,000 Ci/mmol; Perkin-Elmer Life Sciences, Inc., Boston, MA); and 0.5 units AmpliTaq Gold DNA polymerase (Applied Biosystems). The PCR was done at 95°C for 10 minutes followed by 24 cycles at 94°C for 30 seconds, 53°C for 30 seconds, and 72°C for 30 seconds and a final extension step at 72°C for 3 minutes. The PCR primers used were 5′-CTGGCTACCAGGTCTCCTTG-3′ (primer 1) and 5′-CGGCTGGAGACACTGTTGTT-3′ (primer 2).

Table 1. Demographics of study population

	Colorectal cancer	Without colorectal cancer	Total
Gender			
Male	39	30	69
Female	35	42	77
Race/ethnicity			
Caucasian	58	64	122
African American	9	4	13
Hispanic	6	4	10
Asian	1	0	1
Age*			
Mean	43.5	44.5	44
Median	41.5	43	42
Range	23-84	24-84	23-84
MMR mutation			
MLH1	26	26	52
MSH2	48	46	94
Mutation type			
Missense	16	16	32
Truncating/deletion	58	56	114
Ascertainment			
Proband	53	9	62
Relative of proband	21	63	84

*The age of onset is the age at first diagnosis for colorectal cancer patients and the age at blood draw or last contact for unaffected mutation carriers.

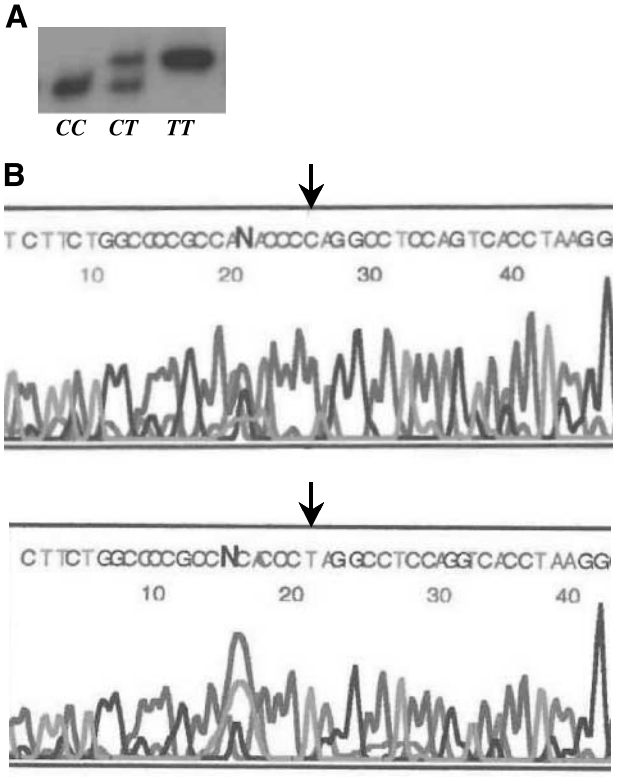

Figure 1. Representative screening for the DNMT3b genotypes. **A.** Patterns detected for each of the three genotypes, *CC, CT*, and *TT*, by SSCP analysis. **B.** Results of nucleotide sequencing analysis of each SSCP band pattern. *Arrows,* location of the nucleotide at which the polymorphism occurs.

For SSCP analysis, the PCR product was mixed with an equal volume of loading buffer containing 95% formamide, 20 mmol/L EDTA, 0.05% xylene cyanol, and 0.05% bromphenol blue; heated at 95°C for 5 minutes; and quickly chilled on ice for at least 5 minutes. The mixture was separated on a mutation detection enhancement gel (FMC BioProducts, Rockland, ME) that was prepared according to the manufacturer's instructions, except that the gel solution was diluted 1:4 with 1× Tris-borate-EDTA. The running buffer was also 1× Tris-borate-EDTA. The gels were vacuum dried and autoradiographed.

Genotyping of the *cyclin D1* and *p53* gene SNPs was carried out as described previously (17, 18).

DNA Sequencing Analysis. The *DNMT3b* genotypes of the three different SSCP band patterns were identified by DNA sequencing analysis (Fig. 1). The PCR product from a representative sample of each SSCP banding pattern was subjected to nucleotide sequence analysis, as described previously, to determine the sequence to which each of the banding patterns corresponded (17). In addition, for purposes of quality control, SSCP genotyping results were confirmed on a fourth of the samples by nucleotide sequence analysis using the ABI 3100.

Statistical Analysis. Kaplan-Meier survival analysis estimates the proportion of the population surviving over the ages of the population. Applying this method to our study allows plotting of the proportion of the population that is cancer-free by the participants' age at the time of evaluation. Such analysis permits visualization and comparison of the dynamics of cancer onset by genotype and is a more powerful method of analysis than a case-control design.

To analyze the data, we defined age of onset for colorectal cancer as the outcome and *DNMT3b* genotype as an independent variable. All statistical analyses were done using Stata 8.0 (Stata Corp., College Station, TX). We tested for Hardy-Weinberg equilibrium using an exact test based on genotypic frequencies (19). We tested the association between age of onset and *DNMT3b* polymorphism by comparing Kaplan-Meier survival curves according to *DNMT3b* genotype and then evaluated the homogeneity of the survival curves by using the log-rank test, Wilcoxon's test, and Fleming-Harrington test. The log-rank test gives equal weight to all failures; the Wilcoxon test emphasizes observations from early-onset patients; and the Fleming-Harrington test emphasizes observations from late-onset patients. The log-rank test is usually the preferred test, but the other test results were presented to show the influence of the polymorphisms at different age points.

We also used the Cox proportional hazards regression model to estimate the association between age-related colorectal cancer risk and *DNMT3b* genotype, adjusting for potential demographic and genetic confounding factors. Hazard ratios (HR) and 95% confidence intervals (95% CI) were calculated from Cox regression analysis to determine the direction and strength of the association, with and without a robust variance correction (20), which adjusts for intrafamilial correlations in time to onset for cancer. We did a sensitivity analysis in which we only included the 84 relatives of probands to assess genotypic effects in this group, which was not influenced by clinical referral patterns.

Results

***DNMT3b* Genotyping.** A 145-bp PCR fragment was generated, and three different genotypes were distinguished by PCR-SSCP analysis and identified by DNA sequencing analysis (Fig. 1). In the study population, the genotypes were in Hardy-Weinberg equilibrium (Pearson $\chi^2_1 = 0.02$; $P = 0.88$; ref. 19).

Subject Demographics. Table 2 summarizes the demographics of the study population by *DNMT3b* genotype, including cancer status, gender, age of onset, and frequencies of the *DNMT3b* alleles. The age of onset values were determined from Kaplan-Meier survival-time data, with the 25th, 50th, and 75th percentiles of cancer-free survival time reported. The median age of onset, defined as the age at which 50% of the population is cancer-free, was 50 years for heterozygotes and 48 years for homozygotes of the variant allele versus 71 years for homozygotes of the wild-type *DNMT3b* allele. When heterozygotes and homozygotes of the variant allele were combined, the median age of onset was 50 years for the combined group.

Of the 72 families, 26 (36.1%) families were represented by two to eight members per family. Because it is possible that genetic or familial factors in addition to *DNMT3b* induce a correlation in family members, we applied a robust variance correction in the Cox regression analysis (20).

There was no significant difference between the age of onset of the subjects with *hMLH1* mutations and those with *hMSH2* mutations as assessed by the log-rank test ($P = 0.90$; data not shown). Similarly, we did not observe a difference in age of onset between subjects with missense mutations and truncating mutations when the data were analyzed by the same procedures ($P = 0.12$; data not shown).

HNPCC Age of Onset and *DNMT3b* Genotype. Kaplan-Meier survival analysis indicated that the median age of onset in patients who were heterozygous (CT) was significantly earlier than that of patients who were homozygous for the wild-type (CC) *DNMT3b* allele, and homozygotes for the variant allele (TT) also developed cancer significantly earlier than wild-type homozygotes (Fig. 2A).

Complete results of the statistical analysis of the population by genotype, including HR and 95% CI, are included in Table 3. The Fleming-Harrington test, which emphasizes

Table 2. Demographics of study population by *DNMT3b* genotype

	CC, *n* (%)	CT, *n* (%)	TT, *n* (%)	Total
Cancer status				
Colorectal cancer patients	12 (16.2)	45 (60.8)	17 (23.0)	74
Unaffected MMR mutation carriers	28 (38.9)	27 (37.5)	17 (23.6)	72
Gender				
Male	17 (24.7)	37 (53.6)	15 (21.7)	69
Female	23 (29.9)	35 (45.4)	19 (24.7)	77
MMR gene mutated				
MLH1	14 (26.9)	23 (44.2)	15 (28.9)	52
MSH2	26 (27.7)	49 (52.1)	19 (20.2)	94
MMR gene mutation type				
Truncation/deletion	33 (28.9)	57 (50.0)	24 (21.1)	114
Missense	7 (21.9)	15 (46.9)	10 (31.2)	32
Ethnicity/race				
Caucasian	36 (29.5)	60 (49.2)	26 (21.3)	122
African American	0	8 (61.5)	5 (38.5)	13
Hispanic	4 (40)	4 (40)	2 (20)	10
Asian	0	0	1 (100)	1
All subjects	40 (27.4)	72 (49.3)	34 (23.3)	146
Allele frequency				
C allele				0.5205
T allele				0.4795
Age of onset for colorectal cancer (y)*				
75% of subjects cancer-free†	47	37	41	39
50% of subjects cancer-free†	71	50	48	50
25% of subjects cancer-free†	—	59	—	63
Time at risk	1,740	3,182	1,486	6,408
Range	23-77	23-84	28-84	23-84

NOTE: All participants were confirmed carriers of a HNPCC-related MMR mutation.

*The age of onset is the age at first diagnosis for colorectal cancer patients and the age at blood draw for unaffected MMR mutation carriers.

† The age of onset values were determined from Kaplan-Meier estimates, with the median age of onset defined as the age at which 50% of the population is cancer-free.

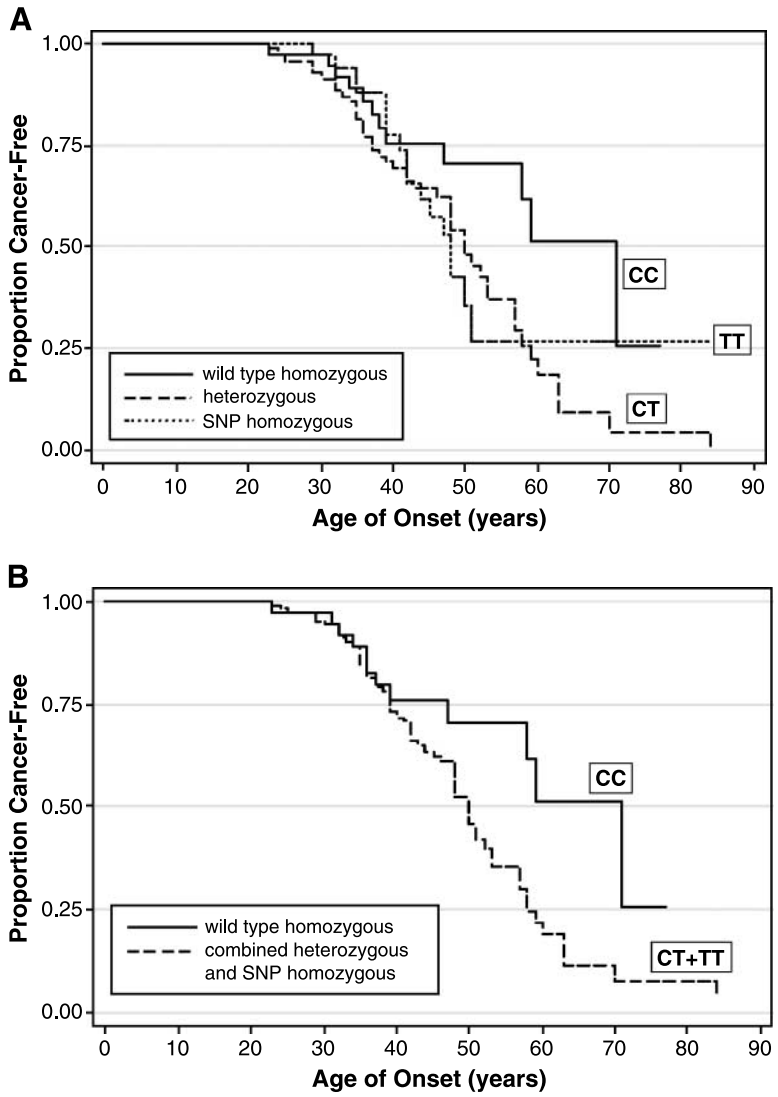

Figure 2. Kaplan-Meier survival analysis by DNMT3b genotype. The plots show the effect of the *DNMT3b* genotypes on age of onset for colorectal cancer in MMR mutation carriers from HNPCC families. *CC,* homozygous wild-type; *CT,* heterozygous; *TT,* homozygous variant allele. **A.** By genotype. **B.** *CT* and *TT* genotypes combined.

observations from late-onset patients, showed a significant difference between the curves ($P = 0.047$), suggesting that the *DNMT3b* polymorphism may manifest its effects later in life. The log-rank test, which gives equal weight to all failures, showed a nearly significant difference between the curves ($P = 0.064$). The Wilcoxon test, which emphasizes observations from early-onset patients, did not ($P = 0.339$), providing supporting evidence for a later effect of the polymorphism.

Sensitivity analysis using only the 84 relatives of probands showed a similar pattern with later onset among CC carriers ($P = 0.078$), for whom the median age of onset could not be estimated because too few events occurred. The ages at which 25% of individuals developed colorectal cancer were 58 years for CC carriers, 50 years for CT carriers, and 45 years for TT carriers.

We combined the heterozygotes and the homozygotes of the variant allele and reanalyzed the data (Fig. 2B). The median age of onset for the combined genotype group was significantly earlier than that of the homozygous wild-type group. Complete results of the statistical analysis of the study population with combined genotypes are included in Table 3. The curves were significantly different by the log-rank test ($P = 0.021$) and Fleming-Harrington test ($P = 0.016$) but not by the Wilcoxon test ($P = 0.155$), again indicating a later effect of the polymorphism.

The Cox proportional hazards regression model was used to evaluate the strength and direction of the association between colorectal cancer age of onset and *DNMT3b* genotype. The robust correction adjusts the variance of the test for homogeneity by genotype by accounting for clustering in time to onset within families. Cox analysis of the combined genotypes showed significant variability in time to onset by genotype with ($P = 0.028$) and without ($P = 0.017$) the robust correction, indicating that time to onset is not correlated within families. The HR indicates that carriers of at least one *DNMT3b* variant allele are 2.03 times more likely ($P < 0.03$, with or without the robust correction) to get colorectal cancer during any age interval than those with the homozygous wild-type genotype. After adjusting for race/ethnicity in the Cox model, the hazard associated with having a risk allele for *DNMT3b* did not change from the unadjusted hazard (2.05 versus 2.03). However, a modifying effect of race/ethnicity cannot be ruled out due to the low number of non-Caucasians in the study.

Genotype data were available for SNPs in the *cyclin D1* and *p53* genes for all subjects examined in this study. Previously, our laboratory has shown that these SNPs also modify age of onset for colorectal cancer (17, 18). These genes were included as covariates in the Cox model. The HR of having a risk allele for *DNMT3b* did not change after adjusting for *p53* genotypes (HR, 2.10; 95% CI, 1.12-3.94) or after adjusting for *cyclin D1* genotypes (HR, 1.96; 95% CI, 1.04-3.68), indicating that the HR

associated with having a risk allele for *DNMT3b* did not vary from the unadjusted HR.

Interestingly, when the analysis was limited to those whose colorectal cancer occurred at age ≥40 years, the median age difference was less, but the results were highly significant without combining genotypes (log-rank test, $P = 0.017$) or with combined genotypes (log-rank test, $P = 0.005$; Table 3). The median age of onset by genotype was 71 years for wild-type homozygotes, 57 years for heterozygotes, and 51 years for SNP homozygotes. In addition, Cox analysis confirmed the difference in age of onset by genotype ($P = 0.028$) and the resulting HRs were significant for both heterozygotes (HR, 3.71; $P = 0.008$) and homozygotes (HR, 4.46; $P = 0.020$) of the variant allele. Limiting the sample to an age of onset of ≥40 years reduced the sample size to 86 MMR mutation carriers, of whom 39 (45.3%) had colorectal cancer. Thus, these results should be confirmed in a larger population. Such results could prove useful in predicting individual HNPCC risk in an age-specific manner.

Discussion

In this study, we found that MMR mutation carriers who also carried at least one T allele for the *DNMT3b* promoter region C-to-T polymorphism had a 2-fold higher risk for colorectal cancer by year than those who were homozygous for the wild-type *DNMT3b* allele. In addition, *DNMT3b* heterozygotes developed colorectal cancer significantly earlier than those who were homozygous for the wild-type allele. It was interesting that the influence of the polymorphism does not appear until around age 40 years. If confirmed in larger studies, such findings might be important for predicting individual HNPCC risk in an age-specific manner.

This study is the first to examine a polymorphism of the *DNMT3b* gene in HNPCC or any colorectal cancer. Our findings are consistent with those of Shen et al. (12), which report the T allele to be an adverse genotype in lung cancer. In their report, the T allele is associated with a >2-fold higher risk for lung cancer (odds ratio, 2.13; 95% CI, 1.47-3.08) in *DNMT3b* heterozygotes (12). Although it was suggested that the influence of the SNP on risk may be due to increased promoter activity, an alternate possibility is that the SNP is in linkage disequilibrium with another functional SNP that influences age-associated risk for HNPCC.

Many tumors exhibit simultaneous inactivation of several tumor suppressor, cell cycle, DNA repair, and metastasis-related pathways by aberrant CpG island-specific promoter hypermethylation (21-23). The pattern of inactivation for many of these genes seems to be tumor specific. Esteller et al. have proposed ''methylotypes'' for single tumor types, including tumors originating from the colon (22, 24). Using DNA from tumor tissues representing 15 major tumor types, Esteller et al. (22) found tumor-specific gene hypermethylation profiles in a study of 12 genes, including genes involved in tumor suppression, cell cycle regulation, apoptosis, DNA repair, and metastasis. Each of the 12 genes contains a 5′ CpG island that is hypermethylated in tumor tissues but unmethylated in corresponding normal tissues (22, 25-27).

CpG island-specific promoter hypermethylation is an epigenetic event that often occurs early in the natural history of human cancer. Hypermethylation of the *APC* gene promoter has been detected in colonic adenomas (28) and *hMLH1* promoter hypermethylation has been detected in colonic adenomas (29) and endometrial hyperplasias (30). *De novo* methylation is mediated by DNA methyltransferases (10). Recent studies have linked aberrant *de novo* hypermethylation of CpG islands to the overexpression of the DNMT3 family, including *DNMT3b* (10, 15). Although the mechanism by which overexpression is associated with hypermethylation is unknown, it is suggested that DNMT3a and DNMT3b might become inappropriately targeted to DNA regions that normally should not be involved with transcriptionally repressive heterochromatin formation (10).

Table 3. Statistical analysis of the study population by *DNMT3b* genotype

	No stratification		Stratified by age			
			<40 y		≥40 y	
n (colorectal cancer/total)	74/146		35/60		39/86	
Tests for homogeneity (*P*)						
Three genotypes						
Log rank	0.068		0.064		0.017	
Wilcoxon	0.350		0.065		0.007	
Fleming-Harrington	0.047		0.229		0.052	
Cox analysis without robust	0.057		0.098		0.010	
Cox analysis with robust	0.079		0.054		0.028	
Combined CT + TT genotypes						
Log rank	0.022		0.342		0.005	
Wilcoxon	0.161		0.239		0.009	
Fleming-Harrington	0.016		0.761		0.055	
Cox analysis without robust	0.018		0.356		0.003	
Cox analysis with robust	0.029		0.281		0.007	
	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
Without robust correction						
Heterozygotes (CT)	2.07 (1.09-3.94)	0.026	1.88 (0.82-4.29)	0.133	3.71 (1.28-10.74)	0.016
SNP homozygotes (TT)	1.88 (0.89-3.98)	0.099	0.82 (0.29-2.33)	0.716	4.46 (1.37-14.48)	0.013
Combined CT + TT	2.02 (1.08-3.77)	0.027	1.44 (0.65-3.20)	0.370	3.88 (1.37-11.04)	0.011
With robust correction						
Heterozygotes (CT)	2.07 (1.10-3.91)	0.024	1.88 (0.90-3.91)	0.091	3.71 (1.41-9.78)	0.008
SNP homozygotes (TT)	1.88 (0.86-4.08)	0.111	0.82 (0.39-1.74)	0.614	4.46 (1.27-15.63)	0.020
Combined CT + TT	2.02 (1.07-3.80)	0.029	1.44 (0.74-2.80)	0.281	3.88 (1.44-10.48)	0.008

In conclusion, our study provides the first evidence that the *DNMT3b* promoter polymorphism is significantly associated with increased age-associated risk in HNPCC families. MMR mutation carriers in HNPCC families have significant variation in their age of cancer onset. The age range for colorectal cancer in this study was 23 to 84 years. We showed that the C-to-T polymorphism in the *DNMT3b* promoter region is associated with a significantly younger age of onset for colorectal cancer, but we know that there are other genetic factors that contribute to this variation as well, such as polymorphisms in *cyclin D1*, N-*acetyltransferase 2*, and *p53* genes (16-18). However, including *cyclin D1* and *p53* genotypes into the Cox model as covariates indicates that the *DNMT3b* results are independent of the *cyclin D1* and *p53* results. Our long-term goal is to determine how different polymorphisms and/or environmental factors additively or synergistically affect age of onset among MMR mutation carriers. Such knowledge could help to provide more efficient cancer screening, resulting in earlier detection and treatment, better response to treatment, and better chances of survival.

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