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ORIGINAL PAPER

O^6-Methylguanine-DNA methyltransferase Leu84Phe and Ile143Val polymorphisms and risk of colorectal cancer in the Nurses' Health Study and Physicians' Health Study (United States)

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Abstract
Objective O^6-methylguanine-DNA methyltransferase (MGMT) removes mutagenic adducts from the O^6-position of guanine in DNA. Unrepaired O^6-methylguanines result in G:C to A:T transitions in mutated *K-ras* and *p53* in colorectal tumors. Two non-synonymous *MGMT* coding region variants, Leu84Phe and Ile143Val, lie in close proximity to the reactive 145Cys residue and to a conserved estrogen receptor interacting helix.
Methods We assessed the association between the *MGMT* Leu84Phe and Ile143Val polymorphisms and risk of colorectal cancer in two nested case–control studies: one each in the Nurses' Health Study (NHS) and the Physicians' Health Study (PHS) cohorts.
Results Among 197 female cases and 2,500 controls from the NHS, the variant 143Val allele was significantly associated with reduced risk of colorectal cancer [odds ratio (OR)=0.52, 95% confidence interval (CI) 0.33–0.80]. In women, statistically significant gene-environment interactions were found between the Leu84Phe polymorphism and alcohol intake (P=0.03), BMI (P=0.04) and postmenopausal hormone use (P=0.03). The Leu84Phe and Ile143Val polymorphisms were not significantly associated with risk of colorectal cancer among 271 male cases and 451 controls from the PHS.
Conclusions Our results suggest that the common Leu84Phe and Ile143Val polymorphisms in *MGMT* influence risk of colorectal cancer in women possibly through modulating estrogen receptor-dependent transcriptional activation, which has previously been shown to occur in response to DNA alkylation damage.

Keywords: Colorectal cancer · O^6-Methylguanine-DNA methyltransferase · Genetic polymorphism · Estrogen receptor

Abbreviations:
NHS Nurses' Health Study
HPFS Health Professionals Follow-Up Study
MGMT O^6-methylguanine-DNA methyltransferase

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NNN *N*′-nitrosonornicotine
NNK 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NDMA *N*-nitrosodimethylamine

Introduction

O^6-Methylguanine-DNA methyltransferase (MGMT) removes mutagenic adducts from the O^6-position of guanine in DNA. MGMT acts alone by means of a suicide mechanism that transfers the alkyl group from the damaged base to an internal cysteine alkyl-acceptor residue located at position 145 [1–3]. Left unrepaired, O^6-methylguanine pairs with thymine during DNA replication resulting in a G:C to A:T transition; such transitions are commonly observed in mutated *K-ras* [4, 5] and *p53* [6] in colorectal tumors. MGMT silencing by promoter hypermethylation occurs in many primary tumors including lymphomas, gliomas, non-small cell lung carcinoma, and head and neck carcinoma [7] and is associated with a higher frequency of colorectal tumors containing G:C to A:T transitions in *K-ras* [4, 5] and *p53* [6]. MGMT activity is also inhibited by the ethanol metabolite, acetaldehyde [8, 9], which occurs in high levels in the colorectal tissue of alcohol drinkers due to bacterial conversion [10]. Teo et al. [11] also showed that post-repair alkylated MGMT interacts with the estrogen receptor (ER), and that this interaction can prevent ER-dependent transcriptional activation that mediates cell proliferation.

O^6-Methylguanine DNA adducts arise from endogenous and exogenous exposure to methylating substances and have been detected in human colorectal DNA at levels found to cause adverse effects in model systems [12]. *N*-nitroso compounds have been shown in animal experiments to be among the most potent group of carcinogens [13], and environmental exposure to these alkylating agents may be a significant determinant of colorectal cancer risk [12]. Exposure to *N*-nitroso compounds derived from tobacco smoke, occupation and diet represent the most common exogenous sources of alkylation damage in humans [13, 14]. Two tobacco-specific nicotine-derived nitrosamines, *N*′-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), are strong adduct forming carcinogens in laboratory animals. The methyl adducts detected in cigarette smokers result in part from exposure to NNK [14]. The most potent adduct forming compound in nitrite containing foods is *N*-nitrosodimethylamine (NDMA), commonly found in cured meats [15]. NDMA is a hepatocarcinogen that causes a rapid accumulation of O^6-methylguanine adducts in animals [16] and is associated with the occurrence of colorectal cancer in humans [15].

Inter-individual variation in MGMT activity has been demonstrated; however, the molecular basis of this variation is unknown [17–20]. Three common, non-synonymous coding region variants, Leu84Phe, Ile143Val, and Lys178Arg, have been reported in the MGMT enzyme [21, 22]. Previous studies have demonstrated genetic linkage of the 143 and 178 polymorphisms [19, 22] and an increased risk of lung cancer in carriers of the variant 143Val allele has been reported [22]. Site-directed mutations at or near the MGMT Cysteine 145 alkyl-acceptor residue have been shown to inactivate or decrease enzyme activity [23, 24].

We assessed the association between *MGMT* Leu84Phe and Ile143Val polymorphisms and risk of colorectal cancer in two nested case–control studies; one each in Nurses' Health Study (NHS) and the Physicians' Health Study (PHS) cohorts. We hypothesized that *MGMT* Leu84Phe and Ile143Val polymorphisms modify risk of colorectal cancer associated with smoking, postmenopausal hormone use, BMI and intake of alcohol, folate and processed meat. Additionally, in an effort to detect novel germline variants that predispose to cancer, we resequenced the four MGMT coding exons in a population-based case series of individuals diagnosed with primary invasive cancer in more than one organ.

Materials and methods

The NHS is an ongoing prospective study of 121,700 US female registered nurses that are predominantly Caucasian-American (96%). Details of the design and follow-up of this cohort have been described previously [25]. Briefly, at enrollment in 1976, the participants, who were 30–55 years old, completed a questionnaire about their health history. Questionnaires have been mailed to the participants every 2 years to update information on lifestyle factors, medication usage and newly diagnosed cases of colorectal polyps, cancer, and other diseases. From 1989 to 1990, blood samples were collected from 32,826 of the NHS participants [26]. As previously detailed [27], women who provided a blood specimen were generally similar to women who did not; however, the proportion of women who were current smokers was lower among women who gave a blood specimen (14.4%) than among women who did not (25.0%). Subsequent follow-up of this subcohort of women has been greater than 96%.

All women for whom a blood sample was taken and who had two confirmed primary incident diagnoses of invasive cancer (excluding non-melanoma skin cancer) between 1976 and 1 June 1996 were eligible for the multiple cancer study; questionable diagnoses and metastatic cancers were excluded. A total of 103 multiple-cancer patients were identified. Prior to screening for *MGMT* mutations, a

physician reviewed the medical records and then all identifying information was masked. Forty-eight of the 103 multiple-cancer patients were selected for *MGMT* screening. We oversampled for individuals with cancers that we believed to be potentially attributed to altered *MGMT* function: colorectal cancer and other gastrointestinal tract cancers, leukemia, lymphoma, and individuals with more than two cancers.

Among eligible women from whom blood samples were collected, 197 incident colorectal cancer cases were documented by the year 2000 and confirmed using medical records and matched on year of birth and month of blood draw to 490 controls who were free from cancer at the time of case assessment. An additional 1,910 women who were controls in a nested case–control study of breast cancer and were free of cancer other than nonmelanoma skin cancer were also genotyped for the two *MGMT* polymorphisms and included in the analysis.

The PHS is a randomized, double-blind trial of aspirin and ß-carotene among 22,071 predominantly Caucasian-American (93%) male physicians, 40–84 years of age in 1982. Men were excluded if they had a history of myocardial infarction, stroke, or transient ischemic attack; cancer except nonmelanoma skin cancer; current renal or liver disease; peptic ulcer or gout; or current use of vitamin A or ß-carotene supplements. Before randomization, blood samples were collected at baseline, in 1982, from 14,916 (68%) of the physicians [28]. The men were subsequently monitored for incident cancer every 6 months in the first year and annually thereafter through mailed questionnaires. By the year 2000, 271 cases of colorectal cancer were identified and confirmed using medical records. A total of 451 men who were free from diagnosed cancer at the time of cancer ascertainment were selected as controls, and were matched to cases on age (±1 year for most and up to ±6 years for some) and on smoking history at baseline (current, former, and never-smokers).

Venous blood samples collected from NHS participants were separated into plasma, buffy coat and red blood cells and stored in liquid nitrogen freezers while the PHS whole blood samples were stored in liquid nitrogen freezers. Genomic DNA was extracted from 50 ml of buffy coat (NHS) or whole blood (PHS) diluted with 150 ml of phosphate-buffered saline and using the QIAmp (Qiagen Inc., Chatsworth, CA) 96-spin blood protocol according to the manufacturer's instructions. Genomic DNA concentrations were calculated in 96-well format using PicoGreen technology (Molecular Probes, Eugene, OR).

The four coding exons (2–5) of *MGMT* were PCR amplified in the 48 multiple-cancer patients with previously described primers [29]. Due to problems with amplification of exon 4, nested PCR was used with another previously described primer [30]. PCR reactions were performed with 25 ng genomic DNA, 50 mM KCl, 10 mM Tris–HCl (pH=8.3), 1.5 mM $MgCl_2$, 200 mM dNTPs, 1% DMSO, and 1.25 U *Taq* polymerase (Promega). Amplification conditions were as follows: 95°C for 1 min; 35 cycles 94°C for 30 s, 51–53°C for 30 s, and 72°C for 30 s; and 72°C for 7 min. Purified PCR products were sequenced in one direction using the Big Dye Terminator cycle sequencing protocol (Perkin-Elmer), electrophoresed on 5% Long Ranger gels (FMC, Rockland, ME), and analyzed on an ABI377 automated DNA sequencer (Perkin-Elmer). Base calling was done using the ABI sequence-analysis software version 3.0. Sequencher v.3.0 (Gene Codes) was used to mark potential differences from GenBank published sequences. Heterozygotes were called at positions where the secondary peak's height was greater than or equal to 50% of the primary peaks height. All flagged differences were confirmed visually.

Genotyping of *MGMT* Leu84Phe (rs12917) and MGMT Ile143Val (rs2308321) was carried out using the *TaqMan* allelic discrimination system (Applied Biosystems, Foster City, CA). *TaqMan* primers and probes are available upon request. Following PCR amplification, end-point fluorescence was read with the Applied Biosystems 7900HT instrument and genotypes were assigned using Allelic Discrimination Software (Applied Biosystems SDS Software v1.7a). Ten percent quality control (QC) samples were included and each analysis included negative controls. Laboratory personnel were blinded to QC and case–control status. Genotyping failures are considered missing data and not analyzed.

For analysis of the main effect of genotype, logistic regression was used to compute odds ratios (ORs) and 95% confidence intervals (CIs) in analyses adjusting for the additional risk factors of family history of colorectal cancer (NHS only), smoking history, aspirin use, BMI, postmenopausal hormone use (NHS only), physical activity (NHS only), and intake of red meat (NHS only), folate (NHS only), multivitamins (PHS only) and alcohol. Risk of colorectal cancer was considered in relation to MGMT genotypes. *MGMT* Leu84Phe and Ile143Val were each categorized into two genotype levels (codon 84: Leu/Leu and Leu/Phe+Phe/Phe; codon 143: Ile/Ile and Ile/Val+Val/Val) and analyzed in combination with smoking (NHS: pack-years of smoking before age 30; PHS: never, past, current), BMI (NHS and PHS: <25, ≥25), alcohol intake (NHS and PHS: drinks per day), µg/day of energy-adjusted folate including supplements (NHS: <300 µg, ≥300 µg/day) based on the cutpoints used for the NHS in Giovannucci et al. [31], postmenopausal hormone use (NHS: never/past, current) and consumption of processed meats (NHS: slices per week), bacon (NHS: pieces per week), and hot dogs (NHS: number per week). Analyses involving consumption of processed meats, bacon, and hot dogs were

additionally adjusted for total caloric intake in the NHS. The *P*-value for interaction was based on the Wald test for the cross-product term in a model containing the main effects of genotype and exposure variable. All exposure information for the NHS cancer was updated from 1990, 1992, 1994, 1996 to 1998 using the latest update of the information prior to diagnosis of disease (except for smoking history, which was cumulatively updated to the latest time period prior to diagnosis of disease). Exposure information was collected at baseline in 1982 for the PHS. A structural image of the Ile143 and Leu84 hydrophobic region was created using PyMol Software (Delano Scientific, San Carlos, CA) and pdb file 1EH6 of the MGMT structure [32].

Results

We first examined the main effect of smoking, postmenopausal hormone use (women only), and intake of alcohol, processed meat, bacon and hot dogs (women only) on risk of colorectal cancer for women and men nested in the NHS and PHS. Women from the NHS did not have an increased risk of colorectal cancer when exposed to ≤10 pack-years of smoking before age 30 (OR=1.06, 95% CI 0.76–1.48) while exposure to >10 pack-years of smoking before age 30 modestly increased risk of colorectal cancer (OR=1.23, 95% CI 0.76–2.00) relative to nonsmokers. Alcohol intake of ≥0.5 drink/day was associated with a moderately increased risk of colorectal cancer (OR=1.34, 95% CI 0.94–1.90) relative to infrequent drinkers. Folate intake of ≥300 μg/day was associated with a significantly decreased risk of colorectal cancer (OR=0.70, 95% CI 0.51–0.97) relative to women with an intake <300 μg/day. Postmenopausal hormone use significantly reduced risk of colorectal cancer for current users (OR=0.62, 95% CI 0.44–0.89) relative to postmenopausal women that were never or past users of postmenopausal hormones. Having a BMI ≥25 did not affect risk of colorectal cancer in women (OR=0.89, 95% CI 0.65–1.21) relative to individuals with a BMI <25. Risk of colorectal cancer was not modified for women consuming ≥1 slices/week of processed meat (OR=1.06, 95% CI 0.73–1.55), ≥2 pieces/week of bacon (OR=0.94, 95% CI 0.56–1.58), or ≥1 hot dog/week (OR=1.06, 95% CI 0.68–1.65), relative to infrequent consumers of these items. Men from the PHS were at a moderately higher, but not significant, risk of colorectal cancer when they were current smokers (OR=1.17, 95% CI 0.66–2.07) while being a past smoker did not increase risk (OR=0.97, 95% CI 0.70–1.35) relative to nonsmokers. Among men, having a BMI ≥25 was associated with a statistically significant increased risk of colorectal cancer (OR=1.37, 95% CI 1.01–1.86) relative to individuals with a BMI <25. Alcohol intake of ≥1 drink/day was not significantly associated with an increased risk of colorectal cancer (OR=1.07, 95% CI 0.76–1.50) relative to infrequent drinkers.

Table 1 presents the main associations between MGMT genotypes and risk of colorectal cancer. The distribution of the *MGMT* Leu84Phe and Ile143Val genotypes conformed to Hardy–Weinberg expectations in controls from both the NHS and PHS and these polymorphisms were not in linkage disequilibrium. Concordance for blinded quality control specimens was 100%. Although the dataset was initially based on matched cases and controls, the stratified analyses required unconditional analysis to optimize power (the results obtained from conditional and unconditional logistic regression were similar).

The addition of 1,910 breast cancer controls did not affect the associations observed between *MGMT* genotype and risk of colorectal cancer (ORs for main effect and *P*-values for interactions). We did not observe a statistically significant association between *MGMT* Leu84Phe and Ile143Val polymorphisms and risk of colorectal cancer for men from the PHS. For women from the NHS, we found no association with the Leu84Phe polymorphism and risk of colorectal cancer. Considering the Ile143Val polymorphism, we observed a significant inverse association among women that carried one copy (RR=0.57, 95% CI 0.37–0.89) or at least one copy (RR=0.52, 95% CI 0.33–0.80) of the variant 143Val allele. For *MGMT* Ile143Val, we observed 28 Ile/Val heterozygotes and no Val/Val homozygotes among 197 colorectal cancer cases in the NHS and this group conformed to Hardy–Weinberg expectations.

We next examined each MGMT polymorphism stratified by smoking history (Table 2), intake of alcohol (Table 3), BMI (Table 4) and postmenopausal hormone use (Table 5). No significant interactions were found between *MGMT* genotype and smoking in either men or women (Table 2). For alcohol intake we found a significant interaction with the Leu84Phe polymorphism in the NHS (*P*=0.03) (Table 3). In women consuming <0.5 drink/day of alcohol, carrying one or two copies of the variant 84Phe allele was inversely associated with risk of colorectal cancer (OR=0.63, 95% CI 0.38–1.03) while consumption of ≥0.5 drink/day of alcohol was associated with a modestly increased risk (OR=1.66, 95% CI 0.92–3.01) relative to Leu84 homozygous individuals that drank <0.5 drink/day of alcohol. We did not find a significant interaction between the *MGMT* Ile143Val polymorphism and alcohol intake in women.

For women, a statistically significant interaction (*P*=0.04) was found between BMI and the Leu84Phe polymorphism (Table 4). Among women with a BMI ≥25, carrying one or two copies of the variant 84Phe allele was inversely associated with risk of colorectal cancer (OR=0.57, 95% CI 0.29–1.10) relative to women with a

BMI <25 who were homozygous for the common Leu84 allele (Table 4). While we did not find a significant interaction between the *MGMT* Ile143Val polymorphism and BMI in women, individuals with a BMI ≥25 carrying one or two copies of the variant 143Val allele had a significantly reduced risk of colorectal cancer (OR=0.38, 95% CI 0.18–0.76) relative to women with a BMI <25 who were homozygous for the common Ile143 allele (Table 4). Our results for the BMI analyses remained the same when we restricted our analyses to postmenopausal women.

A statistically significant interaction (P=0.04) was found between postmenopausal hormone use and the Leu84Phe polymorphism (Table 5). Among postmenopausal women who were never or past users of postmenopausal hormones, the variant 84Phe allele was inversely associated with risk of colorectal cancer (OR=0.60, 95% CI 0.35–1.05) relative to Leu84 homozygotes who were never or past users of postmenopausal hormones (Table 5). The inverse association of postmenopausal hormone use was only observed for individuals that were Leu84 homozygotes (OR=0.52, 95% CI 0.34–0.80) relative to Leu84 homozygotes who were never or past users of postmenopausal hormones (Table 5). In addition, postmenopausal women carrying one or two copies of the variant 143Val allele had a significantly reduced risk of colorectal cancer if they were never or past users of postmenopausal hormones (OR=0.40, 95% CI 0.21–0.74) or current users of postmenopausal hormones (OR=0.37, 95% CI 0.17–0.78) relative to postmenopausal women that were never or past postmenopausal hormone users and were homozygous for the common Ile143 allele (Table 5). We found no significant interactions between *MGMT* genotype and BMI, alcohol intake or smoking history in men (data not shown) or *MGMT* genotype and intake of folate, processed meat, bacon and hot dogs in women (data not shown).

Sequencing of exons 2–5 of MGMT among 48 women with multiple cancers did not yield any novel cancer-associated mutations. Our study confirmed the existence of previously reported non-synonymous polymorphisms at codons 84, 143 and 178 and a polymorphism at codon 53 (C>T) in exon 3 that does not alter the amino acid sequence of MGMT.

3-Dimensional structural modeling of MGMT revealed that the Ile143 and Leu 84 residues pack in a hydrophobic region with three conserved Leucines of a putative estrogen receptor interacting helix (Fig. 1).

Discussion

In the present study we observed a statistically significant inverse association between the variant *MGMT* 143Val allele and risk of colorectal cancer in women but not in men. There was no association between the *MGMT* Leu84Phe polymorphism and risk of colorectal cancer in either men or women. Codon 143 is within the active site region of the MGMT protein being located two amino acids upstream of the Cys145 alkyl-acceptor residue. However, the Isoleucine to Valine amino acid change at codon 143 is conservative in nature (both are non-polar) and do not affect MGMT activity [20, 22, 33]. In previous studies, a marginally significant association was seen with Ile143Val heterozygotes and an increased risk of lung cancer [22] but no association was seen with metastatic melanoma [34].

While we did not observe effect modification of *MGMT* genotype by potential sources of DNA alkylation damage (cigarette smoke and preserved meat intake) we did observe gene–environment interactions between the *MGMT* Leu84Phe genotype and BMI, PMH use and alcohol intake.

Table 1 Associations between *MGMT* genotypes and colorectal cancer risk in the NHS and the PHS

MGMT genotype	NHS			PHS		
	Cases (%)	Controls (%)	OR (95% CI)[a]	Cases (%)	Controls (%)	OR (95% CI)[b]
N*	197	2,500		271	451	
Leu84Phe						
Leu/Leu	147 (79.0)	1,634 (76.5)	1.00 (ref.)	204 (79.4)	330 (76.9)	1.00 (ref.)
Leu/Phe	33 (17.7)	471 (22.0)	0.81 (0.54–1.21)	47 (18.3)	93 (21.7)	0.80 (0.54–1.19)
Phe/Phe	6 (3.2)	32 (1.5)	1.44 (0.49–4.19)	6 (2.3)	6 (1.4)	1.89 (0.59–6.10)
Leu/Phe+Phe/Phe	39 (20.9)	503 (23.5)	0.85 (0.57–1.25)	53 (20.6)	99 (23.1)	0.86 (0.59–1.26)
Ile143Val						
Ile/Ile	163 (85.8)	1,614 (75.0)	1.00 (ref.)	204 (78.5)	348 (80.7)	1.00 (ref.)
Ile/Val	27 (14.2)	487 (22.6)	0.57 (0.37–0.89)	49 (18.9)	76 (17.6)	1.07 (0.71–1.61)
Val/Val	0	50 (2.3)	–	7 (2.7)	7 (1.6)	1.76 (0.60–5.19)
Ile/Val+Val/Val	27 (14.2)	537 (25.0)	0.52 (0.33–0.80)	56 (21.5)	83 (19.3)	1.13 (0.77–1.67)

[a]Unconditional logistic regression including 1,910 breast cancer control subjects in an analysis adjusted for age, family history of colorectal cancer, smoking history, aspirin use, BMI, postmenopausal hormone use, physical activity, and intake of red meat, folate, and alcohol

[b]Unconditional logistic regression adjusted for age, smoking history, aspirin use, BMI, multivitamin use, and alcohol intake

*Numbers do not add to total due to missing genotype data

Table 2 Relationship of smoking and risk of colorectal cancer stratified by *MGMT* genotypes in the NHS and the PHS*

	NHS[a]			*P*-interaction	PHS[b]			*P*-interaction
	Pack-years of smoking before age 30				Smoking history			
	0	≤10	>10		Never	Past	Present	
Leu84Phe								
Leu/Leu	1.00 (ref.) [66; 809][c]	1.20 (0.82–1.76) [62; 591]	1.23 (0.70–2.16) [18; 171]		1.00 (ref.) [80; 125]	0.82 (0.56–1.21) [107; 179]	1.10 (0.54–2.22) [17; 26]	
Leu/Phe+Phe/Phe	1.00 (0.58–1.74) [20; 234]	0.79 (0.42–1.51) [14; 189]	1.16 (0.44–3.04) [5; 56]	0.63	0.60 (0.32–1.13) [18; 45]	0.98 (0.56–1.73) [29; 43]	0.75 (0.26–2.13) [6; 11]	0.37
Ile143Val								
Ile/Ile	1.00 (ref.) [72; 812]	1.26 (0.87–1.83) [70; 565]	1.41 (0.82–2.42) [20; 168]		1.00 (ref.) [76; 134]	0.98 (0.67–1.44) [110; 179]	0.89 (0.46–1.72) [18; 35]	
Ile/Val+Val/Val	0.71 (0.38–1.31) [14; 243]	0.51 (0.25–1.05) [10; 215]	0.53 (0.16–1.76) [3; 60]	0.24	1.04 (0.56–1.93) [22; 36]	1.01 (0.57–1.78) [28; 43]	2.97 (0.79–11.20) [6; 4]	0.34

[a]Unconditional logistic regression adjusted for age, family history of colorectal cancer, aspirin use, BMI, postmenopausal hormone use, physical activity, and intake of red meat, folate, and alcohol

[b]Unconditional logistic regression adjusted for age, aspirin use, BMI, multivitamin use, and alcohol intake

[c][cases; controls]

*Among women, 1 case and 88 controls were missing smoking data and excluded from the analysis

These results, in addition to the disparate results observed between men and women for *MGMT* Ile143Val, suggest that MGMT may influence the risk of colorectal cancer through ER-dependent transcriptional activation, which occurs in response to DNA alkylation damage.

A statistically significant interaction was found between the *MGMT* Leu84Phe polymorphism and alcohol intake in women. Risk was significantly increased among women consuming ≥0.5 drink/day and carrying one or two copies of the variant 84Phe allele. This interaction was not observed in the men analyzed in this study. Alcohol intake may affect colorectal cancer risk partially through an effect on endogenous sex steroid levels [35] and may also be associated with changes in promoter methylation of many genes that influence the development of colorectal cancer [36]. In a recent cross-sectional study of breast cancer risk, Onland-Moret et al. [35] demonstrated that women consuming higher levels of alcohol had increased levels of estrogens when compared to women that did not drink. Additionally, MGMT inactivation by promoter hypermethylation has been observed in many tumors [7] and van Engeland et al. [36] demonstrated that MGMT promoter methylation was slightly higher in colorectal cancer patients with low folate and high alcohol intake compared with patients having high folate and low alcohol intake. Specifically, acetaldehyde inhibits the activity of MGMT [8, 9, 37] and enhances the accumulation of O^6-methylguanine adducts in NDMA treated rats [37]. Bacterial conversion of alcohol to acetaldehyde may lead to profoundly higher acetaldehyde levels in colorectal cancer tissue of alcohol drinkers [38]. We did not observe a significant interaction between alcohol intake and *MGMT* Ile143Val genotype in men or women.

In this study we found a statistically significant interaction between the *MGMT* Leu84Phe polymorphism and BMI in women. A BMI ≥25 was inversely associated with risk of colorectal cancer only among women carrying one or two copies of the variant 84Phe allele. In addition, women with a BMI ≥25 who carried one or two copies of the variant 143Val allele had a significantly reduced risk of colorectal cancer. BMI is a well-established risk factor for colorectal cancer [39–41] and adenoma [42]; however, the association between BMI and colorectal cancer may be different for men and women [43]. Two studies [43, 44] found that the relationship between BMI and colon cancer risk may be modified by menopausal status and estrogen exposure while one recent study [39] did not find the same associations. Terry et al. [44] observed a strong positive relationship between BMI and colon cancer risk in premenopausal but not postmenopausal woman. Slattery et al. [43] reported that BMI was strongly associated with colon cancer risk among premenopausal or postmenopausal women using hormone replacement therapy (PMH) but not

Table 3 Relationship of alcohol consumption and risk of colorectal cancer stratified by *MGMT* genotypes in the NHS and the PHS*

	NHS[a]		*P*-interaction	PHS[b]		*P*-interaction
	Alcohol consumption (drinks/day)			Alcohol consumption (drinks/day)		
	<0.5	≥0.5		<1	≥1	
Leu84Phe						
Leu/Leu	1.00 (ref.) [106; 1,187][c]	1.04 (0.69–1.59) [34; 354]		1.00 (ref.) [140; 232]	1.06 (0.72–1.57) [64; 96]	
Leu/Phe+Phe/Phe	0.63 (0.38–1.03) [20; 369]	1.66 (0.92–3.01) [15; 110]	0.03	0.86 (0.54–1.36) [37; 71]	0.92 (0.47–1.79) [16; 27]	0.98
I1e 143Val						
Ile/Ile	1.00 (ref.) [109; 1,189]	1.34 (0.91–1.98) [43; 341]		1.00 (ref.) [142; 251]	1.11 (0.75–1.65) [62; 94]	
Ile/Val+Val/Val	0.53 (0.32–0.89) [18; 383]	0.65 (0.29–1.45) [7; 120]	0.84	1.21 (0.76–1.94) [38; 56]	1.08 (0.57–2.06) [18; 27]	0.60

[a]Unconditional logistic regression adjusted for age, family history of colorectal cancer, smoking history, aspirin use, BMI, postmenopausal hormone use, physical activity, and intake of red meat, and folate

[b]Unconditional logistic regression adjusted for age, smoking history, aspirin use, BMI, and multivitamin use

[c][cases; controls]

*Among women, 13 cases and 118 controls were missing alcohol consumption data and excluded from the analysis. Among men, 3 controls were missing alcohol consumption data and excluded from the analysis

among postmenopausal women who were not taking PMH. Lin et al. [39] reported that BMI was associated with an elevated risk of colorectal cancer, and the positive relationship was not altered by estrogen exposure among postmenopausal women. The inconsistencies between these studies may be attributable to differences in details of PMH use (duration, dosage, formulation) as well as study design (prospective cohort vs. case–control). BMI may influence colorectal cancer risk by elevating insulin and insulin-like growth factors and possibly by altering endogenous hormone synthesis and metabolism. Obesity is associated with chronic hyperinsulinemia [45, 46] leading to reduced levels of IGF binding protein 1 levels which increases free IGF-1 levels [47, 48]. Obesity is also associated with increased ovarian and adrenal production of androgens, and decreased levels of sex hormone binding globulin leading to increased levels of total and bioavailable estradiol in postmenopausal women [45, 46]. In addition, Slattery et al. [43] hypothesized that estrogen up-regulates IGF-I receptors in the colon, which in turn

Table 4 Relationship of BMI and risk of colorectal cancer stratified by *MGMT* genotypes in the NHS and the PHS*

	NHS[a]		*P*-interaction	PHS[b]		*P*-interaction
	BMI			BMI		
	<25	≥25		<25	≥25	
Leu84Phe						
Leu/Leu	1.00 (ref.) [71; 826][c]	1.11 (0.78–1.58) [75; 768]		1.00 (ref.) [106; 189]	1.24 (0.87–1.78) [98; 141]	
Leu/Phe+Phe/Phe	1.22 (0.74–2.00) [26; 239]	0.57 (0.29–1.10) [13; 253]	0.04	0.74 (0.43–1.26) [24; 58]	1.27 (0.74–2.19) [29; 41]	0.40
Ile143Val						
Ile/Ile	1.00 (ref.) [81; 813]	1.03 (0.73–1.44) [80; 763]		1.00 (ref.) [111; 202]	1.17 (0.82–1.66) [93; 146]	
Ile/Val+Val/Val	0.68 (0.39–1.19) [17; 257]	0.38 (0.18–0.76) [10; 268]	0.19	0.89 (0.51–1.56) [23; 46]	1.59 (0.94–2.70) [33; 37]	0.28

[a]Unconditional logistic regression adjusted for age, family history of colorectal cancer, smoking history, aspirin use, postmenopausal hormone use, physical activity, and intake of alcohol, red meat, and folate

[b]Unconditional logistic regression adjusted for age, smoking history, aspirin use, multivitamin use and alcohol intake

[c][cases; controls]

*Among women, 2 cases and 51 controls were missing BMI data and excluded from the analysis

Table 5 Relationship of postmenopausal hormone use and risk of colorectal cancer stratified by *MGMT* genotypes in the NHS*

MGMT Genotype	PMH use[a]		*P*-interaction
	Never-Past	Current	
Leu84Phe			
Leu/Leu	1.00 (ref.) [95; 819][b]	0.52 (0.34–0.80) [34; 580]	
Leu/Phe+Phe/Phe	0.60 (0.35–1.05) [19; 249]	0.78 (0.43–1.40) [16; 183]	0.04
Ile143Val			
Ile/Ile	1.00 (ref.) [102; 812]	0.63 (0.42–0.94) [43; 571]	
Ile/Val+Val/Val	0.40 (0.21–0.74) [13; 262]	0.37 (0.17–0.78) [9; 199]	0.45

[a]Unconditional logistic regression adjusted for age, family history of colorectal cancer, smoking history, aspirin use, BMI, physical activity, and intake of red meat, folate, and alcohol

[b][cases; controls]

*Among postmenopausal women, 10 cases and 68 controls were missing PMH data and excluded from the analysis

increases susceptibility to obesity-induced increased levels of insulin. Giovannucci [49] proposed that the adverse effects of hyperinsulinemia predominate for premenopausal women because obesity increases insulin concentrations but is a relatively unimportant source of estrogens because ovarian production is high. In postmenopausal women, though, the conversion of androgens to estrogen in adipose tissue is the major source of estrogens [50] and the association between obesity and increased estrogen levels may have a countering effect to insulin/IGF and explain the results of Terry et al. [44].

Hormone replacement has been shown to protect against colorectal cancer in postmenopausal women [51] and we observed a significant interaction between current postmenopausal hormone use and the Leu84Phe polymorphism. The inverse association between current postmenopausal hormone use and risk of colorectal cancer was only observed for individuals that were homozygous for the Leu84 allele while postmenopausal women who were never or past users of postmenopausal hormones carrying one or two copies of the variant 84Phe allele had a reduced risk of colorectal cancer. Additionally, current postmenopausal hormone use was significantly protective among postmenopausal women regardless of *MGMT* Ile143Val genotype; however, postmenopausal women who were current users of postmenopausal hormones and carried one or two copies of the variant 143Val allele had a substantially lower risk of colorectal cancer. The inverse association of the 143Val allele was also present among postmenopausal women who were never or past postmenopausal hormone users.

The mechanism for the protective effect of hormone replacement is unknown and in vitro studies show contradictory effects of estrogens on the proliferation of colorectal cancer cells [52–54]. Exogenous estrogens decrease secondary bile acid production in the colonic epithelium, which reduces chronic irritation of the colonic mucosa [55, 56] and may protect against colorectal cancer [57]. In addition, estrogens have also been shown to increase the expression of vitamin D receptors in a variety of tissues [55, 56] resulting in inhibited neoplastic growth while promoting differentiation of colonic epithelial cells [55, 56]. Finally, methylation-associated inactivation of ER

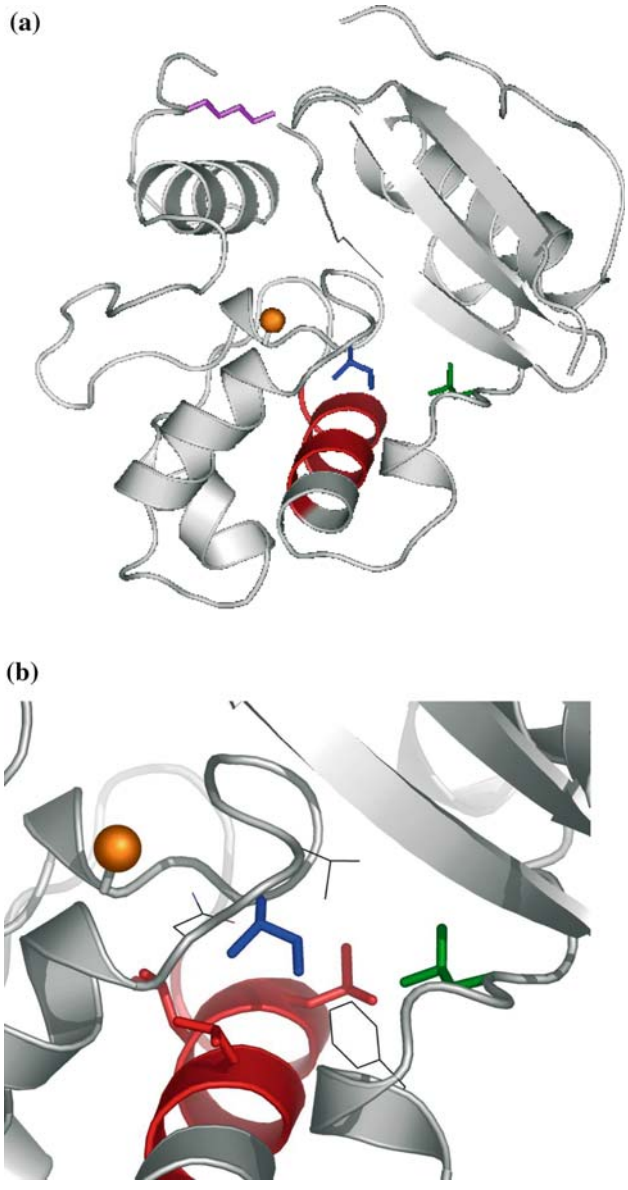

Fig. 1 (**a**) Positions of key structural features and common variable residues in the human MGMT molecule. Key structural features: reactive cysteine (Cys 145, yellow sphere), putative estrogen receptor interacting helix (red). Variable residues (most common side chain is shown): Leucine 84 (green), and linked Isoleucine 143 (blue) and Lysine 178 (purple). (**b**) MGMT Ile143 (blue) and Leu84 (green) pack in a hydrophobic region with three conserved Leucines of a putative estrogen receptor interacting helix (red). The sulfur of the reactive Cysteine is also shown (yellow sphere)

results in deregulated growth of the colonic epithelium suggesting that the ER gene may act as a tumor suppressor in the colon [58]. Estrogens have been shown to reduce ER-gene methylation and inhibit cell proliferation in the colonic epithelium [58]. Specifically, colorectal cells possess the enzymes required for the synthesis and metabolism of estrogens [54] and colorectal cell lines express functional estrogen receptors (ER), with ERβ being the predominant isoform expressed and ERα occurring at very low levels [59].

Recent work in Apc (Min/+) mice (bearing a germline mutation in murine Apc) revealed the effect of ovariectomy and estrogen replacement on tumor formation in colonic epithelium [60, 61]. Weyant et al. [61] found that after ovariectomy, intestinal adenomas in Apc (Min/+) mice increased by 77%. Treatment of ovariectomized mice with estrogen reduced tumor burden to that of the intact Apc (Min/+) mice [61]. Javid et al. [60] showed that enterocyte crypt-villus migration in Apc (Min/+) mice is slowed by ovariectomy and that treatment with estrogen restores crypt-villus migration rate. These results suggest that endogenous estrogens modulate the frequency of epithelial cell turnover and promote high cell turnover, which may protect cells from the genotoxic effects of colonic microenvironment. ERα and ERβ levels in the intestinal tissue also showed changes in relative expression levels in response to ovariectomy and hormone replacement [60, 61]. Normal-appearing intestine of Apc (Min/+) mice showed the highest ERα and lowest ERβ expression while animals that received estrogen treatment showed the lowest ERα and highest ERβ expression [60, 61]. ERβ may be involved in regulating cellular adhesion in the intestinal mucosa and the increased expression of ERβ after estrogen treatment may restore normal levels of adhesion to the colonic epithelium [60]. These results suggest that endogenous estrogens protect against tumor formation and that tumor prevention by estrogen is associated with increased ERβ expression, which may regulate cell migration and adhesion in the intestinal mucosa.

In a model presented by Li and coauthors [11, 62], MGMT switches to being a transcriptional suppressor of ER-dependent signaling upon repair of the O^6-methylguanine lesion. Alkylation of the Cys145 residue of MGMT causes a conformational change and probably exposes an LWKLL motif. This motif, which transcription coactivators also use to bind to nuclear receptors, blocks ER from binding with its coactivator and prevents ER-dependent transcription. The MGMT 3-dimensional structure shows that the Leu84 and Ile143 residues lie in close proximity to the three conserved Leucines of the LXXLL ER-interacting helix. Whether the *MGMT* variants affect response to DNA alkylation damage or affects the regulation of ER-dependent signaling is currently unknown. The proximity of the Leu84 and the Ile143 residues to the ER interacting helix suggests that there could be differential regulation of ER-dependent signaling by the variant 84Phe and 143Val variant residues.

Recently, Margison et al. [20] observed that the 143Val variant did not affect the activity of MGMT on methylated DNA substrate but was more resistant to inactivation by the MGMT pseudosubstrate, O^6-(4-bromothenyl)guanine [20]. Currently, the effect of the 143Val variant allele on the regulation of estrogen receptor-dependent transcriptional activation is unknown. While Inoue et al. [33] have demonstrated that *Escherichia coli* cells carrying 84Phe and wild-type sequences have similar enzymatic and physicochemical properties the effect of the 84Phe variant allele on methylguanine-methyltransferase activity and the regulation of estrogen receptor-dependent transcriptional activation is unknown. In vitro cytotoxicity and repair assays by Ma et al. [34] in *E. coli* showed no effect of the *MGMT* Ile143Val variant on DNA repair activity but suggests that the variant may increase protein expression compared to wild-type protein, although this difference was not quantified. Until a rigorous functional analysis of the Leu84Phe and Ile143Val polymorphisms has been carried out the results presented herein for *MGMT* Leu84Phe and Ile143-Val polymorphisms should be interpreted with caution.

Overall, our results suggest that the common Leu84Phe and Ile143Val polymorphisms in O^6-MGMT influence risk of colorectal cancer in women, and that the effect may be modified by alcohol, BMI and postmenopausal hormone use. In postmenopausal women, the variant *MGMT* 143Val allele significantly reduces risk of colorectal cancer and significantly increases the protective effect of postmenopausal hormone use among current users. Additionally, the variant *MGMT* 84Phe allele significantly interacts with alcohol intake and BMI to affect risk of colorectal cancer in women. In this study we did not observe effect modification of *MGMT* genotype by potential sources of DNA alkylation damage (cigarette smoke and preserved meat intake). Our findings suggest that MGMT may influence the risk of colorectal cancer through ER-dependent transcriptional activation, which occurs in response to DNA alkylation damage.

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