Discovery and Features of an Alkylating Signature in Colorectal Cancer

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ABSTRACT
Several risk factors have been established for colorectal cancer, yet their direct mutagenic effects in patients’ tumors remain to be elucidated. Here, we leveraged whole-exome sequencing data from 900 colorectal cancer cases that had occurred in three U.S.-wide prospective studies with extensive dietary and lifestyle information. We found an alkylating signature that was previously undescribed in colorectal cancer and then showed the existence of a similar mutational process in normal colonic crypts. This alkylating signature is associated with high intakes of processed and unprocessed red meat prior to diagnosis. In addition, this signature was more abundant in the distal colorectum, predicted to target cancer driver mutations KRAS p.G12D, KRAS p.G13D, and PIK3CA p.E545K, and associated with poor survival. Together, these results link for the first time a colorectal mutational signature to a component of diet and further implicate the role of red meat in colorectal cancer initiation and progression.

SIGNIFICANCE: Colorectal cancer has several lifestyle risk factors, but the underlying mutations for most have not been observed directly in tumors. Analysis of 900 colorectal cancers with whole-exome sequencing and epidemiologic annotations revealed an alkylating mutational signature that was associated with red meat consumption and distal tumor location, as well as predicted to target KRAS p.G12D/p.G13D.

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INTRODUCTION

Most tumor mutations are passengers that have little to no functional role in cancer. However, their positional context in the genome may reveal information about the underlying mutational processes (1). Snapshots of these processes, called mutational signatures, were originally deconvoluted using a nonnegative matrix factorization (NMF) approach (2) on a large collection of whole-genome sequencing and whole-exome sequencing (WES) data (3). Mutational signatures may elucidate the roles of mutagens in cancer and inform prevention and treatment efforts. Several studies have been conducted to associate mutational signatures with cellular processes or exposures. These include rare cancer predisposition syndromes (4), environmental agents (5), and microbiota (6). Such association studies have relied on either DNA-sequencing data sets or preclinical models, such as organoids. However, although many lifestyle-related factors have been linked to colorectal cancer (7), larger and more comprehensive data sets are needed to enable the discovery of the associated signatures. Consequently, past efforts have not been able to capture the cumulative effect of putative mutagens, such as dietary components, over decades. In particular, red meat consumption has been consistently linked to the incidence of colorectal cancer (8–10). The suggested mechanism is mutagenesis through alkylating damage induced by N-nitroso-compounds (NOC), which are metabolic products of blood heme iron or meat nitrates/nitrates (11). Nevertheless, this mutational damage is yet to be observed directly in patients’ tumors.

RESULTS

Active Mutational Signatures in Colorectal Tumors and Normal Colonic Crypts

To address this gap, we leveraged a database of incident colorectal cancer cases that had occurred in three U.S.-wide prospective cohort studies, namely the Nurses’ Health Studies (NHS) I and II and the Health Professionals Follow-up Study (HPFS; ref. 12). Study participants (more than 230,000 women and 50,000 men) repeatedly provided data on diet, lifestyle, and other factors without knowing their future colorectal cancer diagnosis, if any. We performed WES on matched primary untreated tumor–normal pairs in 900 patients with colorectal cancer with adequate tissue materials (Fig. 1A; Supplementary Table S1).

NMF signal separation revealed the existence of seven mutational processes (see Methods and Fig. 1B and C; Supplementary Fig. S1). We confirmed the robustness of the deconvolution by using another signature assignment program (SigProfiler; ref. 3); we again found seven mutational processes (Supplementary Fig. S2, left) that are highly similar to the ones obtained using the standard NMF approach (Supplementary Fig. S2, right).

To uncover the etiology of these colorectal signatures (that we name c-signatures), we first used a cosine similarity metric (cossim = 0.95), deficient mismatch repair (dMMR; c-dMMRa/SBS15, cossim = 0.90 and c-dMMRb/SBS26, cossim = 0.90), and exposure to alkylating agents (c-Alkylation/SBS11, cossim = 0.94). c-SBS40 matched the closest to SBS40 (cossim = 0.84), which is a featureless signature with unknown etiology and found in most cancers (3).

We substantiated the etiology of the four mutational processes by integrating clinical, pathologic, and methylation data (Fig. 2A). Tumors harboring a POLE exonuclease domain mutation were significantly enriched in signatures c-POLEa and c-POLEb (P = 2.3 × 10⁻⁵ and P = 1.8 × 10⁻⁴, respectively, Mann–Whitney U test). Similarly, patients with orthogonally assessed microsatellite instability (MSI)–high status were significantly enriched in signatures c-dMMRa and c-dMMRb (P < 2 × 10⁻¹⁶ for both, Mann–Whitney U test). Signature c-Age also displayed a significant association with patients’ age at diagnosis (P = 1.7 × 10⁻², Mann–Whitney U test). Last, we support the etiology of the alkylating-like signature, not previously described in colorectal cancer, by assessing the MGMT (O-6-methylguanine-DNA methyltransferase) promoter methylation status in tumors from the NHS/HPFS cohorts. MGMT is a central gene in the repair of alkylating lesions. Among the sequenced specimens with available MGMT promoter methylation data, we observed that tumors with methylated MGMT promoters were enriched in the signature c-Alkylation (P = 6.6 × 10⁻³, Mann–Whitney U test; Fig. 2A), further supporting that this signature represents the biological consequence of increased alkylating damage. Of note, SBS18, which is associated with MUTYH-associated polyposis (3), is absent in the tumor samples we sequenced. We believe this is the case because of the low occurrence of MUTYH deficiency generally in colorectal cancer (less than 1%; ref. 13), as well as further undersampling of patients with germline predisposition mutations as only healthy individuals were enrolled prospectively in NHS/HPFS.

NMF signal separation in The Cancer Genome Atlas (TCGA) colorectal tumors (n = 540) revealed the existence of seven signatures (Supplementary Figs. S4 and S5 for SigProfiler results) similar to the ones found in NHS/HPFS (Supplementary Fig. S6), thus suggesting the existence of the same underlying mutational processes in all colorectal cancer cohorts. Analysis of the TCGA colorectal tumors (Fig. 2B) substantiated the same etiologies for the POLE signatures c-POLEa and c-POLEb (P = 6.2 × 10⁻⁷ and P = 7.3 × 10⁻⁷, respectively, Mann–Whitney U test), as well as dMMR signatures c-dMMRa and c-dMMRb (P < 2 × 10⁻¹⁶ for both, Mann–Whitney U test). We also observed that TCGA tumors with MGMT promoter methylation were enriched in signature c-Alkylation (P = 9.7 × 10⁻⁵, Mann–Whitney U test). Of note, in TCGA, signature c-Alkylation displayed the highest similarity with SBS30 (cossim = 0.81), followed by SBS11. Conversely, SBS30 was the second most similar signature to the c-Alkylation one in the NHS/HPFS cohorts (Fig. 2C; Supplementary Fig. S3). SBS30 resembles SBS11 (cossim of 0.76, Fig. 2C) and is attributed to base excision repair (BER) deficiency (3), which is also a central pathway in repairing damage from alkylated bases. We nevertheless found no association between germline polymorphisms in NHTL1 and other genes of the BER pathway and the alkylating signature in the TCGA specimens (see Methods and Supplementary...
Figure 1. De novo signature deconvolution in NHS/HPFS colorectal cancers. A, Cohort and data overview. FFPE, formalin-fixed, paraffin-embedded. B, Quality measures for NMF in NHS/HPFS. Arrows indicate the estimated rank of mutational signatures. rss, residual sum of squares. C, The consensus seven signatures found by NMF in NHS/HPFS.

We also estimated the effect size for the Mann–Whitney U tests by calculating the rank-biserial correlation \( r_{rb} \) for each mutational signature and the respective molecular or clinical phenotype shown in Fig. 2B. We observed that the effect sizes were similar for the alkylating signatures and the aging signature (see Methods and Supplementary Fig. S9A and S9B).

Fig. S7). The presence of SBS30 ahead of SBS11 in the TCGA colorectal cancer data set could instead be attributed to a smaller sample size of colorectal cancers in TCGA compared with NHS/HPFS (see “Undersampling Simulations” in Methods and Supplementary Fig. S8). The Fanconi anemia (FA) and translesion synthesis (TLS) DNA damage repair pathways also do not show an association with the alkylating signature (see Methods and Supplementary Fig. S9A and S9B).
Alkylating Mutational Signature and Colorectal Cancer

Figure 2. Active mutational signatures in colonic cells. Proportion of mutations assigned to de novo extracted signatures in colorectal cancers from NHS/HPFS (A) and TCGA (B), segregated by MGMT promoter methylation status, POLE exonuclease mutations, microsatellite instability, and age at diagnosis. Box plot outliers not shown. C, Heat map of the similarity scores between colorectal tumor (from TCGA and NHS/HPFS) signatures, clustered on the x-axis. COSMIC signatures found in either NHS/HPFS or TCGA are bolded. The alkylating normal colon (from EGA) signature is also shown. Clustering has been performed according to cosine similarity.

Interestingly, a previously published survey of mutational signatures in normal colorectal crypts (14) from the European Genome–phenome Archive (EGA) showed the existence of a signature (named SBSC) that we found to be similar to the alkylating one that we observed in NHS/HPFS colorectal cancers (cossim = 0.77; Fig. 2C). The hierarchical clustering of SBSC with the seven signatures deconvoluted from NHS/HPFS and TCGA confirmed the similarity of EGA SBSC with the alkylating imprints (Fig. 2C).

Dietary Patterns of Alkylation Damage

To test whether dietary components contributed to the alkylating signature in colorectal cancer, we leveraged prospectively collected repeated measurements of meat, poultry, and fish consumption in grams per day in the NHS and HPFS cohorts. All available red meat variables showed significant positive associations between prediagnosis intakes and alkylating damage in colorectal cancers (Fig. 3A; overall red meat, \( P = 0.017/7.3 \times 10^{-3} \); processed red meat, \( P = 7.3 \times 10^{-3} \); and processed red meat, \( P = 7.3 \times 10^{-3} \), Mann–Whitney test).
U test). Other dietary variables (fish and chicken intake, Fig. 3B) and lifestyle factors (body mass index, alcohol consumption, smoking, and physical activity in Supplementary Fig. S10) did not show any significant association with the alkylating signature. In addition, no other colorectal cancer mutational process showed a significant association with red meat intake (Supplementary Fig. S11). Of note, MGMT promoter methylation did not differ by red meat consumption (two-sided Mann–Whitney U-test, P = 0.51; Supplementary Fig. S12). When adjusted for red meat intake, there was no difference in alkylating damage between male and female patients with colorectal cancer (two-sided Mann–Whitney U-test, P = 0.27 for patients with high overall red meat consumption).

Previous studies (9, 10) showed a positive association between processed red meat and colorectal cancer incidence in the distal colon. Thus, we also investigated how the alkylating damage might differ by tumor location. We found that, compared with the proximal colon, the distal colorectal specimens exhibited higher alkylating damage in tumors (P = 1.4 × 10^{-4} in NHS/HPFS and P = 1.9 × 10^{-4} in TCGA, Mann–Whitney U-test) and normal crypts (P = 0.022, Mann–Whitney U-test; Fig. 3B).

Carcinogenicity of Alkylating Damage

Mutational processes increase the likelihood of specific driver mutations in certain trinucleotide contexts. To find such driver mutations that associate with the alkylating signature, we devised a simple model (Fig. 4A; see Methods) that predicts the relative likelihood of mutational processes to target colorectal cancer recurrent drivers in non–MSI-high, non–POLE-mutated tumors.

In particular, the alkylating signature appeared to be the dominant one that targets KRAS p.G13D (relative likelihood = 1) and KRAS p.G12D (relative likelihood = 0.91; Fig. 4A). This is due to p.G12D and p.G13D being in trinucleotide contexts (ACC>ATC and GCC>GTC, respectively) mainly targeted by the alkylating signature. PIK3CA p.E545K (TCA>TTA) is also predicted to be predominantly targeted by the alkylating signature (relative likelihood = 0.87). Supporting this, we showed that colorectal cancers having KRAS p.G12D, KRAS p.G13D, or PIK3CA p.E545K-mutant colorectal cancers were enriched with the alkylating signature compared with all other tumors (Fig. 4B, P = 0.013, Mann–Whitney U-test).

Last, we examined patient survival across ordinal alkylating mutational signature quartiles and found that patients whose tumors have high alkylations damage (top quartile) had a worse colorectal cancer–specific survival (log-rank test Prank = 0.036; Fig. 4C; Supplementary Tables S2 and S3). Furthermore, higher alkylating signature contribution was associated with worse colorectal cancer–specific survival in both univariable and multivariable Cox proportional hazards regression analyses (Prank = 0.015 and Prank = 0.036, respectively, Fig. 4D and Supplementary Table S3).

DISCUSSION

Our work demonstrated the presence of a novel alkylating mutational signature, which we deconvoluted directly from WES of colorectal tumors. Interestingly, this signature is highly similar to SBS11, which was originally discovered in patients with prior exposure to temozolomide (1).
Alkylating Mutational Signature and Colorectal Cancer

Temozolomide is an alkylating agent used as a treatment of brain gliomas with MGMT promoter methylation (1) and induces the same lesions as dietary NOCs and in the same proportions (80% of N7-methylguanine and N3-methylguanine, as well as 10% of O6-methylguanine; refs. 15, 16).

Previous attempts have shown the existence of alkylating lesions in normal colorectal mucosa, notably caused by NOCs (17). The latter can be formed endogenously after nitrosylation of heme iron from blood (17, 18) but have also been associated with red meat intake in a small cohort of participants (19). However, these previous studies were based on limited data sets (small sample sizes and/or use of laboratory methylating agents) and lack comprehensive sequencing that would enable the discovery of the full mutational spectrum induced by red meat. Crucially, past efforts have focused on normal colorectal tissues and not examined colorectal cancer. Our analysis reveals the existence of an alkylating signature in colorectal cancer, which is associated with high prediagnosis intake of processed and unprocessed red meat.

Earlier work also hypothesized that the distal colon has increased DNA damage from exposure to dietary carcinogens, as a result of feces storage and water resorption in this portion of the large intestine (20). This is believed to explain the association observed between distal cancer incidence and red meat consumption (9, 10, 20). Consistently, we found an enrichment in tumors and normal crypts in the distal colon and rectum.

In support of the International Agency for Research on Cancer (IARC) Monograph Working Group, which classified processed meat as carcinogenic (8), our results provide molecular evidence of this dietary factor’s mutagenic impact. In addition, our analyses further implicate unprocessed meat intake and suggest MGMT as a factor of susceptibility to red
The existence of a similar alkylating signature in normal colorectal crypts also suggests that alkylating damage is lower than other mutational processes, we showed that alkylating damage may offer future potential therapeutic opportunities. More generally, our study exhibits the potential role of large-scale molecular epidemiologic studies in elucidating cancer pathogenesis (23) and guiding prevention efforts through lifestyle modifications, such as dietary interventions.

**METHODS**

**Study Population, Specimens, and Sequencing**

We used data from three prospective cohort studies in the United States: the Nurses’ Health Study I (NHS1, including 121,701 women ages 30 to 55 years at enrollment who had been followed since 1976), the Nurses’ Health Study II (NHS2, including 116,429 women ages 25 to 42 years who had been followed since 1989), and the HPFS (including 51,529 men ages 40 to 75 years followed since 1986; ref. 12). The study participants had been sent questionnaires biennially to update information on lifestyle factors and newly diagnosed diseases, including colorectal cancer. The follow-up rate had been more than 90% for each follow-up questionnaire cycle in the three cohort studies. The patients were followed until death or end of follow-up (January 1, 2016, for HPFS; June 1, 2016, for NHS1; and June 1, 2015, for NHS2), whichever came first. Study physicians, who were blinded to exposure data, reviewed medical records of 4,855 incident colorectal cancer cases to confirm the disease diagnosis and to collect data on tumor size, tumor anatomic location, and disease stage. Archival formalin-fixed, paraffin-embedded (FFPE) tissue blocks of malignant tissue. Similarly, the association of the signature with cancer driver mutations, such as KRAS and PIK3CA ones, may offer future potential therapeutic opportunities. More generally, our study exemplifies the potential role of large-scale molecular epidemiologic studies in elucidating cancer pathogenesis (23) and guiding prevention efforts through lifestyle modifications, such as dietary interventions.

**Dietary Variables**

Ascertainment of diet was carried out as previously described (9). To assess dietary intake in each cohort, food frequency questionnaires (FFQ) were initially collected in 1980 for NHS and in 1986 for HPFS. For the NHS, a 61-item semiquantitative FFQ was used at baseline (26), which was expanded to approximately 130 food and beverage items in 1984, 1986, and every 4 years thereafter. For the HPFS cohorts, baseline dietary intake was assessed using a 131-item FFQ that was also used for updates generally every 4 years subsequently (27). In particular, unprocessed red meat consumption was evaluated based on forms on the intake of “beef or lamb as main dish,” “pork as main dish,” “hamburger,” and “beef, pork, or lamb as a sandwich or mixed dish.” Processed meat diets included “bacon,” “beef or pork hot dogs”; “salami, bologna, or other processed meat sandwiches,” and “other processed red meats such as sausage, kielbasa, etc.” Consumption of red meat, chicken, poultry, and fish was evaluated in grams per day. For the remainder of our analysis, we considered the top decile of each variable to determine the “high intake” patients and considered the rest as “low-intake” patients, because only the top-decile patients show a substantial difference in overall red meat intake (Supplementary Fig. S13A and S13B). Data were based on the most recent prediagnosis reported intake for each patient.

**MGMT Promoter Methylation, MSI, and POLE Deficiency Status**

**Somatic Variant Calling**

We have used the Cancer Genome Analysis (CGA) WES characterization pipeline (https://github.com/broadinstitute/CGA_Production_Analysis_Pipeline) developed at the Broad Institute of MIT and Harvard to call, filter, and annotate somatic mutations. All analyses were carried out on the human genome build hg19. The pipeline employs the following tools: MuTect (29), ContEst (30), Strelka (31), DeTiN (32), AllelicCapSeg (33), MAFO-NcFilter (34), RealignmentFilter, GATK (35), and PicardTools. WES-specific artifacts are filtered similarly to previous publications (25, 36). Briefly, WES artifacts arise from formaldehyde deamination of cytosines resulting in C-to-T transition mutations, which presents itself as an “Orientation bias” (excess of C>T sites in F1R2 read pairs and an excess of G>A in F2R1 read pairs). In the pipeline we used the “Orientation Bias filter” tool (37) filters out WES-specific artifacts. To further filter spurious single-nucleotide variant calls we used Burrows–Wheeler Aligner BWA-MEM (http://bio-bwa.sourceforge. net/) to realign sequenced reads associated with the mutations to a set of candidate positions.
of sequences derived from the human reference assembly. The Panel of Normal was created using normal samples with less than 1% of cross-sample contamination (as evaluated by Contest; ref. 30) and less than 1% of tumor in normal (as outputted by DeTIN; ref. 32). We illustrate the variant calling pipeline in Supplementary Fig. S14.

**TCGA Data Analysis**

Clinical, methylation, and somatic mutation data from TCGA were downloaded from the Data Coordination Center (DCC) data portal at https://dcc.icgc.org/releases/current/Projects/COAD-US and https://dcc.icgc.org/releases/current/Projects/READ-US (as of March 2020). For consistency, only WES data sets were used. Altogether, we pooled 540 TCGA patients with somatic mutation data, among whom 523 patients also had methylation data.

We evaluated MGMT promoter methylation status using the MGMT-STEP2 prediction model (38). In short, two probes (cg12434587 and cg12981137) were used to predict MGMT promoter methylation. An M value cutoff of 0.358, which empirically maximized the sum of sensitivity and specificity, was then used to discriminate MGMT promoter methylation status (Supplementary Fig. S15).

**Nonnegative Matrix Factorization**

Mutations were deconvoluted into separate signatures based on the number of mutations in each of 96 possible trinucleotide contexts. Deconvolution was carried out with a standard NMF method based on Kullback–Leibler divergence using the “NMF” R package (39). This method is particularly adapted for mutational signature analysis as recent studies demonstrated (40).

A critical parameter in NMF is the estimation of the rank (i.e., the number of expected mutational signatures). To determine this, we performed quality measures on a range of ranks (n = 2 to 10) for the 900 colorectal cancer exomes in the NHS/HPFS cohorts. This showed a sharp increase in the cophenetic (i.e., the stability of the NMF classes) and dispersion (i.e., the reproducibility of the class assignments) metrics after rank = 7. For this rank, we also observed that the residual sum of squares (RSS) reached a lower plateau (Supplementary Fig. S1). A similar rank survey on an independent cohort of 540 colorectal cancer exomes from the TCGA (Supplementary Fig. S4) revealed the same dispersion and cophenetic peaks at rank = 7 and a lower plateau RSS. For the rest of the analysis, we consequently used rank = 7. We confirmed the robustness of these seven signatures by running NMF with different variant allele frequency (VAF) cutoffs (Supplementary Fig. S16). This demonstrates that the signature discovery is not affected by low VAF mutations, which are more likely to represent sequencing artifacts, such as those due to FFPE preservation. SigProfiler was run on NHS/HPFS and TCGA colorectal cancer exomes as previously described (3).

**Undersampling Simulations**

To show that the difference in sample size between TCGA (n = 540) and NHS/HPFS (n = 900) can explain the presence of SBS30 instead of SBS11 in the former cohort, we (i) randomly sampled 540 patients of the 900 from NHS/HPFS; (ii) extracted seven signatures from the 540 patients and found their closest fit among SBS1 (aging signature), SBS10a and SBS10b (POLE signatures), SBS15 and SBS26 (dMMR signatures), and SBS11 and SBS30; and (iii) repeated steps (i) and (ii) a hundred times.

**Crypt Mutational Signature Analysis**

Mutational signatures from normal colonic crypts (14) were used in our analysis. These signatures were extracted from WGS data from 571 crypts from 42 individuals from the EGA (14). Deconvolution was performed using a hierarchical Dirichlet process, which produces results similar to NMF (14).

**Analysis of Recurrent Hotspot Mutations**

To compute the relative likelihood of mutational processes to target a specific hotspot, we (i) localized the trinucleotide context of the hotspot, (ii) extracted the signatures contribution for the specific trinucleotide context, and (iii) normalized the contribution of each signature, such that the sum became 1. Recurrent hotspots were defined as specific point mutations occurring in at least 25 patients.

**TCGA Germline Polymorphisms Analysis**

TCGA genotyping data (Affymetrix SNP 6.0 array platform) were used to select germline variants from genes in the BER, FA, and TLS pathways extracted from the GSEA database (refs. 41, 42; https://www.gsea-msigdb.org/gsea/msigdb/). We imputed autosomal variants for TCGA samples using IMPUTE2 (43), with haplotypes of 1000 Genomes Phase 3 (44) as the reference panel. We used the following criteria to select SNPs with the plink software (45): (i) average imputation confidence score, also called INFO score, ≥0.4; (ii) minor allele frequency ≥5%; (iii) SNP missing rate <5% for best-guessed genotypes at posterior probability ≥0.9; and (iv) Hardy–Weinberg equilibrium P value >1 × 10⁻⁶. After imputation, 2,041 variants were included in our subsequent analysis. We tested for an additive effect (genotype 0,1,2 as a continuous variable) for each SNP and found no association with the alkylating signature [Supplementary Fig. S7 and Supplementary Fig. S9, FDR-adjusted P value (q value) less than 0.1 for all SNPs tested].

**Statistical Analysis**

We used R version 3.6.2 to perform statistical analyses. Significance for two-group comparisons was evaluated by a one-sided Mann–Whitney U test unless otherwise indicated. P < 0.05 was considered statistically significant. For the comparisons of the alkylating signature by age in the NHS/HPFS cohorts and TCGA colorectal cancer database, the patients’ median age (70 and 67 years, respectively) was used as the cutoff.

Eight hundred eighty-two patients with available colorectal cancer survival data were subsequently used for survival analyses. Univariable- and multivariable-adjusted Cox proportional hazards regression analysis as used to calculate the HR of colorectal cancer–specific survival and overall survival according to ordinal alkylating mutational signature quartiles (Q1–Q4). The multivariable Cox regression model initially included sex (female vs. male), age at diagnosis (<60, 60–64, 65–69, and ≥70 years), year of diagnosis (1995 or before, 1996–2000, 2001–2005, and 2006–2014), family history of colorectal cancer (present vs. absent), current smoking status (never smoking, past smoking, 1–14 pack-years, 15–24 pack-years, ≥25 pack-years), alcohol consumption (women: 0–<0.15, 0.15–<2.0, 2.0–<7.5, and ≥7.5 g/day; men: 0 to <1, 1–<6, 6–<15, and ≥15 g/day), tumor location (proximal colon vs. distal colon vs. rectum), CpG island methylator phenotype (high vs. low/negative; ref. 46), KRAS mutation (mutant vs. wild-type; ref. 47), BRAF mutation (mutant vs. wild-type; ref. 47), tumor differentiation (well to moderate vs. poor), disease stage (I/II vs. III/IV), microsatellite instability status (MSI-high vs. non-MSI-high; ref. 46), and long-interspersed nucleotide element 1 (LINE-1) methylation level (continuous; ref. 48). A backward elimination with a threshold P of 0.05 was used to select variables for the final models. Cases with missing data were assigned to the majority category of a given categorical covariate to limit the degrees of freedom, except for cases with missing LINE-1 methylation, for which we assigned a separate indicator variable. We confirmed that excluding the cases with missing information in any of the covariates did not substantially alter results.

**Data Availability**

WES data have been deposited in dbGAP (accession number phs000722). WES quality metrics and a subset of clinical annotations are included in this article. Additional clinical and epidemiology data from the NHS1, NHS2, and HPFS can be requested through the NHS/HPFS consortia.
Code Availability Statement
All analysis scripts are available upon request.

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Authors’ Contributions
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REFERENCES
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