Discovery and Features of an Alkylating Signature in Colorectal Cancer

Carino Gurjao1,2, Rong Zhong3,4, Koichiro Haruki3, Yvonne Y. Li1,2, Liam F. Spurr1,2,5, Henry Lee-Six6, Brendan Reardon1,2, Tomotaka Uga1,7, Xuehong Zhang8,9, Andrew D. Cherniack1,2, Mingyang Song7,8,9,11, Eliezer M. Van Allen1,2, Jeffrey A. Meyerhardt1, Jonathan A. Nowak12, Edward L. Giovannucci7,8,9, Charles S. Fuchs13, Kana Wu9, Shuji Ogino2,3,7,12, and Marios Giannakis1,2

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.

1Department of Medical Oncology, Dana-Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts. 2Broad Institute of MIT and Harvard, Cambridge, Massachusetts. 3Program in MPE Molecular Pathological Epidemiology, Department of Pathology, Brigham and Women’s Hospital and Harvard Medical School, Boston, Massachusetts. 4Department of Epidemiology and Biostatistics and Ministry of Education Key Lab of Environment and Health, School of Public Health, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, China. 5Pritzker School of Medicine, Biological Sciences Division, University of Chicago, Chicago, Illinois. 6Wellcome Sanger Institute, Hinxton, United Kingdom. 7Department of Epidemiology, Harvard T.H. Chan School of Public Health, Boston, Massachusetts. 8Channing Division of Network Medicine, Department of Medicine, Brigham and Women’s Hospital and Harvard Medical School, Boston, Massachusetts. 9Department of Nutrition, Harvard T.H. Chan School of Public Health, Boston, Massachusetts. 10Clinical and Translational Epidemiology Unit, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts. 11Division of Gastroenterology, Massachusetts General Hospital, Boston, Massachusetts. 12Department of Pathology, Brigham and Women’s Hospital and Harvard Medical School, Boston, Massachusetts. 13Yale Cancer Center, Yale School of Medicine, Smilow Cancer Hospital, New Haven, Connecticut.

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C. Gurjao, R. Zhong, and K. Haruki are the co–first authors of this article. K. Wu, S. Ogino, and M. Giannakis are the co–senior authors of this article.

Corresponding Author: Marios Giannakis, Dana-Farber Cancer Institute, 450 Brookline Avenue, Boston, MA 02215-5450. Phone: 617-582-7263; E-mail: Marios_Giannakis@dfci.harvard.edu

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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 nitrites/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) to compare the deconvoluted signatures to reference signatures (see Methods and Fig. 1B and C; Supplementary Fig. S6). SBS30 resembles SBS11 (cossim of 0.81), followed by SBS15, 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, pathology, 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^{-5}, 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^{-5}, 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.
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.

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).

We also estimated the effect size for the Mann–Whitney U tests by calculating the rank-biserial correlation \( r_b \) 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 \( (r_b = 0.14 \text{ and } r_b = 0.16, \text{ respectively}) \) and smaller than the hypermutator dMMR and POLE signatures \( (r_b > 0.8 \text{ for dMMR and POLE signatures in both TCGA and NHS/HPFS}) \).
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 = 0.85). Of note, SBSC matched closely to SBS23, which, similar to SBS30, also resembles SBS11 (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, \( P = 0.017/r_{sb} = 0.14 \); unprocessed red meat, \( P = 7.8 \times 10^{-3}/r_{sb} = 0.016 \); and processed red meat, \( P = 7.3 \times 10^{-3}/r_{sb} = 0.16 \), Mann–Whitney...
Carcinogenicity of Alkylation Damage

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>TTC) 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 alkylation damage (top quartile) had a worse colorectal cancer–specific survival (log-rank test $P_{\text{trend}} = 0.036$; 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 ($P_{\text{trend}} = 0.015$ and $P_{\text{trend}} = 0.036$, 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).
Figure 4. Carcinogenic potency of alkylating damage. A, Relative likelihood of mutational processes to target recurrent hotspots in nonhypermutated colorectal cancer. As hotspots, we considered all point mutations that were present in at least 25 patients with nonhypermutated (non–MSI-high, non–POLE-mutated) colorectal cancer. Each stacked bar represents the relative likelihood of a given signature to target a given hotspot. B, Proportion of mutations assigned to alkylating damage in NHS/HPFS, TCGA colorectal cancers, segregated by KRAS G12D/KRAS G13D/PIK3CA E545K mutation status. Box plot outliers not shown. C, Kaplan-Meier plot illustrating colorectal cancer–specific survival of the patients stratified into quartiles of alkylating signature contribution. D, Forest plot of the association between the colorectal cancer–specific survival and quartiles of alkylating signature contribution in univariable and multivariable Cox regression models.

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
malignant tissue in 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 NHS; 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 tumor and normal colon were collected in a subset of colorectal cancer. We previously showed that in our cohorts, demographic features of cases did not differ appreciably by tissue availability (24). The study protocol was approved by the institutional review boards of the Brigham and Women’s Hospital and Harvard T.H. Chan School of Public Health (Boston, MA) and those of participating registries as required. Written informed consent was obtained from all patients with colorectal cancer.

We prioritized relatively more recent colorectal cancer cases for sequencing to mitigate the potential impact of FFPE artifacts. Given the number of NHS versus HPFS participants (2:1 female/male ratio), we also sequenced relatively more specimens from male patients to obtain more balanced sequencing data. Supplementary Table S4 shows the clinical and pathologic characteristics of the 4,855 patients with colorectal cancer.

WES was carried as previously described (25). Briefly, using guide hematoxylin and eosi–stained slides, tumor areas were selected to extract tumor-enriched DNA from tissue sections of tumor FFPE blocks. Normal DNA was extracted from resection margins or other areas free from tumors. DNA specimens underwent hybrid capture with SureSelect v.2 Exome bait (Agilent Technologies), followed by sequencing on Illumina HiSeq 2000 instruments. The obtained average coverage was 85× in tumors and matched adjacent normal colon tissue (see Supplementary Table S5).

**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,” “ham,” 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 “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**

MGMT promoter methylation analysis in the NHS/HPFS cohorts was carried out using bisulfite conversion and real-time PCR as previously described (28). MSI status was evaluated using 10 microsatellite markers (D2S123, D5S346, D17S250, BAT25, BAT26, BAT40, D18S55, D18S56, D18S67, and D18S487) as formerly detailed (12). MSI status was carried out using bisulfite conversion and real-time PCR as previously described (28). MSI status was evaluated using 10 microsatellite markers (D2S123, D5S346, D17S250, BAT25, BAT26, BAT40, D18S55, D18S56, D18S67, and D18S487) as formerly detailed (12).

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**Somatic Variant Calling**

We have used the Cancer Genome Analysis (CGA) WES characterization pipeline ([https://github.com/broadinstitute/CGA_Production_Analysis_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), ContExt (30), Strelka (31), DeTFIN (32), AllelicCapSeg (33), MAFPoNFilter (34), RealignmentFilter, GATK (35), and PicardTools. FFPE-specific artifacts are filtered similarly to previously published publications (25, 36). Briefly, FFPE 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 F2R1 read pairs). In the pipeline, we use the “Orientation Bias Filter” tool (37) filters out FFPE-specific artifacts. To further filter spurious single-nucleotide variant calls, we used Burrows–Wheeler Aligner BWA-MEM ([http://bio-bwa.sourceforge. net/](http://bio-bwa.sourceforge.net/)) to realign sequenced reads associated with the mutations to a set
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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 DeTN; 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://dec.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-STEP27 prediction model (38). In short, two probes (cg124343S87 and cg12891137) 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 errors. 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-guessted 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. Univariant-and multivariable-adjusted Cox proportional hazards regression analysis was 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–<0.2, 0.2–<0.5, 0.5–<1, 1–<2, 2–<4, 4–<6, 6–<10, 10–<15, ≥15 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.

**Authors’ Disclosures**

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**Authors’ Contributions**

C. Gurjao: Data curation, formal analysis, investigation, visualization, methodology, writing—original draft, writing—review and editing. R. Zhong: Data curation, formal analysis, writing—review and editing. K. Haruki: Data curation, formal analysis, writing—review and editing. Y.Y. Li: Writing—review and editing. L.F. Spurr: Writing—review and editing. H. Lee-Six: Resources, writing—review and editing. B. Reardon: Writing—review and editing. T. Ugai: Writing—review and editing. X. Zhang: Writing—review and editing. A.D. Cherniack: Writing—review and editing. M. Song: Writing—review and editing. E.M. Van Allen: Writing—review and editing. J.A. Meyerhardt: Resources, writing—review and editing. J.A. Nowak: Resources, writing—review and editing. E.L. Giovannucci: Resources, data curation, writing—review and editing. C.S. Fuchs: Resources, funding acquisition, writing—review and editing. K. Wu: Data curation, funding acquisition, writing—review and editing. S. Ogino: Resources, data curation, funding acquisition, writing—review and editing. M. Giannakis: Conceptualization, resources, supervision, funding acquisition, investigation, methodology, writing—original draft, writing—review and editing.

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**REFERENCES**


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Carino Gurjao, Rong Zhong, Koichiro Haruki, et al.


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