Cancer Prevention Research

Research Article

Cancer Risks for the Relatives of Colorectal Cancer Cases with a Methylated *MLH1* Promoter Region: Data from the Colorectal Cancer Family Registry

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Abstract

Methylation of the *MLH1* gene promoter region is an underlying cause of colorectal cancer (CRC) with high microsatellite instability (MSI-H) diagnosed in persons without a germ line mutation in a mismatch repair (MMR) gene (non–Lynch Syndrome CRC). It is unclear whether relatives of CRC cases with *MLH1* methylation have an increased risk of colorectal or other cancers. In this retrospective cohort study, we assessed risk of CRC and other cancers for the first- and second-degree relatives of CRC cases with a methylated *MLH1* gene, by comparing observed numbers of cases with those expected on the basis of age-, sex-, and country-specific cancer incidences (standardized incidence ratios). The cohort consisted of 3,128 first- and second-degree relatives of the 233 *MLH1*-methylated CRC cases with no *MMR* or *MUTYH* gene mutations. The standardized incidence ratio (SIR) for CRC was 1.60 [95% confidence interval (CI), 1.22–2.16] for first-degree relatives and 1.08 (0.74–1.60) for second-degree relatives. The SIR for gastric cancer was 2.58 (1.52–4.71) for first-degree relatives and 4.52 (2.23–10.61) for second-degree relatives and, for ovarian cancer, it was 2.16 (1.29–3.86) for first-degree relatives. The risk of liver cancer was also increased significantly in first-degree relatives but the estimate was on the basis of only two cases. These data imply that relatives of CRC cases with *MLH1* methylation may be at increased risk of CRC and stomach cancer and possibly ovarian and liver cancer, suggesting that there may be a heritable factor for CRC and other cancers associated with *MLH1* methylation in non–Lynch syndrome CRCs. *Cancer Prev Res; 5(2); 328–35.* ©2011 AACR.

Introduction

Colorectal carcinoma (CRC) is a heterogeneous condition that arises via at least two different pathogenetic mechanisms. These mechanisms are distinguished on the basis of the type of DNA damage most prevalent in the tumors. Chromosomal instability (CIN) is characterized by numerous chromosomal abnormalities, widespread LOH, and aneuploidy. Microsatellite instability (MSI-H) is characterized by widespread changes in the length of short regions of repetitive DNA sequences called microsatellites.

Within MSI-H CRCs, there are two subtypes: one resulting from a germ line mutation in 1 of 4 DNA mismatch repair genes (Lynch syndrome) and the other from a somatic epigenetic event in the promoter of a single DNA mismatch repair gene (*MLH1*; refs. 1, 2). The latter subset is known as sporadic MSI-H CRC. Sporadic MSI-H tumors with *MLH1* promoter methylation are also characterized by extensive methylation of multiple gene promoter regions called the CpG island methylator phenotype (CIMP; ref. 3).

A family history of CRC is a well-known risk factor for CRC overall (4), but the role of family history for CRC according to tumor phenotype is not well understood. Findings from studies of CRC with the MSI-H phenotype have been inconsistent, with some reporting an increase in the prevalence of a family history of CRC in MSI-H cancers (5–9), which may be modified by the presence of Amsterdam criteria in the family (6) or proband age (7), and other studies reporting no association between MSI-H CRC and family CRC history (10–12). However, only two studies undertook genetic screening to eliminate Lynch Syndrome families from the study population (5, 9).

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doi: 10.1158/1940-6207.CAPR-11-0419

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We hypothesized that underlying heritable factors may promote a phenotype involving *MLH1* methylation and increase risk of CRC and other cancers in relatives. In the current study, we compared the observed incidence of reported cancers in the relatives of CRC cases with a methylated *MLH1* promoter region, and no *MMR* or *MUTYH* gene mutations, to the number expected for the general population given the age, sex, and country of residence of the relatives. Because the use of molecular profiling of tumors is likely to increase in the future, it is important to understand the familial risk of cancers in this particular subgroup of cases.

Materials and Methods

Study sample

Subjects for this study were enrolled in the Colon Cancer Family Registry (C-CFR), a National Cancer Institute–funded CRC registry, which uses comprehensive and standardized methods for data collection and genotyping. Detailed information about the C-CFR and subject recruitment at the different C-CFR sites can be found in a report by Newcomb and colleagues (13) and at http://epi.grants.cancer.gov/CFR/. For this study, we included incident CRC cases diagnosed between 1997 and 2007 and ascertained from the population-based cancer registries in the United States (Puget Sound, Washington State; the State of Minnesota; Los Angeles, CA; AZ; CO; NH; NC; and HI), Australia (Victoria), and Canada (Ontario). Some recruitment sites stratified the recruitment of cases using a sampling fraction on the basis of their reported family history (for detail, see the study by Newcomb and colleagues; ref. 13). All CRC cases were asked for permission to contact their relatives to seek their enrollment in the Registry. Probands for this study were the CRC cases with a methylated *MLH1* promoter and no *MMR* or *MUTYH* mutations. We obtained informed consent from all participants. The study was approved by the Institutional Review Board at each C-CFR site.

Family history

The baseline questionnaire administered to each proband elicited a detailed history of cancers occurring in first- and second-degree relatives. Standardized procedures were used to collect epidemiologic data from all participants (demographics, selected risk factors, CRC screening tests, personal and family history of cancer, polypectomy, hysterectomy, and other surgeries). Follow-up of all participants, approximately 5 and 10 years after initial recruitment, included a second interview to obtain updated data on selected variables, including family history of cancer. Blood samples, tumor blocks, and pathology reports were collected from all CRC cases. Data for this study were based on both the baseline and follow-up questionnaires. We attempted to verify family history information by comparing reports from multiple individuals within the same family. When available, medical records, death certificates, pathology reports, and tumor tissues were also used to confirm reported cancer diagnoses. Cancers were classified using the International Classification of Diseases of Oncology-3 (ICD-O 3) codes (14).

MLH1 DNA methylation assay

MLH1 DNA methylation was determined as described (15). Briefly, cases were sampled for *MLH1* promoter region methylation testing on the basis of MSI status according to the following strategy: from the population-based series, *MLH1* methylation was measured in all MSI-H and MSI-L cases with sufficient tumor DNA and a random sample of MSS cases. *MLH1* methylation was measured using the MethyLight MLH1-M2Methylight reaction (16) using an ALU control reaction to normalize for bisulfite-converted input DNA, with the modifications described in the study by Poynter and colleagues (15). We classified samples with a percentage of methylated reference (PMR) greater than or equal to 10 as positive for *MLH1* methylation as described (3). An *ALU* control reaction cycle threshold (C_t) value of less than or equal to 24 was used to retain the largest sample size possible for the analysis while minimizing the potential for false negatives as described in the study by Poynter and colleagues (15).

MMR mutation data

Screening for germ line mutations in *MLH1*, *MSH2*, *MSH6*, and *PMS2* was conducted for all population-based probands who had a colorectal tumor displaying evidence of impaired MMR function as evinced by either MSI or by lack of MMR protein expression by immunohistochemistry. Mutation testing was conducted by Sanger sequencing or denaturing high-performance liquid chromatography (dHPLC), followed by confirmatory DNA sequencing. Large duplication and deletion mutations were detected by Multiplex Ligation Dependent Probe Amplification (MLPA) according to the manufacturer's instructions (MRC Holland; refs. 17, 18). All participants who donated a blood sample, and who were relatives of probands with a pathogenic mutation, underwent testing for the same mutation identified in the proband. A pathogenic mutation was defined as a variant that was predicted to result in a stop codon, a frameshift mutation, a large duplication or deletion, or a missense mutation previously reported within scientific literature and databases to be pathogenic.

Tumor subsite

Tumor subsite was classified into right colon (the cecum through the transverse colon), left colon (the splenic flexure through the sigmoid colon), and rectal (the rectosigmoid junction and the rectum).

Statistical analysis

Observation time for each subject started at birth and ended at the age at first diagnosis of cancer or last contact or death, whichever occurred first. We censored each relative at the age of first polypectomy for CRC and at the age of hysterectomy for endometrial cancer, except when it occurred within a year of the diagnosis of the disease.

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To account for stratified sampling on the basis of family history, we gave each subject a probability weight equal to the reciprocal of the family sampling fractions used by each study center.

Increased risk of cancer for relatives were estimated using cancer-specific standardized incidence ratios (SIR) that were calculated by dividing the observed numbers of cancers by the expected numbers. The Jackknife method was used to estimate 95% confidence intervals (CI) by allowing for any correlation of risk between relatives from the same family (19). The observed numbers of cancers were calculated by summing the product of the numbers of observed cancers for each subject by the probability weight. For example, 4 CRC cases with a weight of 0.25 each represented one observed CRC case. The expected numbers of cancers for the study cohort were calculated by multiplying the age-, sex-, and country-specific incidence for the general population with the corresponding observation time in the cohort. Age- and sex-specific cancer incidences in 1988 to 1992 for each country (Australia, Canada, and United States) were obtained from Cancer incidence in five continents (20). The choice of this time period reflects the fact that the majority of the cases (first- and second-degree relatives) were diagnosed before the proband's cancer was diagnosed, and the period of 1988 to 1992 was the closest available data set to the mean calendar year of cancer diagnoses in the relatives.

For relatives with missing ages at cancer diagnoses, age at diagnosis was assumed to be 1 year prior to the last known age or, if last known age was not available, the median age at diagnosis of the specific cancer for the general population. The sex-specific median age for each cancer was obtained from the SEER cancer statistics review, 1996–2000 (21).

SIRs were estimated for cancers of the colon and rectum (ICD-O C18–20), stomach (ICD-O C16), liver (ICD-O C22), pancreas (ICD-O C25), brain (ICD-O C71), kidney (ICD-O C64–65), and lung (ICD-O C34) for both sexes; and endometrium (ICD-O C54–55), ovary (ICD-O C56), and breast (ICD-O C50) for women. To reduce the possibility of including metastatic cancer cases as a primary cancer, cancers of the liver ($n = 3$) and brain ($n = 1$) were excluded if they were reported to have been diagnosed within 1 year of the age at diagnosis of other cancers.

The SIRs of CRCs for relatives were estimated stratified by the characteristics of the CRCs in probands and included (i) age at diagnosis of CRC (<50 and ≥50 years) and (ii) subsite of CRC (left colon, right colon, synchronous tumors on both sides of colon).

All reported statistical tests were two-sided and $P < 0.05$ was considered statistically significant. All statistical analyses were conducted using Stata 11.0 (22).

Estimated cumulative risks (penetrance) of different cancers to age 70 years and their 95% CIs for each sex were calculated by summing over age-specific incidences: incidence$_i$ at age$_i$ multiplied by the estimated SIR, based on the population incidences of United States, using the formula:

$$1 - e^{-\sum_{i=0}^{\text{age}} \text{SIR} \times \text{incidence}_i}$$

Table 1. Baseline characteristics of CRC cases with *MLH1* DNA methylation

	***n* (%)**
Sex	
Female	170 (73.0)
Male	63 (27.0)
Centre	
Ontario	59 (25.3)
USC	26 (11.2)
Australia	13 (5.6)
Mayo	18 (7.7)
Seattle	117 (50.2)
rs1800734	
AA	7 (11.7)
GA	23 (38.3)
GG	30 (50.0)
Not genotyped	173
Subsite of CRC	
Right colon[a]	199 (85.4)
Left colon[b]	23 (9.9)
Rectosigmoid	3 (1.3)
Rectum	4 (1.7)
Both sides of colon[c]	3 (1.3)
Unknown	1 (0.4)
Age at diagnosis of CRC, y	
<50	14 (6.0)
≥50	219 (94.0)
Mean (SD)	65.3 (8.6)
Median (range)	67 (22–87)

[a]Right colon = the cecum through the transverse colon.
[b]Left colon = the splenic flexure through the sigmoid colon.
[c]Synchronous tumors.

Results

Of the 2,470 cases ascertained from the population-based sources of the C-CFR who were assessed for *MLH1* promoter region methylation, 236 (9.6%) had a methylated *MLH1* promoter. Of these, we excluded one proband because s/he carried an *MMR* gene mutation and two probands because they carried a monoallelic mutation in the *MUTYH* gene. Of the remaining 233 probands, 170 (73%) were female. Mean age at diagnosis of CRC was 65.3 (SD, 8.6) years; range, 22 to 87 years. Of the tumors of probands, 199 (85%) were in the right colon, 23 (10%) were in the left colon, 3 (1%) were synchronous tumors in both sides of colon, 7 (3%) were in the rectosigmoid junction and the rectum, and 1 was unknown for tumor site (Table 1).

The 1,681 first- and 1,447 second-degree relatives of the remaining 233 probands with *MLH1* methylation were included in this analysis. Of these, 257 (8%) were from

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Table 2. Relatives of CRC cases with *MLH1* DNA methylation included in the study

Country	Family, *n* (%)	First-degree relatives, *n* (%)	Second-degree relatives, *n* (%)	Combined, *n* (%)
Australia	13 (0.5)	64 (4)	193 (13)	257 (8)
Canada	59 (73.0)	485 (29)	375 (26)	860 (28)
United States	161 (26.5)	1,132 (67)	879 (61)	2,011 (64)
Total	233	1,681	1,447	3,128

Australia, 860 (28%) were from Canada and 2,011 (64%) were from the United States (Table 2). Approximately 30% of all reported CRCs and 8% to 40% of extracolonic cancers were confirmed by pathology report, hospital record, death certificate, or cancer registry.

The risk of CRC was increased by 60% (SIR = 1.60; 95% CI, 1.22–2.16) for the first-degree relatives of *MLH1*-methylated cases (Table 3). For second-degree relatives, there was no evidence of an increased CRC risk (SIR 1.08; 95% CI, 0.74–1.60). In a sensitivity analysis, when we did not censor relatives at age of polypectomy, CRC risk was estimated to be 1.29 (1.00–1.67) for all relatives combined, 1.57 (1.20–2.07) for first-degree relatives, and 1.08 (0.74–1.59) for second-degree relatives.

Risks were increased for gastric cancer for first- and second-degree relatives [2.58 (1.52–4.71) and 4.52 (2.23–10.61), respectively]. The incidence of liver cancer was also increased for first-degree relatives [SIR = 2.74 (1.35–6.45)]. For women, the risk of ovarian cancer was increased for first-degree relatives [SIR = 2.16 (1.29–3.86)]. There was no evidence of increased risks for pancreatic, kidney, brain, endometrial, or breast cancers (Table 3).

There was no statistical evidence that the risk of CRC for relatives differed by proband's age of diagnosis ($P = 0.47$) or the subsite of the proband's tumor ($P = 0.76$; Table 4).

Table 5 shows the cumulative risks to age 70 years for first-degree relatives: CRC, 5% (4%–7%) for males and 3.5% (3%–5%) for females; gastric cancers, 1.4% (0.9%–3%) for males and 0.6% (0.3%–1%) for females; liver cancer, 0.7% (0.3%–2%) for males and 0.2% (0.1%–0.6%) for females, and ovarian cancer, 4% (2%–6%).

Discussion

We assessed the incidence of CRC and other cancers for the relatives of cases with methylation of the *MLH1* promoter region and no *MMR* or *MUTYH* gene mutations. We observed significantly increased risks for colorectal, gastric, liver, and ovarian cancer in first-degree relatives. We found no evidence for increased risks for endometrial, brain, pancreas, kidney, and breast cancers.

Our data suggest that there is a significant increase in the risk of CRC for first-degree relatives of *MLH1*-methylated cases. However, the overall 1.6-fold increase in CRC risk for first-degree relatives in our study is not different ($P = 0.1$) from the excess risk to first-degree relatives of CRC cases in general as estimated by Butterworth and colleagues (OR 2.24; 95% CI, 2.06–2.43; ref. 4). It is also about the same as the increased familial risk in CRCs with a *BRAF* p.V600E mutation (23), which is closely associated with both *MLH1* methylation and CIMP (3). Given the close association between *MLH1* methylation and CIMP, our data suggest that the defect underlying the abnormal hypermethylation of multiple gene promoter regions may include a heritable component. Thus, studies assessing the *MLH1* methylation and CIMP status in tumors of family members of those with *MLH1* methylated and/or CIMP tumors would be of interest.

Ricciardiello and colleagues reported *MLH1* promoter region methylation in 30% of the adenomas of patients with a family history of CRC compared with 9% of those with no family history (24). In a study of young subjects (<40 years), Koh and colleagues observed that 66.7% of subjects with an *MLH1*-methylated colorectal polyp had a positive family history compared with 41.2% in nonmethylated polyps; $P = 0.06$ (25). Subjects with germ line *MMR* gene mutations were not excluded from either study. However, such an increased risk has not been observed in all studies (26, 27).

It is likely that the *MLH1*-methylated cases in this cohort represent a heterogenous group. Evidence from the literature describes a familial syndrome where CRC and colonic precursor lesions develop via the serrated neoplasia pathway (28). This pathway is tightly associated with CIMP, somatic *BRAF* mutation, and *MLH* methylation. Some of the families included in the current cohort may represent cases of this syndrome. In addition, we could not exclude sporadic cases of constitutional *MLH1* epimutations in the proband group (29). Such epimutations are generally not associated with familial transmission. Further pathologic and molecular characterization of the CRCs in the relatives of *MLH1*-methylated cases would allow for more accurate risk estimates.

The high percentage of females among the probands in this study is similar to the overrepresentation of females in sporadic MIS-H, the large majority of which are *MLH1*-methylated tumors (30). It is not clear why such abnormal DNA methylation occurs more frequently in older women compared with older men. It may be due to a decline in estrogen availability with age. Several studies have reported a decrease in the proportion of sporadic MSI-H tumors in women using postmenopausal hormones (30). More studies are necessary to elucidate the underlying biology of this association.

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Table 3. Standardized incidence ratios (SIR) of different cancers for relatives of CRC cases with *MLH1* DNA methylation compared with the general population

	Combined					First-degree relatives					Second-degree relatives				
	Median age[a] (range)	O[b]	E[c]	SIR (95% CI)	*P*	Median age (range)	O	E	SIR (95% CI)	*P*	Median age (range)	O	E	SIR (95% CI)	*P*
Both sexes															
CRC	70 (32–96)	47	35.77	1.30 (1.00–1.69)	0.05	66 (38–95)	25	15.34	1.60 (1.22–2.16)	0.001	71 (32–96)	22	20.43	1.08 (0.74–1.60)	0.67
Gastric cancer	71 (32–95)	22	5.93	3.66 (2.20–6.56)	<0.001	67 (45–92)	7	2.61	2.58 (1.52–4.71)	0.001	71 (32–95)	15	3.32	4.52 (2.23–10.61)	<0.001
Liver cancer	73 (46–87)	4	1.82	2.33 (1.21–5.00)	0.02	74 (46–87)	2	0.82	2.74 (1.35–6.45)	0.01	70 (55–75)	2	1.00	2.00 (0.57–10.72)	0.35
Pancreas cancer	71 (44–89)	6	6.66	0.94 (0.45–2.34)	0.88	73 (44–74)	2	2.93	0.77 (0.27–3.07)	0.67	67 (55–89)	4	3.73	1.07 (0.49–2.82)	0.88
Kidney cancer	61 (50–80)	3	6.04	0.41 (0.16–1.45)	0.11	61 (53–80)	2	2.81	0.71 (0.21–3.82)	0.64	61 (53–80)	1	3.23	0.15 (0.03–1.53)	0.07
Brain cancer	62 (40–84)	5	5.08	0.98 (0.54–1.97)	0.90	63 (54–73)	2	2.45	0.92 (0.31–3.78)	0.90	60 (40–84)	3	2.63	1.05 (0.54–2.22)	0.89
Female															
Endometrial cancer	65 (30–90)	7	7.95	0.85 (0.54–1.40)	0.50	59 (30–75)	4	3.52	1.21 (0.70–2.24)	0.52	65 (63–90)	3	4.43	0.56 (0.26–1.46)	0.19
Ovarian cancer	59 (21–86)	8	5.56	1.43 (0.87–2.52)	0.19	60 (29–86)	6	2.55	2.16 (1.29–3.86)	0.006	59 (21–61)	3	3.01	0.83 (0.30–3.08)	0.75
Breast cancer	62 (16–91)	39	40.83	0.96 (0.70–1.34)	0.81	57 (16–91)	16	18.94	0.83 (0.56–1.28)	0.38	62 (37–86)	23	21.89	1.06 (0.69–1.69)	0.80

[a]Median age in years.
[b]O = observed number of cancers.
[c]E = Expected number of cancers.

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Table 4. Standardized incidence ratios (SIR) of CRC for relatives stratified by characteristics of CRC cases with *MLH1* DNA methylation

	Combined			First-degree relatives			Second-degree relatives		
Characteristics of CRC in probands	**O[a]**	**E[b]**	**SIR (95% CI)**	**O**	**E**	**SIR (95% CI)**	**O**	**E**	**SIR (95% CI)**
Age of onset of proband, y									
<50	4	2.04	1.84 (0.73–4.99)	2	0.55	3.67 (0.81–20.46)	2	1.49	1.17 (0.41–4.14)
≥50	43	33.73	1.27 (0.97–1.67)	23	14.79	1.52 (1.16–2.03)	20	18.94	1.07 (0.71–1.63)
Location of proband's tumor(s)									
Right colon[c]	42	32.31	1.28 (0.98–1.70)	21	13.56	1.55 (1.16–2.10)	21	18.75	1.09 (0.74–1.65)
Left colon[d]	3	1.64	1.52 (0.58–4.90)	1	1.05	0.96 (0.33–3.40)	2	0.59	2.51 (0.55–12.60)
Rectosigmoid and rectum	2	1.59	0.94 (0.10–12.16)	2	0.55	2.72 (0.66–19.84)	0	1.04	—
Both sides of colon[e]	1	0.22	3.45 (1.08–15.98)	1	0.18	4.25 (1.26–20.86)	0	0.04	—

[a]O = observed number of CRC.
[b]E = Expected number of CRC.
[c]Right colon = the cecum through the transverse colon.
[d]Left colon = the splenic flexure through the sigmoid colon.
[e]Synchronous tumors.

Gastric cancer was the most frequent extracolonic cancer diagnosed in relatives of the *MLH1*-methylated cases. The risk of gastric cancer is elevated 3- to 14-fold in Lynch syndrome families (31). In populations with a low gastric cancer risk, the increase is similar to the 2.6-fold (1.7–5.6) increase we observed here. Some studies observed no significant increase in gastric cancer risk in the relatives of patients with sporadic CRC (32–34), whereas one study observed a significant increase of 20% (35), significantly less ($P = 0.01$) than what we observed here.

As with CRC, the MSI-H phenotype is observed in 15% to 33% of gastric cancers, is due to *MLH1* promoter methylation, and is associated with the concurrent methylation of multiple genes in almost all cases (36). This suggests that a common mechanism may underlie the hypermethylation of *MLH1* and other genes in the gastrointestinal tract. Our findings suggest a heritable component associated with an increased risk of gastric cancer for relatives of CRC cases with a methylated *MLH1*. Whether the cancers in relatives of the MLH1-methylated cases are also characterized by *MLH1* methylation or widespread abnormal DNA methylation needs to be assessed in future studies.

Our data are also consistent with a higher risk for ovarian cancers in the first-degree relatives of CRC cases with a methylated *MLH1*. Similar to the findings for gastric cancer, the increased risk for ovarian cancer is in the range of that observed for Lynch syndrome families (31). An association between ovarian and CRCs, with excess risks similar to ours, has been observed in some (37, 38), but not all (39), studies of sporadic CRCs. As in sporadic CRCs, the MSI-H phenotype is present in about 12% of ovarian cancers (40) but the existence of a CIMP is as yet undetermined. Future studies are needed to determine the reliability of this finding.

Primary liver cancer is not associated with Lynch syndrome (31). In this study, the estimates for primary liver cancer are based on very small numbers (2 liver cancers each

Table 5. Cumulative risks (%) and their 95% CIs to age 70 years of different cancers for first- and second-degree relatives of CRC cases with *MLH1* DNA methylation

	Combined	**First-degree relatives**	**Second-degree relatives**
CRC			
Male	4.12 (3.18–5.32)	5.04 (3.87–6.75)	3.43 (2.37–5.04)
Female	2.84 (2.19–3.67)	3.48 (2.67–4.67)	2.36 (1.63–3.48)
Gastric cancer			
Male	2.04 (1.23–3.63)	1.44 (0.85–2.62)	2.51 (1.25–5.80)
Female	0.78 (0.47–1.40)	0.55 (0.33–1.01)	0.97 (0.48–2.26)
Liver cancer			
Male	0.57 (0.30–1.22)	0.67 (0.33–1.57)	0.49 (0.14–2.59)
Female	0.21 (0.11–0.44)	0.24 (0.12–0.57)	0.18 (0.05–0.94)
Ovarian cancer	2.43 (1.49–4.25)	3.65 (2.20–6.44)	1.42 (0.52–5.17)

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in first- and second-degree relatives) and therefore may be due to chance. Although we did not count liver cancers diagnosed within 1 year of another cancer, it remains possible that one or more of the observed liver cancers represents a metastatic tumor from another primary site. Given the relative rarity of primary liver cancer in Western countries, significantly larger studies are needed to see whether the present results are replicated.

The strengths of this study include the relatively large number of *MLH1*-methylated tumors, the large number of relatives of those cases and the retrospective cohort design for estimating cancer risks in relatives. We included all *MLH1*-methylated cases regardless of whether the family met Amsterdam (I or II) criteria. Because some non–Lynch syndrome families meet these criteria too (41, 42) deleting all such families will bias risk estimates downward. The direct sequencing of all *MLH1*-methylated tumors for germ line mutations in *MLH1* and other *MMR* genes allowed us to eliminate Lynch syndrome cases, including those for whom methylation was the "second hit." This study also has some weaknesses. Confirmatory data for family cancers were not available for about 70% of families and in those cases cancer in relatives was determined by the proband's self-report. The specificity of self-reported cancer diagnoses is generally more than 98% suggesting that there are few false positives in such data (43). Finally, we were unable to assess the *MLH1* methylation status in relatives which would have provided additional and more direct support for a familial cancer predisposition involving methylation of the *MLH1* gene promoter and, perhaps, widespread abnormal DNA hypermethylation as well.

In conclusion, we found a 60% increase in the risk of CRC and greater than 2-fold increased risks of gastric, ovarian, and liver cancers in first-degree relatives of cases with a methylated *MLH1* gene and no *MMR* or *MUTYH* gene mutations. The increased risk we observed for CRC was about the same as that predicted for CRC in general. The increased risks for gastric and ovarian cancers were in the range of those seen for Lynch syndrome families. These data suggest that there may be an underlying heritable factor promoting a phenotype involving *MLH1* methylation, and possibly CIMP, in non–Lynch syndrome families. Family members of cases with *MLH1*-methylated CRCs may benefit from increased cancer screening and targeted prevention, especially for gastric and ovarian cancers.

Disclosure of Potential Conflicts of Interest

P.W. Laird is a consultant and scientific advisory board member for Epigenomics, AG, which has a commercial interest in the clinical use of DNA methylation markers. Epigenomics did not contribute to, nor benefit from the research described in this article. The content of the manuscript does not necessarily reflect the views or policies of the National Cancer Institute or any of the collaborating centers in the CFRs, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government or the CFR. No potential conflicts of interests were disclosed by other authors.

Authors' Contributions

A.J. Levine and R.W. Haile conceived the study, participated in its design, and drafted the manuscript. A.K. Win conducted the statistical analysis and participated in drafting the manuscript. D.D. Buchanan, M.A. Jenkins, J.A. Baron, and J.P. Young participated in drafting the manuscript. D.J. Weisenberger, T.I. Long, and P.W. Laird conducted the laboratory analysis for *MLH1* methylation and participated in drafting the manuscript. All authors read and approved the final manuscript.

Acknowledgments

The authors thank the CFR study coordinators and data managers who helped prepare the data set for these analyses (Maggie Angelakos, Terrilea Burnett, Helen Chen, Darshana Daftary Pat Harmon, Heide Miller-Pakvasa, Douglas Snazel, Terry Teitsch, and Allyson Templeton) as well as the participants in the Colon CFR who have generously donated their time for this project. They also thank the principal investigators of all C-CFR sites whose data was used in this project.

Grant Support

This work was supported by the National Cancer Institute, NIH under RFA #CA-95-011 and through cooperative agreements with the Australasian Colorectal Cancer Family Registry (U01 CA097735), the USC Familial Colorectal Neoplasia Collaborative Group (U01 CA074799), the Ontario Registry for Studies of Familial Colorectal Cancer (U01 CA074783), the Seattle Colorectal Cancer Family Registry (U01 CA074794), and the University of Hawaii Colorectal Cancer Family Registry (U01 CA074806) as well as NCI R01 CA112237 (R.W. Haile). A.K. Win was supported by a grant from the Cancer Council Victoria and the Picchi Brothers Foundation, Australia.

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Received August 29, 2011; revised November 8, 2011; accepted November 28, 2011; published OnlineFirst December 5, 2011.

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