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CpG island methylator phenotype, microsatellite instability, *BRAF* mutation and clinical outcome in colon cancer

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Abstract

Background—The CpG island methylator phenotype (CIMP) characterized by widespread promoter methylation is associated with microsatellite instability (MSI) and *BRAF* mutation in colorectal cancer. The independent effect of CIMP, MSI and *BRAF* mutation on patient outcome remains uncertain.

Methods—Utilizing 649 colon cancers (stage I–IV) in two independent cohort studies, we quantified DNA methylation in 8 CIMP-specific promoters [*CACNA1G*, *CDKN2A* (p16), *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3*, and *SOCS1*], as well as MINT1, MINT31, p14, *HIC1*, *IGFBP3*, *MGMT* and *WRN* by MethyLight. We examined MSI, *KRAS* and *BRAF* status. Cox proportional hazard models computed hazard ratios (HRs) for colon cancer-specific and overall mortalities, adjusting for patient characteristics and tumoral molecular features.

Results—After adjustment for other predictors of patient survival, patients with CIMP-high cancers [126 (19%) tumors with ≥6/8 methylated CIMP-specific promoters] experienced a significantly low colon cancer-specific mortality [multivariate HR 0.44, 95% confidence interval (CI) 0.22–0.88], whereas *BRAF* mutation was significantly associated with a high cancer-specific mortality (multivariate HR 1.97, 95% CI, 1.13–3.42). A trend toward a low cancer-specific mortality was observed for MSI-high tumors (multivariate HR 0.70, 95% CI, 0.36–1.37). In stratified analyses, CIMP-high tumors were associated with a significant reduction in colon cancer-specific mortality, regardless of both MSI and *BRAF* status. The relation between CIMP-high and lower mortality appeared to be consistent across all stages. *KRAS* mutation was unrelated to patient outcome.

Conclusion—CIMP-high appears to be an independent predictor of a low colon cancer-specific mortality, while *BRAF* mutation is associated with a high colon cancer-specific mortality.

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Keywords

colorectal cancer; CIMP; methylation; MSI; prognosis

INTRODUCTION

Epigenetic aberrations are thought to be an important mechanism in human carcinogenesis.[1,2] A number of tumor suppressor genes are silenced by promoter CpG island methylation in colon cancers.[2,3] A subset of colon cancers exhibit widespread promoter methylation, referred to as the CpG island methylator phenotype (CIMP),[2,4–6] which is associated with microsatellite instability (MSI).[7,8] CIMP-high colon cancers have been associated with older age, cigarette smoking, proximal tumor location, female gender, poor differentiation, *BRAF* mutation, wild-type *TP53*, inactive β-catenin/*WNT* and stable chromosomes,[8–19] and many of these associations are independent of MSI status.[9,16–19]

Among patients with colon cancer, MSI has generally been associated with good prognosis in most,[20] though not all studies.[21] On the other hand, the presence of *BRAF* mutations in tumors has been characteristically associated with an inferior patient survival.[22] In contrast, results for CIMP have been conflicting.[22–28] These inconsistent results likely reflect differences in patient cohorts, methylation markers examined, and the variable inclusion of data on other potentially confounding molecular events, such as MSI and *BRAF* in multivariate analysis models.

We therefore examined both genetic and epigenetic alterations among colon cancer patients participating in two large prospective cohort studies, to assess the independent effect of CIMP, MSI and *BRAF* mutation on patient outcome. Furthermore, to assess CpG island methylation, we utilized quantitative DNA methylation assays (MethyLight technology) on a validated expanded panel of 8 markers that appears to well characterize the presence or absence of CIMP-high in colorectal cancers.[8,29]

METHODS

Study population

We utilized the databases of two independent prospective cohort studies; the Nurses' Health Study (N=121,700 women followed since 1976),[30,31] and the Health Professional Follow-up Study (N=51,500 men followed since 1986).[31] On each biennial follow-up questionnaire, participants were asked whether they had a diagnosis of colon cancer during the previous 2 years. When a participant (or next of kin for decedents) reported colon cancer, we sought permission to obtain medical records. Study physicians, while blinded to exposure data, reviewed all records related to colon cancer, and recorded the date of cancer diagnosis, AJCC (American Joint Committee on Cancer) stage and tumor location. For nonresponders, we searched the National Death Index to discover deaths and ascertain any diagnosis of colon cancer that was a primary cause of death or a secondary diagnosis. Approximately 96% of all incident colon cancer cases were identified through these methods. We collected paraffin-embedded tissue blocks from hospitals where patients underwent resections of primary colon cancers.[31] Tissue sections from all cases in this study were reviewed by a pathologist (S.O.). Tumor grade was categorized as high (≤50% glandular area) or low (>50% glandular area). We excluded rectal cancers and cases that were preoperatively treated with radiation and/or chemotherapy. Based on availability of tissue samples, we included a total of 649 colon cancer cases (283 from the men's cohort and 366 from the women's cohort) diagnosed up to 2002. Written informed consent was obtained from all study subjects. This study was approved by

the Human Subjects Committees at Brigham and Women's Hospital and Harvard School of Public Health.

Measurement of mortality

Patients were observed until death or June 2006, whichever came first. Ascertainment of deaths included reporting by the family or postal authorities. The names of persistent nonresponders were searched in the National Death Index. The cause of death was assigned by physicians blinded to information on lifestyle exposures and molecular changes in colon cancer. In rare patients who died as a result of colon cancer not previously reported, we obtained medical records with permission from next of kin. More than 98% of deaths in the cohorts were identified by these methods.

Genomic DNA extraction and sequencing of *KRAS* and *BRAF*

Genomic DNA from paraffin-embedded tissue was extracted, and whole genome amplification was performed by PCR using random 15-mer primers.[32] PCR and sequencing targeted for *KRAS* codons 12 and 13, and *BRAF* codon 600 were performed as previously described.[32, 33]

Real-time PCR (MethyLight) for quantitative DNA methylation analysis

Bisulfite treatment on genomic DNA and subsequent real-time PCR (MethyLight)[34] were validated and performed as previously described.[35] We quantified DNA methylation in 8 CIMP-specific promoters (*CACNA1G*, *CDKN2A* (*p16*), *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3* and *SOCS1*)[8,29] (which were selected from screening of 195 CpG islands in the human genome[8,17]), as well as *HIC1*, MINT1, MINT31,[36] *MGMT*,[35] *IGFBP3* and *WRN*.[25] The PCR condition was initial denaturation at 95°C for 10 min followed by 45 cycles of 95°C for 15 sec and 60°C for 1 min.

CIMP-high was defined as ≥6/8 methylated markers using the 8-marker CIMP panel, CIMP-low/0 as ≤5/8 methylated markers, and CIMP-0 as 0/8 methylated markers, according to the previously established criteria.[29]

Microsatellite Instability (MSI) Analysis

MSI status was determined using D2S123, D5S346, D17S250, BAT25, BAT26,[37] BAT40, D18S55, D18S56, D18S67 and D18S487 (i.e., 10-marker panel).[29] A "high degree of MSI" (MSI-high) was defined as the presence of instability in ≥30% of the markers, and "microsatellite stability" (MSS) as no unstable marker or instability in <30% of the markers. When tumors with instability in <30% of the markers (i.e., "MSI-low") was compared to tumors with no unstable marker, "MSI-low" did show no prognostic value (data not shown). Thus, we combined "MSI-low" tumors into MSS tumors in further analyses.

Statistical analysis

Cox proportional hazard models were used to calculate hazard ratios (HRs) of death according to molecular features in tumor (i.e., MSI, CIMP and *BRAF* mutation), adjusted for age, sex, year of diagnosis, tumor stage, tumor location, tumor grade, and the molecular variables. In the analyses for colon cancer-specific mortality, death as a result of colon cancer was the primary end point and deaths as a result of other causes were censored. Age and year of diagnosis were used as continuous variables, and all of the other variables were used as categorical variables. When information on tumor location (1.4% missing), tumor stage (7.4% missing), *KRAS* (0.3% missing) or *BRAF* (2.8% missing) was missing, we assigned a separate ("missing") indicator variable and included those cases in the multivariate analysis model. We confirmed that excluding cases with a missing variable did not significantly alter results (data

not shown). An interaction was assessed by including the cross product of two variables of interest in the analysis model. The Kaplan-Meier method was used to describe the distribution of colon cancer-specific and overall survival time, and the log-rank test was performed to test the null hypothesis of no difference in survival time distributions among all subtypes. The chi square test was used to examine an association between categorical variables. The t-test assuming unequal variances was used to compare mean ages. All analyses used SAS version 9.1 (SAS Institute, Cary, NC) and all p values were two-sided.

RESULTS

CpG island methylator phenotype (CIMP) and microsatellite instability (MSI)

Among 649 colon cancer patients with available tissue specimens, there were 281 deaths, including 163 colon cancer-specific deaths. Among all patients, 121 (19%) demonstrated MSI-high; 126 (19%) were CIMP-high (≥6/8 methylated CIMP-specific markers;[29] i.e., *CACNA1G*, *CDKN2A*, *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3* and *SOCS1*); 238 (37%) demonstrated a mutation in *KRAS*; and 105 (17%) demonstrated a mutation in *BRAF*. Tumors were distributed bimodally according to the number of methylated CIMP markers (Figure 1), and *BRAF* mutations were common in CIMP-high tumors while *KRAS* mutations were common in CIMP-low tumors (1/8–5/8 methylated markers). We assessed baseline patient characteristics according to MSI and CIMP status (Table 1).

MSI-high tumors were more likely to originate in the proximal colon and possess *BRAF* mutations and CIMP-high status, and less likely to present with stage III or IV disease. CIMP-high tumors were also more likely to originate in the proximal colon and possess *BRAF* mutations.

When we jointly classified tumors by MSI and CIMP status, MSS (microsatellite stable) CIMP-high tumors had a greater prevalence of stage IV disease (36%; 13/36; p=0.0004, compared to all other subtypes) when compared to MSI-high CIMP-high tumors (4%; 3/84), MSI-high CIMP-low/0 tumors (6%; 2/32) or MSS CIMP-low/0 tumors (14%; 65/452). In addition, *BRAF* mutations were found in 62% (53/86) of MSI-high CIMP-high tumors, 57% (21/37) of MSS CIMP-high tumors, none (0/32) of MSI-high CIMP-low/0 tumors, and 6% (31/476) of MSS CIMP-low/0 tumors.

Molecular features in colon cancer and patient survival

We assessed the influence of MSI, CIMP, and *BRAF* mutation on patient survival, independent of the clinical and other tumoral variables (Table 2).

Compared to patients with MSS tumors, those with MSI-high tumors experienced a significant reduction in colon cancer specific mortality in a univariate analysis [hazard ratio (HR) 0.38, 95% confidence interval (CI), 0.22–0.66]; however, in the multivariate model that adjusted for CIMP, *KRAS*, *BRAF* and patient characteristics, the effect of MSI-high was attenuated. This attenuation in the effect of MSI-high was principally the result of adjusting for tumor stage; when we simply adjusted for tumor stage, the HR for colon cancer-specific mortality for MSI-high tumors was 0.73 (95% CI, 0.42–1.28).

In addition, compared to CIMP-0, CIMP-high tumors were associated with a non-significant reduction in colon cancer specific mortality in a univariate analysis (HR 0.88, 95% CI, 0.57–1.38), which became statistically significant after adjusting for other molecular and patient characteristics (multivariate HR 0.44, 95% CI, 0.22–0.88). The greater beneficial effect of CIMP-high status in the multivariate model was principally the result of adjusting for *BRAF* mutational status; when we simply adjusted for *BRAF* status, the HR for colon cancer-specific mortality for CIMP-high tumors was 0.45 (95% CI, 0.26–0.79).

In both univariate and multivariate analyses, *BRAF* mutation was associated with a significant increase in colon cancer-specific mortality (multivariate HR 1.97, 95% CI, 1.13–3.42). In contrast, *KRAS* mutation was not associated with patient outcome. Of note, the aforementioned molecular events did not significantly influence all-cause mortality.

Although statistical power was diminished for individual patient subsets, the influence of CIMP status on colon cancer-specific mortality appeared similar among patients with either early (I and II) or advanced (III and IV) pathologic stages of disease (p for interaction =0.93). Compared to CIMP-low/0 tumors, the multivariate HR for colon cancer-specific mortality in CIMP-high tumors was consistently low across all stages (I to IV) (Table 3).

In contrast, any apparent effect of CIMP-high on overall mortality was limited to patients with stage III/IV disease; the multivariate HR for all-cause mortality was 1.49 (95% CI, 0.66–3.34) for stage I/II patients and 0.58 (95% CI, 0.29–1.15) for stage III/IV patients.

Next, we examined whether the effect of CIMP or *BRAF* mutation on survival differed between the cohort studies. The effect of CIMP-high did not significantly differ between the male cohort (Health Professionals Follow-up Study) and the female cohort (Nurses' Health Study; p for interaction =0.59). Likewise, the effect of *BRAF* mutation did not significantly differ between the male cohort and the female cohort (p for interaction =0.35).

To eliminate potential confounding effect of HNPCC (hereditary nonpolyposis colorectal cancer), we identified 19 possible or suspected HNPCC cases [i.e., MSI-high CIMP-low/0 tumors (none of which turned out to be *BRAF*-mutated) with any of the followings: (1) positive family history of colorectal cancer in at least one first-degree relative; (2) loss of MLH1 without evidence of *MLH1* methylation; (3) loss of PMS2 without evidence of MLH1 loss; (4) loss of MSH2 and/or MSH6]. After we excluded these 19 cases, multivariate Cox regression analysis showed following results for colon cancer-specific mortality: HR for MSI-high, 0.68 (95% CI, 0.34–1.35); HR for CIMP-high, 0.41 (95% CI, 0.20–0.83); HR for *BRAF* mutation, 1.85 (95% CI, 1.12–3.06). These results were similar to Table 2.

We compared different CIMP panels consisting of different sets of markers. Multivariate HRs for colon cancer-specific mortality in CIMP+ vs. CIMP− were as follows: HR 0.57 (95% CI, 0.32–1.01) by a panel consisting of *CACNA1G, IGF2, NEUROG1, RUNX3* and *SOCS1*;[8] HR 0.81 (95% CI, 0.55–1.20) by a panel consisting of *CDKN2A* (p16), MINT1, MINT31, *MLH1* and p14;[26] HR 0.74 (95% CI, 0.46–1.20) by a panel consisting of *CDKN2A, HIC1,* MINT1, MINT31 and *MLH1;* HR 0.54 (95% CI, 0.30–0.99) by a panel consisting of *CACNA1G, CDKN2A, CRABP1, MLH1* and *NEUROG1*;[17] HR 0.65 (95% CI, 0.37–1.12) by a panel consisting of *CACNA1G, CDKN2A, CRABP1, IGF2, MLH1, NEUROG1, RUNX3, SOCS1, IGFBP3,* MINT1, MINT31, *MGMT* and *WRN*.[25] These results indicate that a variation in methylation markers in CIMP panels can result in a variation in associations with patient outcome, which may, at least in part, explain the discrepancy of different studies on CIMP and patient outcome. In the current study, we utilized the validated 8-marker panel in light of our prior published work.[29]

Combined MSI/CIMP status and patient survival

We further stratified patients according to both MSI and CIMP status to assess the joint effect on patient outcome (Table 4), because molecular classification based on MSI and CIMP status is increasingly important.[38,39]

Compared to patients whose tumors were both MSS and CIMP-low/0, those with CIMP-high tumors experienced a significant reduction in colon cancer-specific mortality, regardless of MSI status. A combination of MSI and CIMP determinations might differentiate ~24% [(38

+33+88)/649] of tumors (either CIMP-high or MSI-high) with good prognosis (HR estimates 0.17–0.40) from the other ~76% of tumors (MSS CIMP-low/0).

Combined MSI/*BRAF* status and patient survival

Similarly, we stratified patients according to both MSI and *BRAF* status to assess joint effect on patient outcome. Compared to patients whose tumors were both MSS and *BRAF*-mutated, those with MSI-high/*BRAF*-wild-type tumors showed a significant reduction in colon cancer-specific mortality (Table 4). Notably, there was no protective effect of MSI-high among *BRAF*-mutated tumors; compared to MSS *BRAF*-mutated tumors, the multivariate HR for colon cancer-specific mortality among MSI-high *BRAF*-mutated tumors was 1.09 (95% CI, 0.48–2.51).

Combined CIMP/*BRAF* status and patient survival

We also stratified patients according to both CIMP and *BRAF* status to assess the joint effect on patient outcome. Colon cancer-specific survival at 5 years was 45% for patients with CIMP-low/0 *BRAF*-mutated tumors, 80% for CIMP-low/0 *BRAF*-wild-type tumors, 74% for CIMP-high *BRAF*-mutated tumors, and 86% for CIMP-high *BRAF*-wild-type tumors (multi-group log rank $p<0.0001$; Figure 2A). Similarly, overall survival at 5 years was lower in CIMP-low/0 *BRAF*-mutated tumors than the other subtypes (multi-group log rank $p=0.0015$; Figure 2B).

In a multivariate analysis, when compared to patients with CIMP-low/0 *BRAF*-mutated tumors, those with CIMP-high tumors demonstrated a significantly lower colon cancer-specific mortality regardless of *BRAF* status (Table 4). Moreover, the adverse effect of *BRAF* mutation on patient survival was not apparent when tumors also demonstrated CIMP-high.

DISCUSSION

In this cohort of patients with colon cancer, we examined the effect of the CpG island methylator phenotype (CIMP), microsatellite instability (MSI), and *BRAF* mutation on patient outcome. CIMP-high status was independently associated with a low cancer-specific mortality, whereas *BRAF* mutation was associated with a significant increase in cancer-specific mortality. Consistent with other studies,[20] we found that MSI-high tumors showed a trend towards an association with longer survival. Of note, the adverse effect of *BRAF* mutation appeared to be limited to tumors that were not CIMP-high. Although our observations need to be confirmed by other independent studies, the associations of CIMP and *BRAF* mutation with clinical outcome were consistent across the two independent prospective cohort studies in this analysis.

The relationship between CIMP, MSI, and *BRAF* mutations in colon cancer is complex. In our cohort, 70% of *BRAF*-mutated tumors exhibited CIMP-high, and 70% of CIMP-high tumors exhibited MSI-high. Among patients who do not manifest hereditary nonpolyposis colorectal cancer (HNPCC), MSI-high is often the consequence of promoter methylation (and subsequent silencing) of *MLH1*, a DNA mismatch repair gene.[8] In fact, CIMP and *BRAF* tests are used to exclude HNPCC among patients who exhibit MSI-high, since HNPCC seldom exhibits CIMP or *BRAF* mutation.[8,40,41]

Studying epigenetic and/or genetic alterations is increasingly important in cancer research.[3, 42–44] To decipher the apparently complex effect of CIMP and *BRAF* mutation on patient survival, we utilized a validated expanded panel of 8 methylation markers for CIMP diagnosis in colorectal cancer.[8,29] To determine DNA methylation status at each locus, we used a quantitative method that appears to reproducibly differentiate high-level from low-level methylation.[35] Our validated criteria for CIMP-high are based on the bimodal distribution of tumors according to the number of methylated CIMP markers, and the observation that

CIMP-high is associated with *BRAF* mutation while CIMP-low is associated with *KRAS* mutation.[33,45] Our large sample size from the two independent cohort studies enabled us to estimate the frequencies of specific molecular features (e.g., CIMP-high, etc.) and cancer death rates at the population level.

Although prognostic factors have been extensively investigated for colon cancer,[20–22,46–48] previous studies of CIMP and survival in colon cancer have yielded somewhat inconsistent results.[22–28] Some studies suggested an adverse effect of CIMP on survival of patients with MSS tumors.[23,25,26] However, accumulating evidence has been suggested that MSI-high tumors are associated with good prognosis regardless of CIMP status,[22,23] which is in agreement with our current study (Table 4). *BRAF* mutation has been associated with worse survival in MSS tumors, but there was little prognostic value of CIMP in multivariate analysis.[22,27] Our findings of good prognosis in CIMP-high tumors appear to differ from the data in the previous studies.[23,25,26] These discrepant observations might have resulted from differences in patient cohorts, methylation markers, criteria for CIMP, and/or the variable inclusion of other potential confounders (such as *BRAF* mutation) in multivariate analysis models. In particular, we have previously observed worse prognosis associated with CIMP-high tumors in stage IV colorectal cancer in small phase I/II clinical trials.[25] The possible reasons for the discrepant results are as follows: 1) A selection bias in the small clinical trials with only 5 CIMP-high tumors might have caused this discrepancy. 2) Data in our previous study[25] with only 5 CIMP-high tumors might simply be the result by chance in the setting of a small patient population. A p value by the log-rank test is calculated by the Mantel-Haenszel chi-square test that can offer a far more accurate p value with a large sample size. Thus, we would emphasize the importance of a large sample size in any clinical study. Because of the use of the expanded CIMP marker panel (including the 5 new markers described by Weisenberger et al.[8]) in the current study, good prognosis might be specifically associated with CIMP-high tumors defined by these new CIMP makers. Our observations of good prognosis in CIMP-high tumors appeared to be consistent across all stages (I to IV), further supporting that CIMP-high tumor is a biologically indolent subtype. In addition, we found that, after jointly examining CIMP and *BRAF* status, CIMP-high predicted a lower colon cancer-specific mortality (regardless of *BRAF* status) compared to CIMP-low/0 *BRAF*-mutated tumors, whereas the deleterious effect of *BRAF* mutation was not as evident in patients with CIMP-high tumors.

In our cohorts, data on cancer treatment are limited. Nonetheless, it is unlikely that chemotherapy use differed according to tumoral CIMP, MSI or *BRAF* status, especially since such data were not typically available to patients or treating physicians. It still remains a possibility that differential response to chemotherapy according to a specific molecular variable (e.g., MSI) might confound our findings. Further studies are necessary to examine whether response to chemotherapy may be differentially influenced by specific molecular features in colon cancer. In addition, beyond cause of mortality, data on cancer recurrences were not available in these cohorts. Nonetheless, given the median survival for metastatic colon cancer was approximately 10 to 12 months during much of the time period of this study,[49] colon cancer-specific mortality should be a reasonable surrogate for cancer-specific outcomes.

Despite the apparent effects of CIMP, MSI, and *BRAF* mutation on colon cancer-specific mortality, the influence of these tumoral events on overall mortality was markedly attenuated, which may have reflected the inclusion of earlier stage (I and II) patients in our analysis. In fact, when we limited our analysis to patients with either stage III or IV cancer, we observed similar effects of CIMP on both cancer-specific and all-cause mortality. Moreover, when we jointly classified patients according to both CIMP and *BRAF* status, we observed similar trends for cancer-specific and overall mortality among the entire patient cohort (Table 4 and **Figure 4**).

In conclusion, this large prospective study of colon cancer patients suggests that CIMP-high is independently associated with a low cancer-specific mortality. While *BRAF* mutation is associated with worse survival, CIMP-high appears to eliminate the adverse effect of *BRAF* mutation. Finally, while our data confirm the extensive body of evidence supporting a better prognosis for patients with MSI-high tumors, the good prognosis associated with MSI-high was abrogated in the presence of a *BRAF* mutation. Our finding that CIMP-high is an independent predictor of cancer survival may have significant clinical implications, although it needs to be confirmed by additional independent studies. Future studies to validate our observations should consider a joint examination of CIMP, MSI and *BRAF* mutation to decipher the role of these molecular features in biological and clinical behavior of colon cancer.

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Abbreviations

AJCC
: American Joint Committee on Cancer

CI
: confidence interval

CIMP
: CpG island methylator phenotype

HNPCC
: hereditary nonpolyposis colorectal cancer

HPFS
: Health Professionals Follow-up Study

HR
: hazard ratio

MSI
: microsatellite instability

MSS
: microsatellite stable

NHS
: Nurses' Health Study

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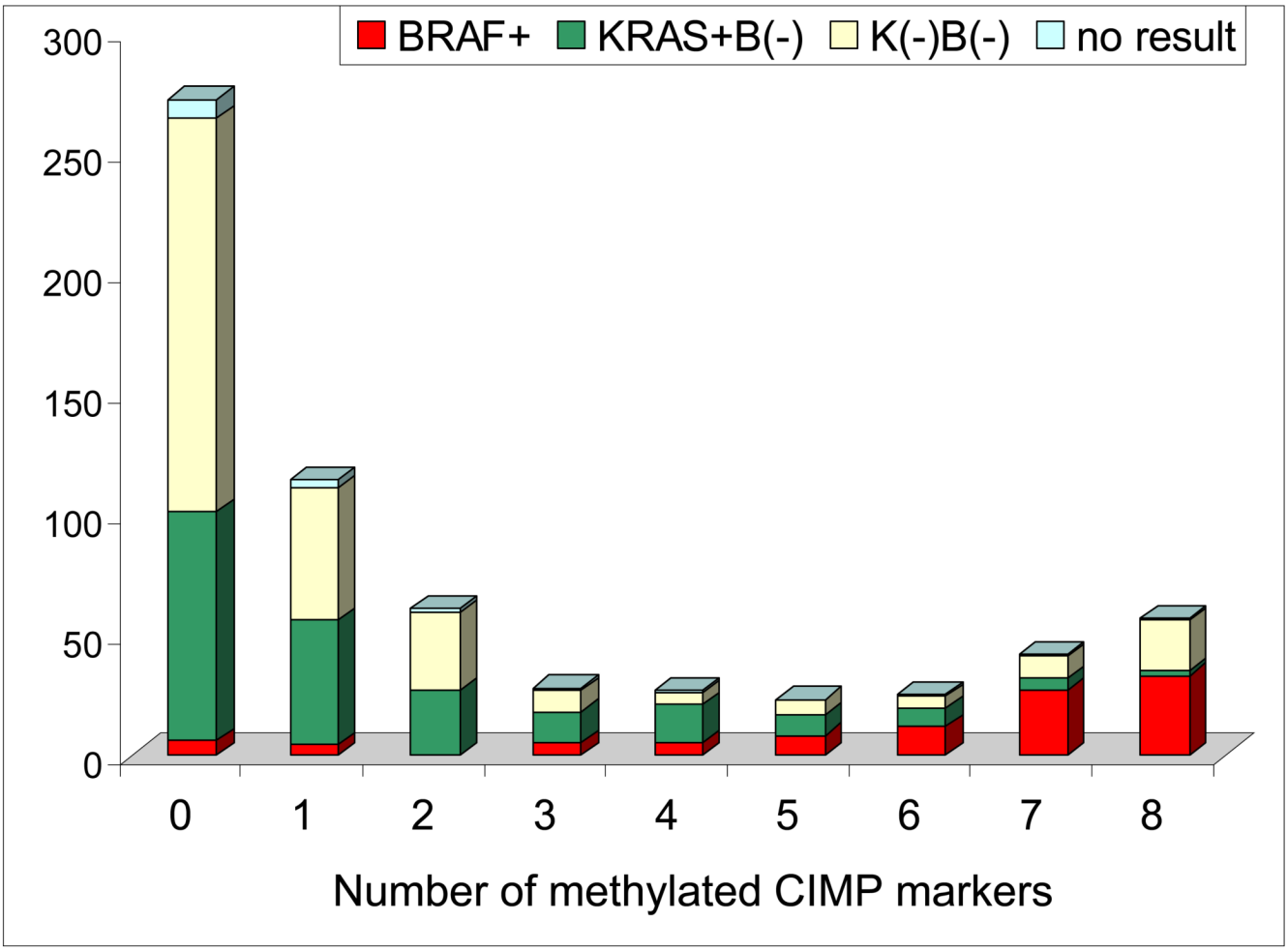
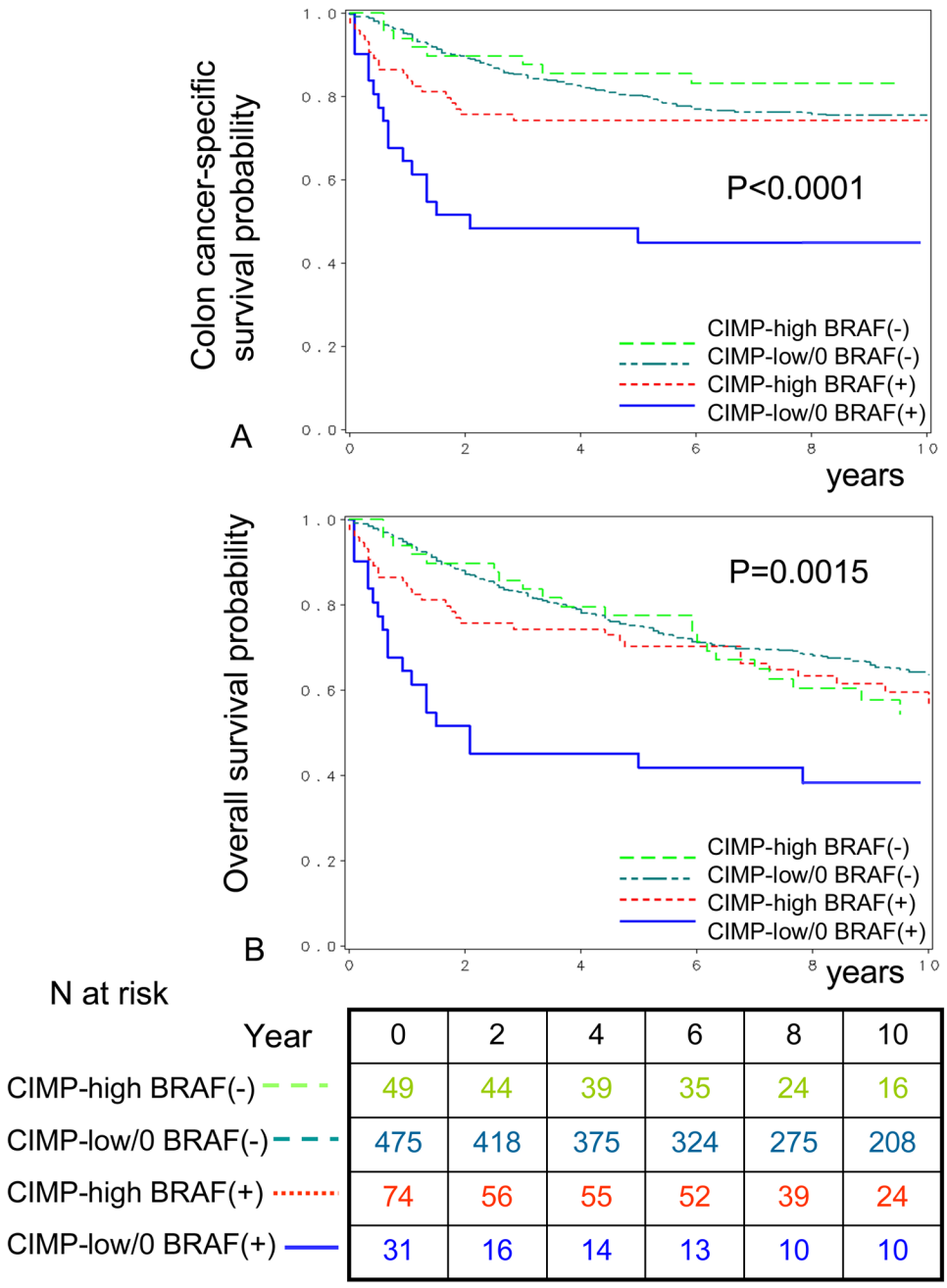


Figure 1. Distribution of colon cancers and *BRAF* and *KRAS* mutations according to the number of methylated CIMP markers

A bimodal distribution of tumors is evident, and *BRAF* mutation is common in heavily methylated tumors, while *KRAS* mutation is common in tumors with fewer methylated markers. B(-), *BRAF* wild-type; CIMP, CpG island methylator phenotype; K(-), *KRAS* wild-type.



N at risk

Year	0	2	4	6	8	10
CIMP-high BRAF(-)	49	44	39	35	24	16
CIMP-low/0 BRAF(-)	475	418	375	324	275	208
CIMP-high BRAF(+)	74	56	55	52	39	24
CIMP-low/0 BRAF(+)	31	16	14	13	10	10

Figure 2. Kaplan-Meier survival curves in colon cancer according to combined CIMP/*BRAF* status

A. Colon cancer-specific survival. B. Overall survival.

CIMP-low/0 *BRAF*(+) tumors demonstrate shorter survival than the other 3 CIMP/*BRAF* types. P values indicate statistical significance of a deviation of any one of the curves from the null hypothesis.

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Table 1

Clinical and molecular characteristics of colon cancer according to MSI or CIMP status.

Clinical or molecular feature	All cases	MSI-high	MSS	CIMP-high(6–8 methylated promoters)	CIMP-low(1–5 methylated promoters)	CIMP-0(0 methylated promoters)
Total N	649	121	528	126	252	271
Sex						
Male (HPFS)	283 (44%)	43 (36%)	240 (45%)	40 (32%)	125 (50%)	118 (44%)
Female (NHS)	366 (56%)	78 (64%)	288 (55%)	86 (68%)	127 (50%)	153 (56%)
		P=0.05		P=0.004		
Mean age ±SD	66.5 ±8.2	68.1 ±7.3	66.2 ±8.4	69.5 ±6.8	66.4 ±8.2	65.3 ±8.5
		P=0.01#		P<0.0001# *		
Year of diagnosis						
Prior to 1990	101 (16%)	13 (11%)	88 (17%)	9 (7.1%)	43 (17%)	49 (18%)
1990 to 1999	473 (73%)	95 (79%)	378 (71%)	102 (81%)	184 (73%)	187 (69%)
2000 to 2002	75 (12%)	13 (11%)	62 (12%)	15 (12%)	25 (9.9%)	35 (13%)
		P=0.23		P=0.14		
Tumor location^						
Proximal	371 (58%)	102 (85%)	269 (52%)	114 (90%)	153 (62%)	104 (39%)
Distal	269 (42%)	18 (15%)	251 (48%)	12 (9.5%)	94 (38%)	163 (61%)
		P<0.0001		P<0.0001		
Tumor stage						
I	137 (21%)	19 (16%)	118 (22%)	14 (11%)	55 (22%)	68 (25%)
II	216 (33%)	70 (58%)	146 (28%)	66 (52%)	64 (25%)	86 (32%)
III	165 (25%)	22 (18%)	143 (27%)	24 (19%)	69 (27%)	72 (27%)
IV	83 (13%)	5 (4.1%)	78 (15%)	16 (13%)	40 (16%)	27 (10%)
Unknown	48 (7.4%)	5 (4.1%)	43 (8.1%)	6 (4.8%)	24 (9.5%)	18 (6.6%)
		P<0.0001		P<0.0001		
Tumor grade						
Low	574 (89%)	86 (71%)	488 (93%)	86 (68%)	232 (92%)	256 (95%)
High	72 (11%)	35 (29%)	37 (7.0%)	40 (32%)	19 (7.6%)	13 (4.8%)
		P<0.0001		P<0.0001		
KRAS mutation						
(−)	409 (63%)	102 (84%)	307 (58%)	109 (87%)	131 (52%)	169 (63%)

Clinical or molecular feature	All cases	MSI-high	MSS	CIMP-high(6–8 methylated promoters)	CIMP-low(1–5 methylated promoters)	CIMP-0(0 methylated promoters)
(+)	238 (37%)	19 (16%)	219 (42%)	17 (13%)	120 (48%)	101 (37%)
		P<0.0001		P<0.0001		
BRAF mutation						
(−)	526 (83%)	65 (55%)	461 (90%)	49 (40%)	221 (90%)	256 (97%)
(+)	105 (17%)	53 (45%)	52 (10%)	74 (60%)	24 (9.8%)	7 (2.7%)
		P<0.0001		P<0.0001		
CIMP						
CIMP-0	271 (42%)	12 (9.9%)	259 (49%)	-	-	-
CIMP-low	252 (39%)	21 (17%)	231 (44%)	-	-	-
CIMP-high	126 (19%)	88 (73%)	38 (7.2%)	-	-	-
		P<0.0001				

%indicates the proportion of tumors with a specific clinical or molecular feature in a given subtype.

^Proximal colon includes cecum to transverse colon, and distal colon includes splenic flexure to sigmoid colon.

#The t-test assuming unequal variances was used to compare mean ages.

*Compared to combined CIMP-low/CIMP-0.

CIMP, CpG island methylator phenotype; HPFS, Health Professionals Follow-up Study; MSI, microsatellite instability; MSS, microsatellite stable; NHS, Nurses' Health Study; SD, standard deviation.

Table 2

Survival analysis on colon cancer patients according to molecular features in tumor

Molecular feature		Total N	Colon cancer-specific mortality			Overall mortality		
			Deaths/person-years	Univariate HR (95% CI)	Multivariate HR (95% CI)	Deaths/person-years	Univariate HR (95% CI)	Multivariate HR (95% CI)
MSI	MSS	528	149/4653	1 (referent)	1 (referent)	238/4653	1 (referent)	1 (referent)
	MSI-high	121	14/1101	0.38 (0.22–0.66)	0.70 (0.36–1.37)	43/1101	0.75 (0.54–1.04)	0.89 (0.58–1.38)
CIMP	CIMP-0	271	70/2556	1 (referent)	1 (referent)	106/2556	1 (referent)	1 (referent)
	CIMP-low	252	66/2183	1.09 (0.78–1.53)	0.78 (0.54–1.11)	116/2183	1.27 (0.98–1.66)	1.01 (0.77–1.33)
	CIMP-high	126	27/1016	0.88 (0.57–1.38)	0.44 (0.22–0.88)	59/1016	1.35 (0.98–1.85)	0.78 (0.47–1.29)
KRAS	(−)	409	98/3604	1 (referent)	1 (referent)	170/3604	1 (referent)	1 (referent)
mutation	(+)	238	65/2137	1.10 (0.81–1.51)	1.06 (0.73–1.54)	111/2137	1.10 (0.86–1.40)	0.98 (0.74–1.28)
BRAF	(−)	526	127/4821	1 (referent)	1 (referent)	229/4821	1 (referent)	1 (referent)
mutation	(+)	105	36/775	1.73 (1.20–2.51)	1.97 (1.13–3.42)	52/775	1.46 (1.08–1.98)	1.20 (0.79–1.80)

The multivariate analysis model includes age, year of diagnosis, sex, tumor location, stage, tumor grade, and statuses of MSI, CIMP, KRAS and BRAF.

CI, confidence interval; CIMP, CpG island methylator phenotype; HR, hazard ratio; MSI, microsatellite instability; MSS, microsatellite stable.

Table 3

Stage-specific hazard ratio (HR) for colon cancer-specific mortality in CIMP-high tumors compared to CIMP-0 tumors.

AJCC tumor stage	Total N	Multivariate HR (with 95% CI) for colon cancer- specific mortality in CIMP-high tumors compared to CIMP-0 tumors
Stage I	82	0*
Stage II	152	0.76 (0.20–2.82)
Stage III	96	0.52 (0.17–1.59)
Stage IV	43	0.47 (0.18–1.21)
Stage missing	24	0*

The multivariate analysis model includes the CIMP variable (CIMP-high vs. CIMP-0) stratified by stage, age, year of diagnosis, sex, tumor location, tumor stage, grade, MSI, CIMP, *KRAS* and BRAF.

* 95% CI was not shown because there was no death in patients with stage I and stage-missing CIMP-high tumors.

AJCC, American Joint Commission on Cancer; CI, confidence interval; CIMP, CpG island methylator phenotype; HR, hazard ratio.

Table 4

Combined MSI/CIMP status, MSI/*BRAF* status or CIMP/*BRAF* status and patient survival in colon cancer

Molecular feature	N	Colon cancer-specific mortality		Overall mortality	
		Deaths/person-years	Multivariate HR (95% CI)	Deaths/person-years	Multivariate HR (95% CI)
MSS CIMP-low/0	490	134/4384	1 (referent)	218/4384	1 (referent)
MSS CIMP-high	38	15/269	0.40 (0.20–0.83)	20/269	0.62 (0.34–1.13)
MSI-high CIMP-low/0	33	2/355	0.17 (0.02–1.22)	4/355	0.22 (0.07–0.69)
MSI-high CIMP-high	88	12/746	0.30 (0.14–0.64)	39/746	0.77 (0.48–1.24)
MSS *BRAF*(+)	52	25/365	1 (referent)	28/365	1 (referent)
MSS *BRAF*(−)	461	121/4162	0.74 (0.41–1.36)	200/4162	1.12 (0.67–1.89)
MSI-high *BRAF*(+)	53	11/410	1.09 (0.48–2.51)	24/410	1.27 (0.68–2.36)
MSI-high *BRAF*(−)	65	3/659	0.18 (0.05–0.61)	18/659	0.65 (0.35–1.21)
CIMP-low/0 *BRAF*(+)	31	17/199	1 (referent)	20/199	1 (referent)
CIMP-low/0 *BRAF*(−)	477	116/4410	0.46 (0.25–0.86)	193/4410	0.56 (0.33–0.95)
CIMP-high *BRAF*(+)	74	19/577	0.35 (0.16–0.79)	32/577	0.49 (0.25–0.98)
CIMP-high *BRAF*(−)	49	8/411	0.30 (0.11–0.82)	25/411	0.63 (0.30–1.31)

The multivariate analysis model includes age, year of diagnosis, sex, tumor location, stage, tumor grade, *KRAS* status, as well as *BRAF* status in the top analysis (for MSI/CIMP types), CIMP status in the middle analysis (for MSI/*BRAF* types) or MSI status in the bottom analysis (for CIMP/*BRAF* types).

CI, confidence interval; CIMP, CpG island methylator phenotype; HR, hazard ratio; MSI, microsatellite instability; MSS, microsatellite stable.