Advances in Brief

CpG Island Methylator Phenotype Is an Independent Predictor of Survival Benefit from 5-Fluorouracil in Stage III Colorectal Cancer[1]

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Abstract

Purpose: **The CpG island methylator phenotype (CIMP) is observed in approximately 30% of colorectal cancer (CRC) cases and is characterized by the concurrent methylation of multiple CpG islands in tumor DNA. This phenotype (CIMP+) is more frequently observed in tumors with proximal location, microsatellite instability, and normal *p53*. Because it has previously been observed that each of these features is associated with a good survival benefit from 5-fluorouracil (5-FU)-based adjuvant chemotherapy, we investigated in the present study whether CIMP+ has independent predictive value.**

Experimental Design: **CIMP+ status was evaluated in 103 stage III CRCs from patients treated with surgery alone and for an additional 103 cases from patients treated with surgery and adjuvant 5-FU-based chemotherapy. The two cohorts were randomly pair-matched for age, sex, and tumor site, and the median length of follow-up time was 39 months.**

Results: **CIMP+ status predicted survival benefit from 5-FU treatment independently of microsatellite instability and *p53* mutation status (relative risk = 0.22; 95% confidence interval, 0.06–0.84; $P = 0.027$). Unmeasured, high-risk confounding factors could only account for this association if they were unequally distributed between the two patient cohorts by a factor of at least 2-fold.**

Conclusions: **CIMP+ has independent predictive significance for the survival benefit from 5-FU chemotherapy in CRC. This molecular marker should be incorporated into prospective clinical trials of fluorouracil-based therapies to confirm its clinical value.**

Introduction

Transcriptional silencing of tumor suppressor genes associated with the hypermethylation of CpG dinucleotide "islands" located within promoter regions is thought to be an important epigenetic mechanism for carcinogenesis (1). The simultaneous hypermethylation of multiple genes including *p16*, *THBS1*, *IGF-2*, and *HIC-1* is referred to as CIMP+[3] (2, 3) and is observed in approximately 20–40% of colorectal tumors (3–5). In a proportion of these tumors, the DNA mismatch repair gene *hMLH1* is hypermethylated (6, 7). This is associated with a lack of *hMLH1* expression and consequently with widespread instability in microsatellite sequences, in particular large mononucleotide repeats such as BAT-26. Sporadic CRCs with the CIMP+ or MSI+ phenotypes share several important biological features including frequent location in the proximal colon (2, 4, 5, 8–10), poor histological differentiation (4, 5, 9, 10), and wild-type *p53* (3–5, 9). These common properties suggest that CIMP+ and MSI+ CRCs develop along a similar pathway, possibly involving serrated adenomas and hyperplastic polyps as precursors (11, 12).

In earlier studies (13, 14), we reported that stage III CRC patients with MSI+ tumors showed good survival benefit from 5-FU-based chemotherapy, an observation subsequently confirmed in stage IV patients (15). MSI+ was not entirely sensitive as a predictive marker in our study, however, because a survival benefit from 5-FU was still apparent in the MSI− patient group. This led us to hypothesize that the larger but closely related CIMP+ patient group might account for the majority (if not all) of the survival benefit seen with 5-FU chemotherapy (13). Our reasoning was that widespread DNA hypermethylation, a characteristic of CIMP+ tumors, may be a surrogate marker for aberrant folate/methyl group metabolism in neoplastic cells. Such tumors could be more sensitive to antifolate treatments such as 5-FU in comparison with tumors that have apparently normal methylation patterns. In the present study, we have therefore investigated the predictive value of CIMP+ by comparing the survival of stage III CRC patients treated with or without 5-FU.

Patients and Methods

Tumor Series. A total of 891 stage III CRC cases were diagnosed at the Sir Charles Gairdner Hospital between 1985

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[3] The abbreviations used are: CIMP, CpG island methylator phenotype; MSI, microsatellite instability; 5-FU, 5-fluorouracil; CRC, colorectal cancer; RR, relative risk; CI, confidence interval.

and 1999 (14). This spans the time period during which 5-FU-based adjuvant chemotherapy was being introduced in Western Australia for the management of stage III CRC. Adjuvant chemotherapy was given to 270 (30%) patients according to the standard Mayo regimen (5-FU/leucovorin). This comprised at least two cycles of chemotherapy, and for the majority of patients the full six cycles were completed. Patients were separated into categories based on 5-year age intervals, gender, and site of tumor origin. The latter two factors have been shown to influence the survival benefit from 5-FU in CRC (13, 16). Within these groups, adjuvant-treated and nontreated patients were pair-matched at random. A total cohort of 125 matched pairs was selected for DNA methylation analysis. All tumors had negative surgical margins, and patients showed no signs of metastatic disease at the time of surgery. All cases were diagnosed at a single pathology laboratory (Hospital and University Pathology Service/Pathcenter) associated with the Sir Charles Gairdner Hospital. This laboratory maintained relatively constant reporting practices during the 1985–1999 study period. Five cases were classified as T_4 lesions, and all others were classified as T_3. The study included 48 rectal, 24 sigmoid, 24 descending colon, 17 transverse colon, 47 ascending colon, and 46 cecal tumors. Four patients with rectal cancer received postoperative radiotherapy.

Disease-specific survival information was obtained on all 206 patients by examination of hospital and West Australian Health Department records. The median follow-up time was 39 months (range, 1–172 months), with 119 patients (58%) dying as a result of recurrent disease by the end of the study. Survival data for 19 (9%) patients who died from other causes were censored at the time of death. It has been estimated that net migration out of the state of Western Australia is 0.4% per year, equating to approximately 1 case/year of the 206 cases investigated in this series. However, this rate would be expected to be considerably lower for older individuals and particularly for those diagnosed with cancer. The Sir Charles Gairdner Hospital Human Research Ethics Committee gave approval for this study.

CIMP+ Molecular Analysis. There is currently no consensus definition for CIMP+, although Toyota *et al.* (2) have suggested that investigation of between two and four type "C" (cancer-specific) CpG loci is sufficient for the accurate evaluation of this phenotype. Methylation-specific PCR was used to determine the methylation status of CpG islands located within the *p16* promoter (4, 5, 10, 17), the MINT-2 clone (3, 4), and the *MDR1* promoter (4, 9). DNA amplification of all three CpG loci was successful for 103 matched pairs, equating to an overall success rate of approximately 90%. CIMP+ was arbitrarily defined as the presence of two or more of these sites showing methylation. Of the 206 tumors successfully analyzed in this study for CIMP+ status, the majority (83%) were sourced from formalin-fixed and paraffin-embedded archival tissue blocks. The remaining cases were in the form of unfixed tissue samples taken shortly after surgical resection and stored frozen at –80°C. Our group has previously evaluated the MSI+ and *p53* mutation status of the tumors included in this study (14). MSI+ status was determined by screening for deletions in the BAT-26 mononucleotide repeat (18), whereas screening for *p53* mutations in exons 5–8 inclusive was performed by single-strand conformational polymorphism analysis (19).

Table 1 Characteristics of CRC patients in the two treatment cohorts

Feature (*n*)	Surgery (%)	Surgery + 5-FU (%)	*P*
Total (206)	103	103	
Mean age (yrs)	61.4	60.4	0.48
Mean follow-up (mo)	44.3	47.9	0.54
Year of surgery			
1985–1989 (17)	17	0	
1990–1994 (106)	58	48	
1995–1999 (83)	28	55	<0.01
Sex			
Female (74)	37 (50)	37 (50)	
Male (132)	66 (50)	66 (50)	1.0
Site			
Rectum (49)	24 (49)	25 (51)	
Distal colon (47)	24 (51)	23 (49)	
Proximal (110)	55 (50)	55 (50)	1.0
Histological grade			
Well/moderate (162)	78 (48)	84 (52)	
Poor (41)	23 (56)	18 (44)	0.36
Nodal involvement			
1 or 2 nodes (96)	44 (46)	52 (54)	
≥3 nodes (110)	59 (54)	51 (46)	0.26
p53			
Wild-type (110)	51 (46)	59 (54)	
Mutant (76)	43 (57)	33 (43)	0.17
MSI			
− (165)	79 (48)	86 (52)	
+ (28)	12 (43)	16 (57)	0.62
CIMP			
− (139)	65 (47)	74 (53)	
+ (67)	38 (57)	29 (43)	0.18

Statistical Analyses. Multivariate Cox proportional hazard test with matched-pair stratification and Kaplan-Meier analyses were used to evaluate differences in survival between patient groups. Regression sensitivity was determined by analyzing the effect of unmeasured binary confounders as described by Lin *et al.* (20) Statistical analyses were performed using the Stata 7.0 (Stata Corporation, College Station, TX) software package. All *P*s are two-sided.

Results

The clinical, pathological, and molecular features of the patient cohorts treated by surgery alone or by surgery and 5-FU are shown in Table 1. There are no significant differences between these groups, with the exception of the year of surgery. All patients who received chemotherapy were diagnosed after 1989, whereas 17 (16%) of the patients treated by surgery alone were diagnosed between 1985 and 1989.

Methylation of *p16*, *MDR1*, and MINT-2 was detected in 36%, 25%, and 38% of tumors, respectively. Using a definition of two or more sites showing methylation, 33% of tumors (67 of 206 tumors) in this series were classified as CIMP+. This phenotype was significantly associated with proximal location in the colon, poor histological grade, MSI+, and normal *p53* status, but not with age, gender, or extent of nodal involvement (Table 2). Of the 67 CIMP+ tumors, 21 (31%) were of poor histological grade compared with 20 of 136 (15%) for the

Table 2 Associations between CIMP+ and clinicopathological or molecular features

Feature (*n*)	CIMP− (%)	CIMP+ (%)	*P*
Total (206)	139 (67)	67 (33)	
Sex			
Female (74)	50 (68)	24 (32)	
Male (132)	89 (67)	43 (33)	0.98
Age[a]			
<62 yrs (105)	70 (67)	35 (33)	
≥62 yrs (101)	69 (68)	32 (32)	0.80
Site			
Rectum (49)	42 (86)	7 (14)	
Distal colon (47)	40 (85)	7 (15)	
Proximal (110)	57 (52)	53 (48)	<0.005
Grade			
Well/moderate (162)	116 (72)	46 (28)	
Poor (41)	20 (49)	21 (51)	<0.005
Nodal involvement			
1 or 2 nodes (89)	59 (66)	30 (34)	
≥3 nodes (94)	63 (67)	31 (33)	0.9
p53			
Wild-type (110)	65 (59)	45 (41)	
Mutant (76)	61 (80)	15 (20)	<0.005
MSI			
Negative (165)	120 (73)	45 (27)	
Positive (28)	8 (29)	20 (71)	<0.005

[a] Median age of patients was 62 years.

CIMP− tumors ($P = 0.005$). Similarly, 20 of 65 (31%) CIMP+ tumors were MSI+ compared with only 8 of 128 (6%) CIMP− tumors ($P < 0.0001$).

The prognostic value of CIMP+ is shown in Fig. 1 for each of the two treatment cohorts. For patients treated by surgery alone (Fig. 1*A*), CIMP+ was associated with worse prognosis compared with CIMP− (RR = 1.65; 95% CI, 1.00–2.72; $P = 0.05$). However a trend for better survival of CIMP+ patients was observed in the cohort treated with surgery plus 5-FU (Fig. 1*B*), possibly due to an interaction between CIMP+ and chemotherapy as described below.

In agreement with results from randomized clinical trials (16, 21), the absolute 5-year survival benefit associated with the use of 5-FU in this study was approximately 11% (RR = 0.62; 95% CI, 0.43–0.90; $P = 0.012$). When analyzed according to CIMP+ status, almost all of the long-term benefit from 5-FU treatment was attributable to the CIMP+ patient group (Fig. 2*B*), with no long-term survival benefit apparent for CIMP− patients (Fig. 2*A*; RR = 0.96; 95% CI, 0.62–1.49; $P = 0.86$). Multivariate analysis for the matched pairs revealed that CIMP+ was predictive for survival benefit independent of MSI+ and *p53* mutation status (RR = 0.22; 95% CI, 0.06–0.84; $P = 0.027$). Neither MSI+ nor *TP53* status was found to have independent predictive value in a multivariate analysis model that included CIMP+ (data not shown).

Sensitivity analyses revealed that an unmeasured, high-risk confounding factor could only account for the predictive value associated with CIMP+ if it was present with at least twice the frequency in the cohort treated by surgery alone compared with that treated with 5-FU (Table 3). The RR associated with this confounding factor would also need to be greater than 3.0.

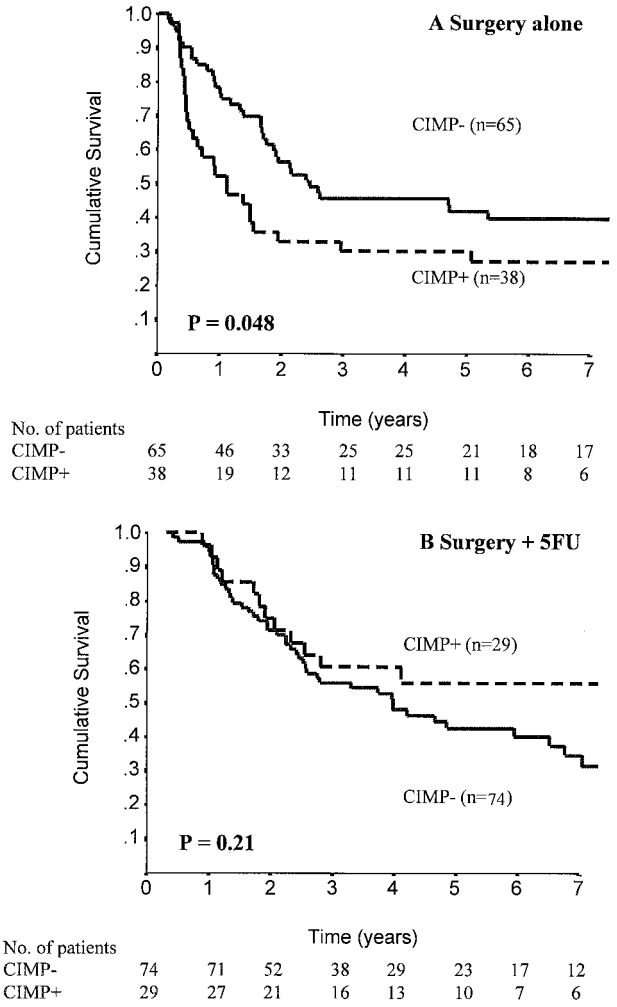

Fig. 1 Prognostic values for CIMP+ in stage III CRC patients treated with surgery alone (*A*) or with surgery and 5-FU (*B*). *P*s shown are from the log-rank test.

Discussion

Clinical trials have established that adjuvant chemotherapy with 5-FU-based regimes is associated with small but significant improvements in the survival of stage III CRC patients (21). Until recently, there was no evidence to suggest that different subgroups of CRC patients defined by gender or anatomical location of the tumor obtain different benefit from this treatment. However, data from two recent publications, one a retrospective cohort study (13) and the other a prospective, randomized study (16), indicate that the level of survival benefit from 5-FU may vary according to gender and tumor site. Female patients and patients with colon tumors appear to derive more benefit than male and rectal cancer patients, respectively. The underlying molecular basis for this differential response to 5-FU is not known. Based on the observation that patients with MSI+ tumors appear to gain considerable benefit from 5-FU, we have previously suggested that the closely related CIMP+ tumors may represent the 5-FU-chemoresponsive subgroup (13). In the current study, we therefore investigated the predictive value of

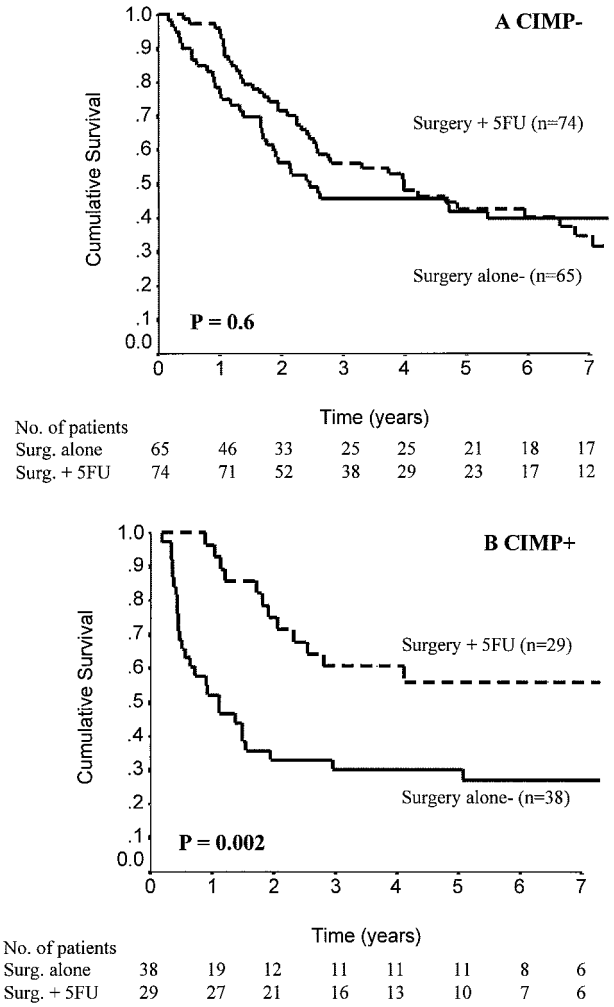

Fig. 2 Predictive values of CIMP+ (*A*) and CIMP− (*B*) for the survival benefit from 5-FU. *P*s shown are from the log-rank test.

CIMP+ by comparing the survival of two age-, sex-, and site-matched patient cohorts: one treated by surgery alone; and the other treated by surgery and 5-FU/leucovorin chemotherapy.

5-FU-based chemotherapy for stage III CRC was introduced over a relatively short time period during the early to mid-1990s in Western Australia, and therefore patients in adjuvant-treated and nontreated cohorts were likely to have received comparable surgical procedures, pathological diagnosis, and postsurgical management. In support of this, the survival rate for patients treated by surgery alone in the early period (1985–1992) was not significantly different from that of more recent patients (1993–1999). As shown in Table 1, the two treatment cohorts also demonstrated similar clinicopathological and molecular characteristics. The absolute survival benefit associated with 5-FU treatment in this study, 11% after 5-years of follow-up, is similar to that reported for randomized clinical trials (16, 21).

Although a consensus has yet to be reached for the classification of CIMP+, the definition used in the present work identified a tumor subgroup with characteristics similar to those

Table 3 Sensitivity assessment for the predictive value of CIMP+

Prevalence of UBC[a] in surgery alone cohort (%)	Prevalence of UBC in surgery + 5-FU cohort (%)	UBC RR	Predictive value of CIMP+ adjusted for UBC
0	0	1	0.22 (0.06–0.84)
40	20	3	0.28 (0.08–1.00)
90	50	3	0.30 (0.08–1.09)
40	20	2	0.25 (0.07–0.91)
90	50	2	0.27 (0.08–0.98)

[a] UBC, unmeasured binary confounder.

reported by other workers (2–5, 8, 9). These include associations with proximal tumor location, poor histological grade, wild-type *p53*, and MSI+ (Table 2).

The prognostic value of CpG island methylation has been investigated previously. Liang *et al.* (22) studied 84 stage III CRC patients and found an association between *p16* methylation and shortened survival. Also, a recent study of 426 cases of stage I-IV CRC reported that patients with CIMP+ tumor have worse prognosis (5). However, two other reports did not find prognostic value for *p16* methylation (23) or CIMP+ (4). In the present work, we observed that CIMP+ was associated with worse survival for patients treated with surgery alone, but not for patients treated with surgery and chemotherapy. Patient treatment information should therefore always be considered when interpreting data on molecular prognostic markers.

The present investigation is the first to report on the predictive value of CIMP+. The novel finding of the present study is that CRC patients with CIMP+ tumors could account for the majority and perhaps all of the long-term survival benefit associated with the use of 5-FU chemotherapy (Fig. 2). The predictive significance of CIMP+ was independent of two other molecular markers, MSI+ and *p53*, that also have predictive value for survival benefit from 5-FU in CRC (14, 15, 24). Sensitivity analyses revealed that unidentified confounder variables are unlikely to explain the association between CIMP+ and apparent survival benefit from 5-FU (Table 3), although this possibility cannot be completely excluded. Statistical evaluation of unmeasured binary confounding variables has previously been used to estimate the benefit from 5-FU chemotherapy in elderly, stage III CRC patients (25).

It should be noted that approximately 40% of patients with CIMP+ tumors died from recurrent CRC despite the use of 5-FU (Fig. 2*B*), indicating that this phenotype is not entirely specific for the prediction of response to treatment. The use of other combinations of CpG islands to define CIMP+ may yield stronger predictive information than that observed with the current panel of *p16*, MINT-2, and *MDR1*. Additional predictive factors might also be the level of expression of genes involved in 5-FU metabolism, including thymidylate synthetase, dihydropyrimidine dehydrogenase, and thymidine phosphorylase (26–28). The levels of genomic hypomethylation or of intratumoral folate intermediates could also be associated with the degree of response to antifolate therapies.

The predictive value of CIMP+ proposed here should be validated in prospective clinical trials that include a patient cohort treated by surgery alone. Because 5-FU is now recommended as standard treatment for all stage III patients, such

trials are only feasible with stage II patients. An alternate approach would be to carry out molecular screening for CIMP+ in archival tumor specimens from previous clinical trials of 5-FU. CIMP+ is associated with the transcriptional silencing of specific genes including *hMLH1* and *p16*, and consequently this phenotype may show characteristic protein expression patterns. If these can be accurately identified, it could allow immunohistochemical analysis of gene expression as an alternative to DNA analyses to identify the CIMP+ subgroup of CRC.

Strong links have been demonstrated between folate metabolism and changes in DNA methylation (29). We hypothesize that the DNA hypermethylation observed in CIMP+ tumors could be a surrogate marker for more widespread aberrations in cellular folate and methyl group metabolism. Such changes might render CIMP+ tumor cells more sensitive to antifolate therapies including 5-FU and leucovorin. Comparison of the level of folate intermediates between CIMP− and CIMP+ tumors may shed more light on this possibility. Another explanation for the apparent chemosensitivity of CIMP+ tumors is that the transcriptional silencing associated with this phenotype inactivates genes required for cell survival in the presence of 5-FU.

Proximal (13) and colonic (16) tumors appear to gain the majority of survival benefit observed from 5-FU in CRC patients. In the present study of 206 cases, 48% of proximal tumors were CIMP+ compared with only 14–15% of distal colon or rectal tumors (Table 2). In a recent study of 417 consecutive stage I-IV CRC cases, 37% of proximal tumors compared with only 9% of distal tumors were classified as CIMP+ using a definition of 3 or more CpG sites methylated out of 5 examined (5). The tumor site difference in CIMP+ frequency becomes even greater (8-fold) if only heavy methylation (3 of 3 sites methylated) is considered (4). In addition to proximal tumor location, we have also shown that females appear to gain more benefit from 5-FU than males (13). Previous studies have shown that *p16* methylation (10), heavy methylation (4), and methylation of ≥3 of 5 CpG sites (5) are all more common in tumors from female patients. In the current study using a definition of ≥2 of 3 sites methylated for CIMP+, we did not find a gender difference in CIMP+ frequency (Table 2). However, this may be due to the selected nature of the current patient cohort in comparison with nonselected series used in previous studies. In particular, the median age of patients in this study was 7 years younger than that seen in a large consecutive series from our institute (14).

In conclusion, the present study provides evidence that CIMP+ is a predictive factor for survival benefit from 5-FU chemotherapy in CRC patients independently of MSI+ and *p53* status. Confirmation of these findings may lead to the improved selection of CRC patients to receive adjuvant 5-FU chemotherapy. Additional studies are required to determine whether other combinations of methylated CpG islands or classification criteria for CIMP+ have stronger or more specific predictive value. Although indirect, the observed correlation between higher CIMP+ frequency in proximal tumors and greater survival benefit from chemotherapy is suggestive of a causal link. Comparisons of the cellular folate pool and of gene expression patterns between CIMP+ and CIMP− tumors may help to explain the apparent chemosensitivity of tumors with aberrant DNA methylation.

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