

CHFR Promoter Methylation Indicates Poor Prognosis in Stage II Microsatellite Stable Colorectal Cancer

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CHFR Promoter Methylation Indicates Poor Prognosis in Stage II Microsatellite Stable Colorectal Cancer

Arjen H.G. Cleven¹, Sarah Derks¹, Muriel X.G. Draht¹, Kim M. Smits^{1,2}, Veerle Melotte¹, Leander Van Neste¹, Benjamin Tournier⁴, Valerie Jooste⁵, Caroline Chapusot⁴, Matty P. Weijenberg³, James G. Herman⁶, Adriaan P. de Bruïne¹, and Manon van Engeland¹

Abstract

Purpose: Data on the prognostic significance of promoter CpG island methylation in colorectal cancer (CRC) are conflicting, possibly due to associations between methylation and other factors affecting survival such as genetic alterations and use of adjuvant therapy. Here, we examine the prognostic impact of promoter methylation in patients with CRC treated with surgery alone in the context of microsatellite instability (MSI), *BRAF* and *KRAS* mutations.

Experimental Methods: One hundred and seventy-three CRCs were analyzed for promoter methylation of 19 tumor suppressor and DNA repair genes, the CpG island methylator phenotype (CIMP), MSI, the exon 15 V600E *BRAF* mutation and *KRAS* codon 12 and 13 mutations.

Results: Unsupervised hierarchical clustering based on methylation status of 19 genes revealed three subgroups: cluster 1 [CL1, 57% (98/173) of CRCs], cluster 2 [CL2, 25% (43/173) of CRCs], and cluster 3 [CL3, 18% (32/173) of CRCs]. CL3 had the highest methylation index (0.25, 0.49, and 0.69, respectively, $P < 0.01$) and was strongly associated with CIMP ($P < 0.01$). Subgroup analysis for tumor stage, MSI, and *BRAF* status showed no statistically significant differences in survival between CL1, CL2, and CL3 nor between CIMP and non-CIMP CRCs. Analyzing genes separately revealed that *CHFR* promoter methylation was associated with a poor prognosis in stage II, microsatellite stability (MSS), *BRAF* wild-type (WT) CRCs: multivariate Cox proportional HR = 3.89 [95% confidence interval (CI), 1.58–9.60, $P < 0.01$; $n = 66$] and HR = 2.11 (95% CI, 0.95–4.69, $P = 0.068$, $n = 136$) in a second independent population-based study.

Conclusions: *CHFR* promoter CpG island methylation, which is associated with MSI, also occurs frequently in MSS CRCs and is a promising prognostic marker in stage II, MSS, *BRAF* WT CRCs. *Clin Cancer Res*; 20(12); 3261–71. ©2014 AACR.

Introduction

Accurate staging of colorectal cancer (CRC) is essential for optimal disease management. Although patients with the same stage can demonstrate considerable variation in outcome, the tumor—node—metastasis (TNM) staging system remains the gold standard for predicting prognosis and guiding clinical management of CRC.

Adjuvant chemotherapy is recommended for all stage III CRC patients. In Europe, the majority of stage II CRC patients undergo surgery alone, despite the recognition that a subgroup with a poor prognosis would probably benefit from adjuvant chemotherapy. Molecular classification of CRC might aid in selecting patients with CRC who could benefit from adjuvant therapy.

CRC is characterized by (epi)genetic alterations of genes controlling the hallmarks of cancer (1–4). Frequently observed alterations affecting these pathways include chromosomal instability (CIN), microsatellite instability (MSI), coding sequence mutations in *APC*, *TP53*, *KRAS*, and *PIK3CA* (3, 4) and promoter CpG island hypermethylation (5). A distinct subset of CRCs, characterized a greater degree of promoter CpG island methylation, is associated with proximal location, poor differentiation, MSI, and *BRAF* mutations (6–11) and is referred to as the CpG island methylator phenotype (CIMP; refs. 10, 12).

CIMP has previously been associated with CRC prognosis. However, published reports are inconsistent, perhaps due to confounding factors such as MSI, *BRAF*, and *KRAS* mutations, variations in use of adjuvant chemotherapy

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Translational Relevance

Here, we present *CHFR* promoter CpG island methylation as a prognostic biomarker for stage II, microsatellite stability, *BRAF* wild-type CRCs in two independent population series. This finding could aid in the identification of high-risk stage II colorectal cancer (CRC) patients. The use of adjuvant chemotherapy for stage II colon cancer is still controversial because parameters to accurately identify stage II CRC patients at risk for recurrence are lacking. Currently, these patients are being identified by clinicopathological parameters such as the T stage, number of lymph nodes examined, tumor differentiation, tumor perforation, vascular, lymphatic and perineural invasion, and tumor budding. However, some of these parameters are subject to interobserver variability and lack reproducible scoring systems. *CHFR* methylation can facilitate better selection of high-risk stage II CRC patients for adjuvant therapy.

(13–16), and methodologic differences, such as different CIMP definitions (12, 17).

The aim of this study was to explore the role of CIMP and frequently methylated promoter CpG islands on prognosis of CRC, eliminating the influence of genetic alterations and adjuvant chemotherapy.

Material and Methods

Study population

For the study population, we used CRC material from patients that were entered in two clinical studies in the Netherlands between 1979 and 1981. One trial was designed to compare patient survival after treatment for CRC by conventional surgery ($n = 119$) or the no-touch isolation technique ($n = 117$; ref. 18). The two treatment groups were comparable with regard to patient characteristics. The survival between these groups was not statistically different between both groups. One hundred and fourteen cases with tumor material were available for analysis. From a second clinical study, designed to compare outcome in rectal cancer patients with and without preoperative radiotherapy (19), only the patients in the no-preoperative radiotherapy arm ($n = 114$) were included. From this group, tumor material from 59 rectum cases was available. At the time these studies were conducted, adjuvant chemotherapy was not yet standard practice. None of the patients in the study population received chemotherapy. Tumor stage was defined according to the UICC-TNM staging system and American Joint Committee on Cancer classifications, Cancer Staging Sixth Edition. For both studies, follow-up took place every 3 months during the first 3 years and every 6 months between 3 years and 5 years after initial diagnosis and surgery. Standard protocols were followed, with routine blood counts and chemistry studies (including carcinoembryonic antigen levels) at each visit and liver ultrasound, chest X-ray, and colonoscopy annually, to evaluate

recurrence of disease and disease-related death. After 5 years of follow-up, only time and cause of death were registered. Follow-up was complete for all patients. Failure was defined as death due to recurrent disease, excluding postoperative mortality within 30 days, and non-disease-related death. For molecular analyses, tumor tissues from 173 (114 + 59) tissue samples of patients with primary CRC were available.

Independent validation population of CRCs

A second, independent population of 734 CRC cases, derived from the prospective Netherlands Cohort Study on diet and cancer which started in 1986 with the enrolment of 120,852 healthy individuals between 55 years and 69 years old from 204 municipalities throughout the Netherlands, was used to validate survival data. From 1989 to 1994, 925 incident CRC cases (ICD-O: 153.0–154.1) were identified by computerized linkage with the Netherlands Cancer Registry and PALGA, a nationwide network and registry of histopathology and cytopathology (20). Information on tumor localization, tumor staging, differentiation grade, and incidence date was available through the Netherlands Cancer Registry. Vital status until May 2005 was retrieved from the Central Bureau of Genealogy and the municipal population registries and could be obtained for all cases. Causes of death were retrieved through linkage with Statistics Netherlands. Paraffin-embedded tumor tissue was collected from 54 pathology registries; tissue blocks for 734 (90%) of the CRC cases contained sufficient DNA for analyses. Details of this cohort have been described

Table 1. Clinicopathological characteristics of the CRC populations

	Study population ($n = 173$)	Validation population ($n = 569$)	<i>P</i>
Age			
Mean age (SD)	67.8 (11.8)	63.1 (4.1)	<0.001
Gender			
Male	82 (47%)	303 (53.3)	0.177
Female	91 (53%)	266 (46.7)	
Tumor location			
Right-sided colon	62 (36%)	210 (37.2)	<0.001
Left-sided colon	52 (30%)	253 (44.9)	
Rectum	59 (34%)	101 (17.9)	
CRC stage			
I	4 (2%)	172 (30.9)	<0.001
II	100 (58%)	201 (36.2)	
III	50 (29%)	127 (22.8)	
IV	19 (11%)	56 (10.1)	
Event frequency ^a	64 (38%)	210 (37.1)	0.774
Median follow-up time	4.8 y	8.9 y	<0.001

^aCRC-specific death. The Pearson χ^2 test was used. Fisher exact test was used if there were less than five objects in any category.

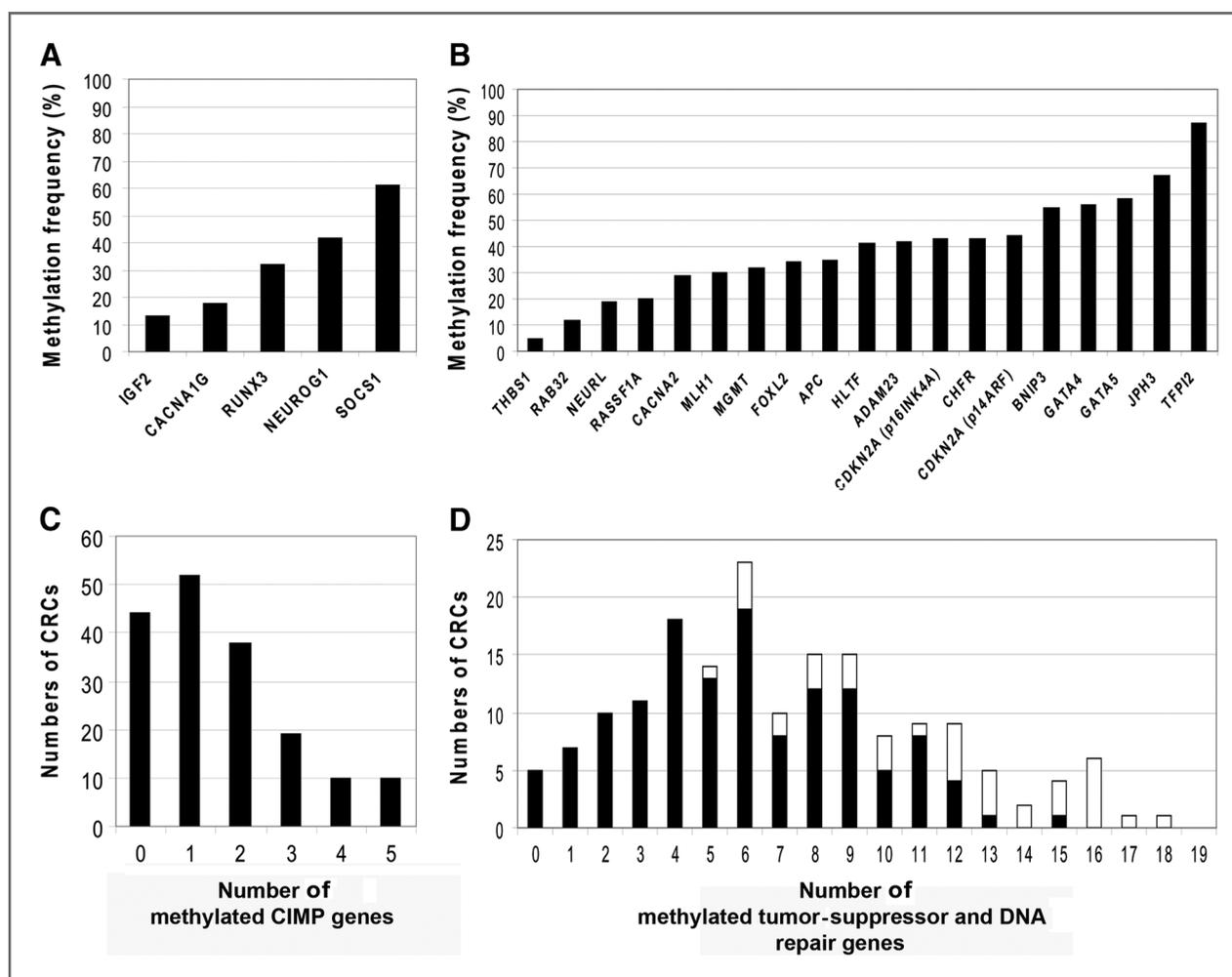


Figure 1. A, methylation frequencies of the five CIMP genes in the study population (black bars indicate percentages). B, methylation frequencies of 19 additional tumor suppressor- and DNA repair genes in the study population (black bars indicate percentages). C, distribution of CRC cases according to the number of methylated CIMP genes (black bars indicate number of cases). D, distribution of CRC cases according to the number of methylated tumor suppressor and DNA repair genes (black bars indicate number of cases CIMP⁺, white bars indicate number of cases CIMP⁻).

previously (21). For 714 cases, therapy data were available. Of these 714 cases, 145 patients received chemo- or radiotherapy, leaving 569 cases not treated with additional therapies that will be used for analyses.

Clinical pathologic characteristics are provided for both populations (Table 1). In the validation study, younger patients ($P < 0.001$), more left-sided tumors ($P < 0.001$) and stage I tumors ($P < 0.001$), were diagnosed and the median follow-up time was longer in the validation population: 8.9 years compared with 4.8 years ($P < 0.001$). However, event frequencies were comparable between both studies, making the validation population suitable for validation of prognostic markers.

Promoter CpG island methylation, MSI and BRAF and KRAS analysis

Genomic DNA was extracted from CRC tissues using PureGene Genomic DNA Isolation Kit (Gentra Systems) according to the manufacturer's protocol.

Promoter CpG island methylation of genes reported to be methylated in CRC (2, 5, 22, 23): *mutL homolog 1, colon cancer, nonpolyposis type 2 (Escherichia coli; MLH1)*, *cyclin-dependent kinase inhibitor 2A (CDKN2A; p16INK4 and p14ARF)*, *O-6-methylguanine-DNA methyltransferase (MGMT)*, *Ras association (RalGDS/AF-6) domain family member 1 (RASSF1A)*, *adenomatous polyposis coli (APC)*, *helicase-like transcription factor (HLTF)*, *GATA-binding protein 4 (GATA4)*, *GATA-binding protein 5 (GATA5)*, *checkpoint with forkhead and ring finger domains (CHFR)*, *ADAM metalloproteinase domain 23 (ADAM23)*, *Rab32, member RAS oncogene family (RAB32)*, *junctional protein 3 (JPH3)*, *forkhead box L2 (FOXL2)*, *BCL2-adenovirus E1B 19kDa interacting protein 3 (BNIP3)*, *neutralized homolog (Drosophila; NEURL)*, *calcium channel, voltage dependent, α -delta subunit 1 (CACNA2)*, *thrombospondin 1 (THBS1)*, *tissue factor pathway inhibitor 2 (TFPI2)*, and the CIMP genes *calcium channel, voltage-dependent, T type, α -1G subunit (CACNA1G)*, *insulin-like growth factor-II (somatomedin*

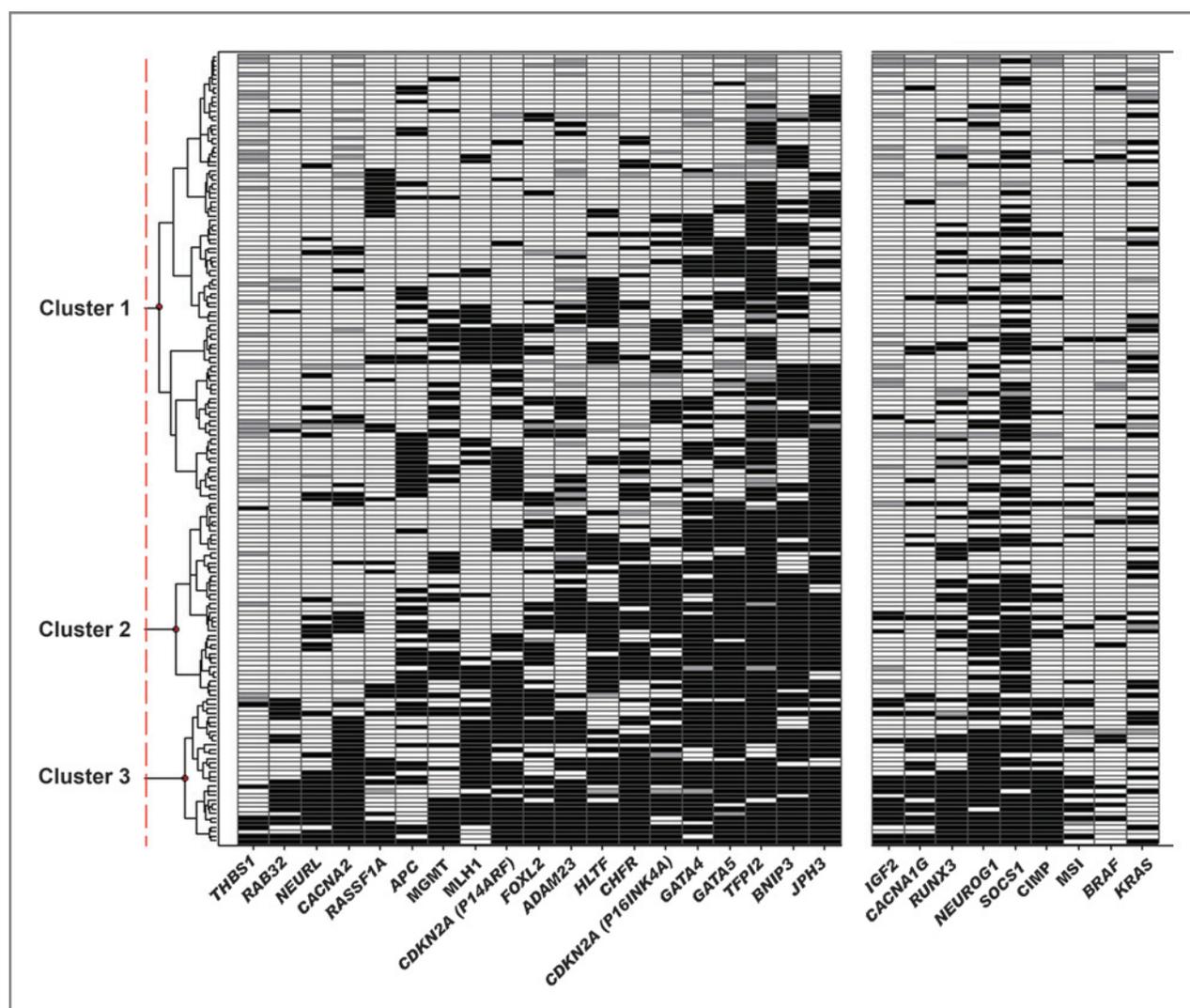


Figure 2. CRC clusters CL1 (57%), CL2 (25%), CL3 (18%) obtained by unsupervised hierarchical clustering of promoter CpG island methylation of *MLH1*, *CDKN2A* (p16INK4A), *CDKN2A* (p14ARF), *MGMT*, *RASSF1A*, *APC*, *HLTF*, *GATA4*, *GATA5*, *CHFR*, *ADAM23*, *RAB32*, *JPH3*, *FOXL2*, *BNIP3*, *NEURL*, *CACNA2*, *THBS1*, *TFPI2*. A black box indicates a methylated gene, a white box indicates an unmethylated gene, and a gray box indicates a failed PCR. After clustering, identification of patients was done for individual CIMP markers (*CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3*, and *SOCS1*), CIMP, MSI, *BRAF*-, and *KRAS* mutations as visualized. Black box indicates positive, white box indicates negative, and gray box indicates missing value.

A, *IGF-II*), neurogenin 1 (*NEUROG1*), runt-related transcription factor 3 (*RUNX3*), and suppressor of cytokine signaling 1 (*SOCS1*) were determined using sodium bisulfite modification of genomic DNA (EZ DNA Methylation Kit, ZYMO research Co.). To facilitate methylation-specific PCR (MSP) analysis on DNA retrieved from formalin-fixed, paraffin-embedded tissue, nested MSP was performed as described elsewhere (24, 25). Primers and PCR conditions are provided in Supplementary Table S1.

MSI was determined by a pentaplex PCR, using the mononucleotide MSI markers BAT-26, BAT-25, NR-21, NR-22, and NR-24, as previously described (26). MSI was defined positive when three or more of five markers (BAT-26, BAT-25, NR-21, NR-22, and NR-24) showed allelic size variants.

The common V600E *BRAF* mutation in exon 15 was analyzed by semi-nested PCR and subsequent restriction

fragment length polymorphism (RFLP) analysis, *KRAS* mutations were analyzed as described previously (27, 28).

Data analysis

A consensus marker panel to analyze CIMP in CRC has not been established yet (12). We analyzed CIMP using the marker panel proposed by Weisenberger and colleagues (11). CRCs were defined as CIMP when ≥ 3 of 5 analyzed markers (*CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3*, and *SOCS1*) were methylated. Unsupervised clustering (Spotfire DecisionSite for Functional Genomics), based on the similarity of methylation of 19 CpG islands that have been reported to be methylated in CRC (CIMP genes excluded), was performed by using half-square Euclidian distance (Wards method linkage rule; refs. 29, 30). Methylation index (MI = number of methylated promoter CpG islands divided

Table 2. Correlations between CIMP, CL1, CL2, and CL3 and MSI and *BRAF* mutation in the study population

	MSI	MSS	BRAF M	BRAF WT	CIMP
CIMP	12/19 (63%)	26/145 (18%) <0.01	7/14 (50%)	32/143 (22%) <0.05	
CL1	3/19 (16%)	94/150 (63%)	5/14 (36%)	83/147 (57%)	6/39 (15%)
CL2	4/19 (21%)	38/150 (25%)	3/14 (21%)	39/147 (27%)	8/39 (21%)
CL3	12/19 (63%)	18/150 (12%) <0.01	6/14 (43%)	25/147 (17%) 0.06	25/39 (64%) <0.01

NOTE: CIMP using the markers of Weisenberger et al. (11). CL1, CL2, and CL3, groups of CRCs identified by unsupervised hierarchical clustering based on methylation patterns of 19 tumor suppressor and DNA repair genes. The Pearson χ^2 test was used. The Fisher exact test was used if there were less than five objects in any category.

Abbreviation: M, mutant.

by number of promoter CpG islands successfully analyzed) was calculated using the promoter CpG islands of 19 tumor suppressor- and DNA repair genes as well as the CIMP panel. To assess normality for the distribution of CRC cases according to the number of methylated CIMP genes and number of methylated tumor suppressor genes, we calculated the sample skewness divided by the standard error of skewness. Normality was rejected if the ratio was less than -2 or more than $+2$. In addition, quantile–quantile plots were generated to compare the shapes of the distributions. Differences between methylation, clinicopathological, and molecular characteristics were determined by the Pearson χ^2 test and the Fisher exact test in cases with <5 subjects in any category. Kaplan–Meier curves were used to evaluate the relationship between promoter CpG island methylation and patient survival in the overall population and stratified for tumor stage, MSI, and *BRAF* mutation status. For graphical reasons, to increase comparability of the curves in the two populations, Kaplan–Meier curves were cutoff at 10

years of follow-up for the validation population. For Cox proportional hazard analyses, maximal follow-up periods were used. Statistical differences between groups were assessed by use of the log-rank test. The endpoint for analyses was cancer-specific survival starting from the day of surgery to the time of death due to CRC. Independent variables predicting survival were evaluated in a multivariate model using Cox Regression analyses. The Cox regression model including CIMP, CL1, CL2, CL3, *CHFR* promoter CpG island methylation, age, gender, tumor location, differentiation grade, and TNM stage was used to assess the prognostic influence of these variables. All *P* values (two sided) <0.05 were considered statistically significant. SPSS 15.0 and Stata 11.2 were used for data analyses.

Results

(Epi)genetic characterization of CRCs

MSI, *BRAF*, and *KRAS* mutations were detected in 11% (19/169), 9% (14/161), and 26% (41/157) of CRCs of the

Table 3. Cox proportional hazard models for MSS, *BRAF* WT, stage II CRCs

	Study population (n=66)			Validation population (n = 136)		
	n	CRC mortality, HR (95% CI)	P	n	CRC mortality, HR (95% CI)	P
CHFR M ^a	28	3.89 (1.58–9.60)	<0.01	44	2.11 (0.95–4.69)	0.06
Age, y		1.02 (0.97–1.07)	0.41		1.06 (0.95–1.17)	0.31
Gender (female ^b)	34	0.53 (0.23–1.27)	0.16	60	1.59 (0.71–3.58)	0.26
Tumor location						
Left-sided colon ^c	24	0.52 (0.20–1.38)	0.19	54	1.08 (0.43–2.71)	0.87
Rectum ^c	25	0.29 (0.09–0.93)	0.04	11	3.23 (0.90–11.66)	0.07
Differentiation grade poor ^d	26	1.52 (0.69–3.36)	0.30	10	0.92 (0.08–17.38)	0.95

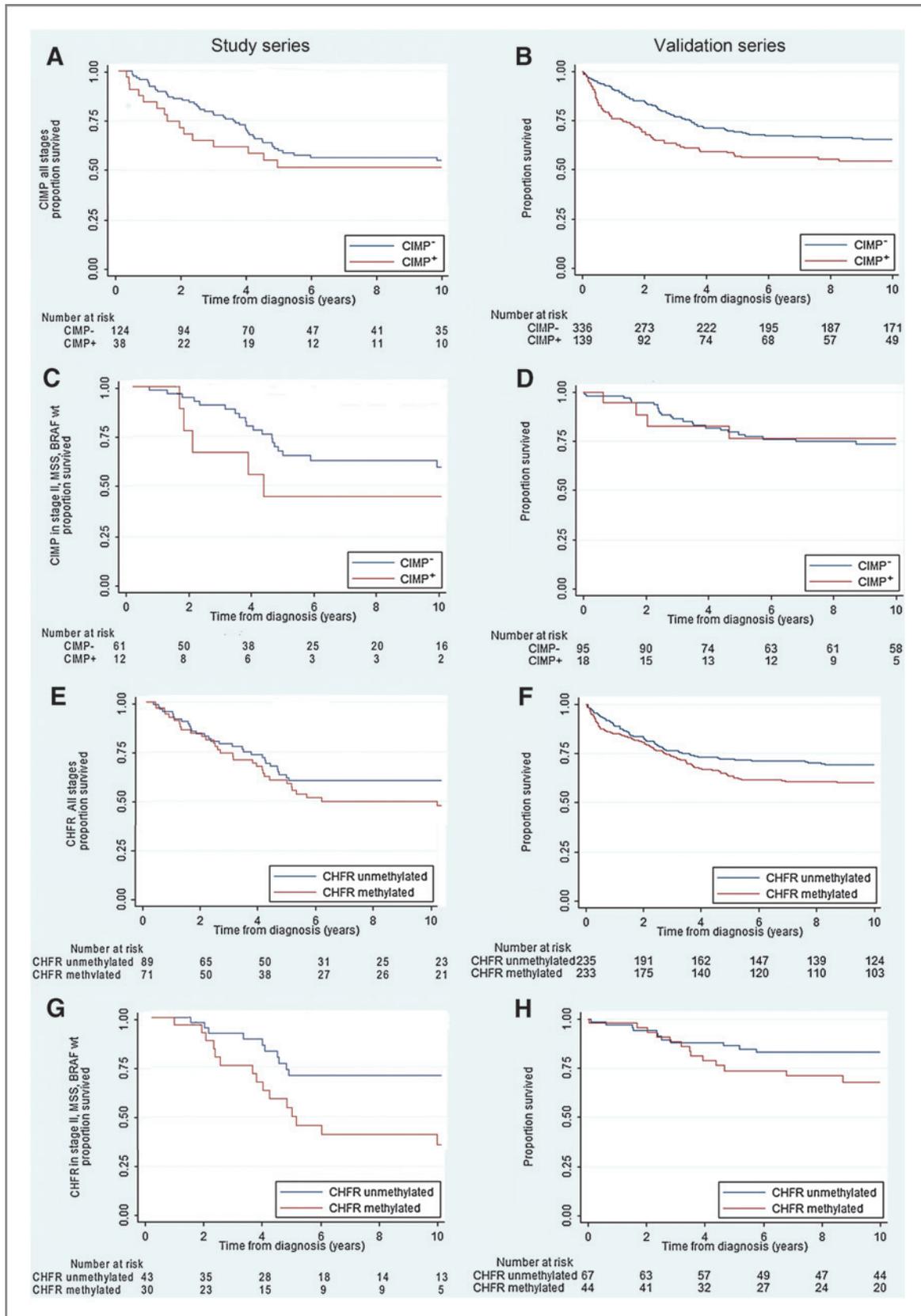
Abbreviations: H, hazard ratio=relative risk; M, methylated.

^aReference group = *CHFR* unmethylated cases.

^bReference group = male.

^cReference group = right-sided colon.

^dReference group = well/moderate.



study population, respectively (Supplementary Table S2), which is in accordance with previously reported frequencies (3, 4, 31).

The methylation frequency for CIMP genes ranged from 13% to 61%: *IGF2* [13% (20/154)], *CACNA1G* [18% (30/170)], *RUNX3* [32% (52/163)], *NEUROG1* [42% (70/165)], and *SOCS1* [61% (103/170); Fig. 1A]. Twenty-three percent (39/168) of CRCs were classified as CIMP (Supplementary Table S3). This is consistent with previous publications with reported frequencies of CIMP between 18% to 25% (11, 32, 33). The distribution of the number of CIMP markers methylated for each tumor resembles a normal distribution (Fig. 1C; sample skewness/standard error of skewness = 0.187; which was confirmed by a quantile–quantile plot analysis, data not shown).

We sought to more broadly examine the methylation of CpG islands in CRC to determine whether the five genes used to define CIMP optimally separated phenotypic differences in colorectal tumors. The frequency of promoter CpG island methylation in 19 additional tumor suppressor genes ranged from 5% (*THBS1*) to 87% (*TFPI2*); (Fig. 1B and Supplementary Table S2), of which many correlated strongly. We compared the distribution of tumors according to the methylation status of the 19 additional genes (Fig. 1D). This resembles a normal distribution (sample skewness/standard error of skewness = 0.185; confirmed by quantile–quantile plot analysis, data not shown) and reveals that CRCs with a high number of methylated genes are predominantly characterized by CIMP (white bars). This suggests that the Weisenberger CIMP markers are sensitive but not completely specific in identifying CRCs with a high frequency of promoter CpG island methylation.

We assessed whether subgroups of CRCs could be identified using methylation patterns of the 19 non-CIMP genes. Unsupervised hierarchical cluster analysis identified three clusters of CRCs, CL1 [57% (98/173)], CL2 [25% (43/173)], and CL3 [18% (32/173); Fig. 2 and Supplementary Table S3]. CL3 showed the highest number of methylated genes (MI; mean MI, CL1 = 0.25; CL2 = 0.49; CL3 = 0.69; $P < 0.001$).

Associations between genetic, epigenetic, and clinicopathological characteristics

CIMP was highly associated with promoter CpG island methylation of 16 of 19 additional genes. Furthermore, CRCs grouped in CL3 were most often classified as CIMP ($P < 0.01$; Fig. 2; Table 2). CIMP was associated with MSI, *BRAF* mutations, and CL3 ($P < 0.01$, $P = 0.05$, and $P < 0.01$, respectively; Table 2). Sixty-nine percent (26/38) of CIMP CRCs and 60% (18/30) of CL3 CRCs are microsatellite stability (MSS; Table 2). Mutations in *KRAS* were neither

associated with CIMP nor with the identified clusters (data not shown).

As expected, CIMP and MSI were associated with right-sided tumor location, as were CL3 CRCs ($P = < 0.01$, $P = < 0.01$, and $P = < 0.01$, respectively; Supplementary Tables S2 and S3). We did not observe previously reported association between sex, age, and CIMP or with our newly defined CL3 tumors.

The aim of this study was to evaluate the prognostic influence of promoter CpG island methylation using CIMP and to extend this analysis to include 19 additional tumor-suppressor and DNA repair genes. As tumor stage, MSI, *BRAF*, and *KRAS* mutations have in some reports influenced survival, we analyzed whether these alterations were of prognostic significance in the study population. As expected, disease outcome was significantly influenced by tumor stage, with stage I having an improved overall survival compared with other tumor stages ($P < 0.01$, data not shown). We also observed that wild-type (WT) *BRAF* tumors showed an improved overall survival compared with *BRAF*-mutated tumors ($P = 0.04$, data not shown). MSI was also associated with improved overall survival as compared with MSS within CRC stage II, although this association is not statistically significant ($P = 0.07$, data not shown). *KRAS* mutations were of no prognostic value, neither in the overall population nor in the separate stages (data not shown). Therefore, survival analyses were also performed for subgroups based on stage, MSI status, and *BRAF* mutation status.

No statistically significant survival differences between CIMP and non-CIMP CRCs were observed in the overall study population ($P = 0.381$; Fig. 3A), or when specifically examining stage II ($P = 0.16$; Fig. 3C) or stage III (data not shown) MSS, *BRAF* WT CRCs. The same was observed for CL1, CL2, and CL3 CRCs (data not shown). Examining the 569 patients of the validation population not treated with adjuvant therapy showed a statistically significant association between CIMP and prognosis in the overall population ($P = 0.004$; Fig. 3B) but neither in stage II MSS *BRAF* WT CRCs ($P = 0.955$; Fig. 3D) nor the stage III MSS *BRAF* WT CRCs (data not shown). Extending these analyses using the full follow-up period of the validation population, did not alter these conclusions ($P = 0.0087$ for the overall and $P = 0.867$ for stage II, MSS *BRAF* WT cases; data not shown). We next sought to determine whether one possible explanation for additional variation in previous studies was that specific CpG island methylation, underlying any prognostic importance, was imperfectly associated with CIMP. When analyzing all 19 (or 24 if including CIMP) genes in a univariate analysis, only *APC* and *CHFR* methylation, although the latter was positively associated with MSI ($P = 0.02$) and

Figure 3. Survival curves for CIMP-positive and CIMP-negative CRCs for the overall study population (A, $P = 0.381$) and validation population (B, $P = 0.004$). Survival curves for stage II, MSS, and *BRAF* WT CRCs for the study population CIMP⁺ ($n = 12$) and CIMP⁻ ($n = 61$), $P = 0.16$ (C) and of the validation population CIMP⁺ ($n = 18$) and CIMP⁻ ($n = 95$), $P = 0.95$ (D). Survival curves for the overall population of *CHFR* unmethylated ($n = 89$) versus *CHFR* methylated ($n = 71$), $P = 0.26$ (E); and for the validation population *CHFR* unmethylated ($n = 235$) versus *CHFR* methylated ($n = 233$), $P = 0.06$ (F). Survival curves for stage II, MSS, and *BRAF* WT tumors for *CHFR* unmethylated ($n = 43$) and *CHFR* methylated ($n = 30$) CRCs, $P = 0.02$ (G); and for the validation population *CHFR* unmethylated ($n = 67$) and *CHFR* methylated ($n = 44$) CRCs, $P = 0.10$ (H).

CIMP ($P < 0.01$; Supplementary Table S4), were associated with a worse prognosis in stage II MSS, *BRAF* WT CRCs (HR = 2.63; 95% confidence interval (CI), 1.21–5.68, $P = 0.01$ and HR = 2.59; (95% CI, 1.16–5.76, $P = 0.02$, respectively; Fig. 3G; Supplementary Table S5). For stage III MSS *BRAF* WT CRCs, associations with worse prognosis were found for *RASSF1A*, *THBS1*, and *CACNA1G* (HR = 3.89; 95% CI, 1.23–12.3, $P = 0.02$; HR = 26.5; 95% CI, 1.66–423, $P = 0.02$ and HR = 5.62; 95% CI, 1.49–21.3, $P = 0.01$; Supplementary Table S5). These effects were not observed in the overall study population (Supplementary Table S5). Because of the small numbers of cases, the number of comparisons examined to find significance and the broad confidence intervals raised concerns, we decided to validate these results in an independent validation population. Cox proportional hazard analysis confirmed an association with prognosis only for *CHFR* methylation in stage II, MSS, *BRAF* WT CRCs (HR = 2.11; 95% CI, 0.95–4.69, $P = 0.068$; Fig. 3F and H; Table 3). Again, extending the Kaplan Meier (KM) analyses to maximal follow-up did not alter these conclusions (data not shown). The Cox regression multivariate model within the study population of MSS, *BRAF* WT, stage II CRCs shows that *CHFR* methylation was associated with a poor prognosis (HR = 3.89; 95% CI, 1.58–9.60, $P = < 0.01$) and was, in this study, a better predictor of survival than differentiation grade (HR = 1.52; 95% CI, 0.69–3.36, $P = 0.30$; Table 3).

Discussion

Data on the prognostic significance of promoter CpG island methylation, and CIMP in particular, are conflicting in CRC (13–16). These inconsistencies might be caused by factors affecting the course of the disease, such as genetic alterations, adjuvant therapy, and differences in methodologic study approaches. Here, we analyzed the prognostic value of promoter CpG island methylation in CRCs from patients not treated with (neo)adjuvant therapy taking into account the confounding role of clinicopathological and genetic (MSI and *BRAF*) characteristics. Previously reported associations between CIMP and proximal tumor location, MSI, and *BRAF* mutations' (10, 11, 16) as well as the identification of three subgroups of CRCs (9, 34–36) based on promoter methylation profiles could be confirmed. In addition, the prognostic role of tumor stage, MSI, and *BRAF* (37, 38) could be confirmed in the study and the validation series.

We could not observe a statistically significant association of CIMP or CL3 with prognosis, neither overall nor when analyzed for stage or microsatellite status. These conclusions are valid for the subgroups of CRC defined by analyzing CIMP with the markers proposed by Weisenberger and colleagues (11) and also for unsupervised clustering of the methylation data for the 19 CpG islands that we added to the analysis. Because also other CIMP definitions have been used, caution with generalizing these conclusions is warranted (17). The statistically significant prognostic effect of CIMP in the validation

series indicates that the prognostic role of CIMP in CRC is still unclear and that large, independent studies are needed to answer this question. In addition, until the underlying biologic cause for CIMP is being identified, an accurate definition remains hard to establish. Our evidence for imperfect correlation of highly methylated tumors using the independent 19 genes with CIMP illustrates this.

However, our data confirm the concept that based on CpG island methylation, three subgroups of CRC can be identified, independently of the specific markers used. The subgroup characterized by extensive promoter CpG island methylation is strongly, although not perfectly, associated with CIMP and its reported clinicopathological characteristics.

Performing subgroup analysis for established confounders including age, gender, tumor location, differentiation grade, TNM stage, MSI, and *BRAF* mutation status, promoter methylation of only 1 of 24 genes, namely *CHFR*, showed a strong association with poor prognosis in stage II MSS CRCs in the study population (HR = 3.89; 95% CI, 1.58–9.60, $P < 0.01$) and the validation series (HR = 2.11; 95% CI, 0.95–4.69, $P = 0.068$). Because of the assumption that many biomarker data are false, and the fact that we studied many variables without adjustment for multiple testing, these data should be considered exploratory and hypothesis generating. Validation of the effect of *CHFR* promoter CpG island methylation in an independent prospective cohort study as well as a small pilot study recently published by an independent team pointing into the same direction (39), underscores the potential of *CHFR* promoter CpG island methylation as a prognostic marker in CRC. Additional supporting evidence for the effect of *CHFR* methylation comes from a collection of CRCs collected by the Ferdinand Cabanne Biological Resources Centre from Dijon, France (40, 41). In this study, we observed that *CHFR* methylation, although not statistically significant, was associated with a worse overall survival (data not shown). Independent validation of our results in large series of CRCs will provide the best evidence for the clinical value of *CHFR* methylation as a marker for prognosis in stage II, MSS, *BRAF* WT CRCs. However, currently no studies that analyzed *CHFR* methylation at the same genomic location (42), in a comparable subgroup of CRCs, using the same endpoints are available, preventing the use of meta-analysis for combined analyses of these data.

A dominant effect of MSI over DNA methylation about prognosis has been shown previously in CRC by Ward and colleagues, for a "CIMP-like" phenotype (43). The mechanisms underlying this paradox are still not clear. An explanation could be the increased lymphocytic infiltrate that is observed in MSI CRCs (44) or the extent of instability making MSI tumor cells less fit to metastasize (45, 46). The prognostic effect of *CHFR* in MSS stage II CRCs is surprising, as we previously reported a strong association between *CHFR* promoter CpG island methylation, MSI, and *MLH1* promoter methylation in CRC (47). However, because a

significant subset of MSS CRCs also has *CHFR* promoter methylation, this could be of clinical relevance.

The biologic role of *CHFR* also points to a role in cancer progression and metastasis. *CHFR* is a tumor suppressor gene which is inactivated by promoter CpG island methylation in a variety of solid tumors (47–51). It encodes an ubiquitin ligase that regulates both entry into metaphase and chromosome segregation later in mitosis to maintain genomic stability (52, 53). *CHFR* inactivation has been hypothesized to be associated with CIN, although conflicting data have been reported (54, 55). Although both *CHFR* and *MLH1* contribute to genomic integrity, they function through different mechanisms. *CHFR* deficiency triggers mild CIN and *MLH1* deficiency leading to MSI (56). Recent data from Oh and colleagues 2009 (57) indicate that, *in vitro*, *CHFR* binds and downregulates HDAC1 thereby downregulating cyclin-dependent kinase inhibitor 1 and the metastasis suppressors, KAI1 and Cadherin-1. This eventually results in cell-cycle arrest and a less invasive phenotype (58). In addition to a potential role for *CHFR* as prognostic biomarker, *CHFR* promoter methylation has been proposed as biomarker for response to microtubule inhibitor taxanes in endometrial (59), cervical (59), oral (60), and gastric cancer (61). Although taxanes are not implemented in CRC treatment because they failed to demonstrate a significant clinical benefit in phase II trials (62), CRCs with *CHFR* promoter methylation might benefit from taxanes.

In summary, although our study failed to demonstrate a consistent prognostic effect of CIMP, we identified promoter CpG island methylation of *CHFR* as a prognostic biomarker in stage II, MSS, *BRAF* WT CRCs in two independent populations.

These data indicate that the evaluation of novel CRC biomarkers requires subgroup analysis reflecting the under-

lying biology and warrant large-scale validation and clinical trials designed to evaluate the value of *CHFR* promoter CpG island methylation as a prognostic marker in stage II, microsatellite stable, *BRAF* WT CRC.

Disclosure of Potential Conflicts of Interest

L. Van Neste is employed as scientific director in MDxHealth. M. van Engeland has commercial research grant from MDxHealth. No potential conflicts of interest were disclosed by the other authors.

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