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

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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 exons 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...
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.

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.
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 homolog1, 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 metallopeptidase domain 23 (ADAM23), Rab32, member RAS oncogene family (RAB32), junctophilin (JPH3), forhead box L2 (FOXL2), BCL2-adenovirus E1B 19kDA interacting protein 3 (BNIP3), neutralized homolog (Drosophila; NEURL), calcium channel, voltage dependent, a2-delta subunit 1 (CACNA2), thrombospondin 1 (THBS1), tissue factor pathway inhibitor 2 (TFPI2), and the CIMP genes calcium channel, voltage-dependent, T type, a-1G subunit (CACNA1G), insulin-like growth factor II (somatomedin
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 by number of analyzed CpG islands) was used to assess the methylation level of CpG islands in CRCs.

![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, HLF, GATA4, GATA5, CHFR, ADAM23, RAB32, JPH3, FOXL2, BPI13, 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.](https://example.com/figure2.png)
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 $\chi^2$ 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 cut off 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 study population (Table 2). Correlations between CIMP, CL1, CL2, and CL3 and MSI and $BRAF$ mutation in the study population are shown in Table 2. The Pearson $\chi^2$ test was used. The Fisher exact test was used if there were less than five objects in any category.

### Cox proportional hazard models for MSS, $BRAF$ WT, stage II CRCs

Table 3 presents the Cox proportional hazard models for MSS, $BRAF$ WT, and stage II CRCs in the study and validation populations. The abbreviations used are: H, hazard ratio = relative risk; M, methylated.

<table>
<thead>
<tr>
<th>CIMP</th>
<th>MSI</th>
<th>MSS</th>
<th>BRAF M</th>
<th>BRAF WT</th>
<th>CIMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>12/19 (63%)</td>
<td>26/145 (18%)</td>
<td>7/14 (50%)</td>
<td>32/143 (22%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$&lt;0.01$</td>
<td>$&lt;0.05$</td>
<td>$&lt;0.01$</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>3/19 (16%)</td>
<td>94/150 (63%)</td>
<td>5/14 (36%)</td>
<td>83/147 (57%)</td>
<td>6/39 (15%)</td>
<td></td>
</tr>
<tr>
<td>4/19 (21%)</td>
<td>38/150 (25%)</td>
<td>3/14 (21%)</td>
<td>39/147 (27%)</td>
<td>8/39 (21%)</td>
<td></td>
</tr>
<tr>
<td>12/19 (63%)</td>
<td>18/150 (12%)</td>
<td>6/14 (43%)</td>
<td>25/147 (17%)</td>
<td>25/39 (64%)</td>
<td></td>
</tr>
<tr>
<td>$&lt;0.01$</td>
<td>$0.06$</td>
<td>$&lt;0.01$</td>
<td></td>
<td></td>
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</tbody>
</table>

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

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

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

$^a$Reference group = CHFR unmethylated cases.

$^b$Reference group = male.

$^c$Reference group = right-sided colon.

$^d$Reference 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% (TPP12; 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 instability (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).](www.aacrjournals.org)
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 confirmed an association with prognosis only for 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 (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 a 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 underlying 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
I. 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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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.H.G. Cleven, K.M. Smits, L. Van Neste, V. Joost, M.P. Weisenberg, J.G. Herman, A.P. de Bruine, M. van Engeland
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.H.G. Cleven, V. Joost
Study supervision: A.H.G. Cleven, M.P. Weisenberg, A.P. de Bruine, M. van Engeland

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