Published OnlineFirst March 2, 2010; DOI: 10.1158/1078-0432.CCR-09-2594

Imaging, Diagnosis, Prognosis

Clinical Cancer Research

The Role of the CpG Island Methylator Phenotype in Colorectal Cancer Prognosis Depends on Microsatellite Instability Screening Status

Anna M. Dahlin[1], Richard Palmqvist[1], Maria L. Henriksson[1], Maria Jacobsson[1], Vincy Eklöf[1], Jörgen Rutegård[2], Åke Öberg[2], and Bethany R. Van Guelpen[1]

Abstract

Purpose: The aim of this study was to relate the CpG island methylator phenotype (CIMP; characterized by extensive promoter hypermethylation) to cancer-specific survival in colorectal cancer, taking into consideration relevant clinicopathologic factors, such as microsatellite instability (MSI) screening status and the *BRAF* V600E mutation.

Experimental Design: Archival tumor samples from 190 patients from the Northern Sweden Health and Disease Study (NSHDS) and 414 patients from the Colorectal Cancer in Umeå Study (CRUMS), including 574 with cancer-specific survival data, were analyzed for an eight-gene CIMP panel using quantitative real-time PCR (MethyLight). MSI screening status was assessed by immunohistochemistry.

Results: CIMP-low patients had a shorter cancer-specific survival compared with CIMP-negative patients (multivariate hazard ratio in NSHDS, 2.01; 95% confidence interval, 1.20-3.37; multivariate hazard ratio in CRUMS, 1.48; 95% confidence interval, 1.00-2.22). This result was similar in subgroups based on MSI screening status and was statistically significant in microsatellite stable (MSS) tumors in NSHDS. For CIMP-high patients, a shorter cancer-specific survival compared with CIMP-negative patients was observed in the MSS subgroup. Statistical significance was lost after adjusting for the *BRAF* mutation, but the main findings were generally unaffected.

Conclusions: In this study, we found a poor prognosis in CIMP-low patients regardless of MSI screening status, and in CIMP-high patients with MSS. Although not consistently statistically significant, these results were consistent in two separate patient groups and emphasize the potential importance of CIMP and MSI status in colorectal cancer research. *Clin Cancer Res; 16(6); 1845–55. ©2010 AACR.*

Colorectal cancer (CRC) is one of the most common malignancies in western countries and has a lethal outcome in ~40% of cases. Colorectal tumorigenesis involves the accumulation of genetic and epigenetic events, which show substantial variation. Research has therefore focused on identifying and characterizing subgroups of CRC based on molecular features of the tumor, with the hope of discovering new targets for treatment and for refining the prediction of disease outcome. The CpG island methylator phenotype (CIMP) is one such feature, which might prove valuable for more accurate identification of CRC patients with a high risk of cancer-related death and who might potentially benefit from additional, and perhaps some day tailored, treatment.

Hypermethylation of cytosine- and guanine-rich stretches of DNA, called CpG islands, in the promoter region of genes causes transcriptional silencing and has been implicated in carcinogenesis (1). Frequent promoter hypermethylation defines CIMP in CRC (2), which has been associated with microsatellite instability (MSI) in sporadic tumors, the *BRAF* V600E mutation, low frequencies of *p53* mutations, proximal tumor location, poor differentiation, mucinous histology, infiltrating lymphocytes, female sex, and higher age at diagnosis (2–11). This phenotype, recently specified as CIMP-high (8, 12), or CIMP1 (13), is relatively well established and is believed to occur in approximately 15% to 20% of CRC (14, 15). In contrast, a proposed CIMP-low (8, 12), or CIMP2 (13), subgroup, with less frequent, but possibly more age-related, promoter hypermethylation and somewhat different clinicopathologic features (8, 12, 13), is more controversial and has been reported to occur in 20% to 45% of CRC (14, 15).

Although promoter hypermethylation in multiple genes has been related to a poor prognosis in CRC in some previous studies (16, 17), several reports have noted a better

Authors' Affiliations: [1]Department of Medical Biosciences, Pathology, and [2]Department of Surgical and Perioperative Sciences, Surgery, Umeå University, Umeå, Sweden

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding Author: Anna M. Dahlin, Department of Medical Biosciences, Pathology, Umeå University, Building 6M, 2nd Floor, SE-90185 Umeå, Sweden. Phone: 46-90-785-44-31; Fax: 46-90-785-44-84; E-mail: anna.dahlin@medbio.umu.se.

doi: 10.1158/1078-0432.CCR-09-2594

Published OnlineFirst March 2, 2010; DOI: 10.1158/1078-0432.CCR-09-2594

Translational Relevance

This study relates the CpG island methylator phenotype (CIMP) to cancer-specific survival in colorectal cancer (CRC) patients, taking into account relevant clinicopathologic factors such as microsatellite instability (MSI) screening status and the *BRAF* V600E mutation. The main findings, that the role of CIMP in prognosis may depend on MSI status, contribute to the growing knowledge and highlight the complexity of the field of molecular markers in CRC. The potential importance of CIMP and other novel parameters for refining patient prognosis is increasingly being recognized. There is hope that such factors may one day be used to identify patients who might benefit from specific treatments to improve response to therapy and prevent unnecessary side effects. The study thus emphasizes the potential importance of considering subgroups based on CIMP and MSI status in CRC research.

prognosis or null associations (5, 6, 18–26). A poor prognosis in CIMP-high CRC patients might result from other factors closely related to CIMP, such as the *BRAF* V600E mutation, rather than to an effect of the phenotype itself (20, 22, 26, 27). MSI is another prime candidate, given its tight association with CIMP, but MSI is predictive of a better prognosis (28). In addition, a shorter survival in patients with multiple promoter hypermethylation has frequently been attributed to the microsatellite stable (MSS) subgroup (6, 19, 20, 22, 26, 29, 30), although other studies have not found this association (27, 31). The interpretation of studies investigating CIMP in CRC survival is also complicated by the different methods and gene panels used to assess CIMP status, but taking these issues into consideration does not explain the lack of consistency in findings.

The aim of the present study was to relate gene-specific promoter hypermethylation and CIMP status to cancer-specific survival in two separate patient groups, including a total of 604 CRC patients, taking into consideration clinicopathologic factors associated with CIMP.

Materials and Methods

Study design and patient selection. The CRC cases included in the present study were from two separate patient groups. The study initially included patients from the prospective, population-based Northern Sweden Health and Disease Study (NSHDS) and was subsequently expanded to include the Colorectal Cancer in Umeå Study (CRUMS).

NSHDS comprises three cohorts: the Västerbotten Intervention Project (VIP), the Northern Sweden WHO Monitoring of Trends and Cardiovascular Disease (MONICA) Study, and the local Mammography Screening Project (MSP). The NSHDS cohort protocols and CRC case selection principles used in the present study have previously been described in detail (32). To summarize, the subjects recruited to the cohorts of NSHDS include men and women ages 40, 50, and 60 y in VIP; men and women ages 25 to 74 y in MONICA; and women ages approximately 50 to 70 y in MSP. Within these cohorts, a total of 226 CRC cases (diagnosed between 1985 and 2002) were identified and selected for a previous nested case-referent study (32). After exclusions for insufficient or unavailable tumor tissue sample, 194 patients from NSHDS, including all stages of disease, were available for methylation analysis. Thirty-two (17.9%) NSHDS patients (10 stage II and 22 stage III patients) received adjuvant chemotherapy. Preoperative radiotherapy was administered to 64.4% of the rectal cancer cases.

Sample collection in CRUMS had a consecutive intent. Inclusion criteria were primary CRC patients (all stages of disease) for whom curative or palliative tumor resective surgery was done between 1995 and 2003 at Umeå University Hospital, Sweden. Exclusion criteria were insufficient or unavailable tumor tissue sample, insufficient clinical information, and simultaneous inclusion in NSHDS, leaving 414 eligible tumors for methylation analysis. Fifty-one (12.5%) CRUMS patients (2 stage I, 15 stage II, and 34 stage III patients) received adjuvant chemotherapy. Preoperative radiotherapy was administered to 56.2% of the rectal cancer cases.

In both NSHDS and CRUMS, tumor characteristics were extracted from pathology reports and patient records, and all diagnoses were reassessed and verified by a pathologist (R.P.). Patients were followed up until January 2008 in NSHDS and spring 2005 in CRUMS. Vital status and cancer-specific survival were obtained from the Swedish population registry and patient records, respectively.

DNA extraction and* BRAF *V600E genotyping. Tumor tissue resected at surgery was formalin fixed and paraffin embedded by routine methods, and all samples were stored under comparable conditions. All analyses described below were done at a single laboratory. DNA was extracted and purified using the Nucleon kit (GE Healthcare) according to the manufacturer's instructions. DNA for the CIMP analyses was extracted from tumor tissue samples, which, when necessary, were macrodissected to increase the proportion of tumor cells.

The Taqman allelic discrimination assay used for the detection of *BRAF* V600E mutation (reagents from Applied Biosystems) has been described in detail elsewhere (33).

Bisulfite modification and methylation analysis. All bisulfite modification and methylation analyses were done at a single laboratory. Purified DNA (0.5 μg) was bisulfite treated and purified using the EZ DNA methylation kit (Zymo Research) according to the manufacturer's instructions and eluted in 30 μL elution buffer. Bisulfite-treated DNA was further diluted 1:10 before use in MethyLight reactions. For samples with an unsuccessful MethyLight analysis, a lesser dilution was initially tested, and if necessary, the entire protocol was rerun with a new 1.0 μg sample of DNA.

Published OnlineFirst March 2, 2010; DOI: 10.1158/1078-0432.CCR-09-2594

The MethyLight method (quantitative real-time PCR) and primer and probe sequences used in this study have previously been described in detail (34) and have been shown to do well in analyses of paraffin-embedded tissue samples (35). For all DNA samples, one reaction was run for each of the eight genes included in the CIMP panel (*CDKN2A*, *MLH1*, *CACNA1G*, *NEUROG1*, *RUNX3*, *SOCS1*, *IGF2*, and *CRABP1*). To account for the amount of input bisulfite-treated DNA, one reaction amplifying the repetitive *ALU* sequence was also run for each DNA sample. Human Genomic DNA (Promega) was methylated by treatment with CpG methyltransferase (M. *Sss*I; New England Biolabs), as previously described (34), and bisulfite modified using the EZ DNA methylation kit according to the manufacturer's instructions. This DNA served as a methylated reference in control reactions done to account for the efficiency of DNA amplification and for use in standard curve reactions. C_t values were converted to quantity using the standard curve, and the percent of methylated reference value was calculated for each gene by the following equation: (quantity of the gene-specific reaction of the sample/quantity of the *ALU* reaction of the sample)/(mean quantity of the gene-specific reaction for the methylated reference sample/mean quantity of the *ALU* reaction for the methylated reference sample) (34).

Samples were considered positive for methylation when an exponential amplification curve was present and generated a percent of methylated reference of >10 (34). Four NSHDS samples and no CRUMS samples with C_t for *ALU* >22 were considered uninformative and thus excluded from further analyses in this study. The *ALU* probe was purchased from Applied Biosystems. All other probes were from Biosearch Technology or Thermo Electron. Primers were purchased from Thermo Electron or DNA Technology A/S. Real-time PCRs were done in a HT7900 unit (Applied Biosystems), and the results were analyzed with the SDS 2.1 software (Applied Biosystems).

Immunohistochemistry and MSI screening status. Immunohistochemical analyses were done using standard procedures. Primary monoclonal p53 (Ab-6, dilution 1:400, Oncogene Research Products in NSHDS; Ab-6, 1:200, Calbiochem in CRUMS), MLH1 (clone G168-15, 1:50, BD Biosciences), MSH2 (clone FE11, 1:50, Oncogene Research Products), MSH6 (clone 44, 1:50, BD Biosciences), and PMS2 (clone A16-4, 1:25, BD Biosciences) were used. Antigen retrieval treatment was executed in microwave oven in EDTA (pH 8.0; for MLH1, MSH2, MSH6, and PMS2) or citrate (pH 6.0; p53 in NSHDS) or in pressure cooker (2100 retriever, Biocare Medical) in Diva Decloaker (for p53 in CRUMS; Biocare Medical) and using a semiautomatic staining machine (intelliPATH FLX, Biocare Medical for p53 in CRUMS; Ventana ES, Ventana, Inc. for all other antibodies). For each antibody, samples were evaluated for nuclear staining, which was done at a single laboratory. Cases without internal positive control staining, such as lymphocytes, were considered uninformative. Cases were considered to have overexpression of p53 if ≥25% of tumor cells showed nuclear staining for p53 protein (36). Abnormal expression of p53 detected by immunohistochemistry is closely related to mutational status of the gene (36) but does not reflect the functional status of the p53 protein product. Tissue samples with tumor cells lacking nuclear staining for MLH1, MSH2, MSH6, or PMS2 were considered to have a positive MSI screening status, hereafter referred to as MSI. Negative MSI screening status based on the immunohistochemical staining is hereafter referred to as MSS.

Statistics. Clinicopathologic characteristics in subgroups were compared using Kruskal-Wallis tests for continuous variables and χ^2 tests, or Fisher's exact tests when observed or expected frequencies were less than five, for categorical variables. For cancer-specific survival analyses, Kaplan-Meier plots were used, and differences between groups were tested by log-rank tests. Cancer-specific events were defined as death with known disseminated or recurrent disease, and cases were censored at the end of follow-up or at time of death by other causes. Patients in CRUMS who died with postoperative complications within 1 mo after surgery ($n = 16$) were excluded from the survival analyses. Deaths due to postoperative complications were not recorded in NSHDS, but only four patients died within 1 mo of surgery. To take into consideration other clinicopathologic factors, multivariate Cox proportional hazard models were used. Factors that were associated to cancer-specific survival ($P < 0.10$), and affected hazard ratios (HR) for CIMP status by >10% in bivariate analyses, were included in the multivariate analyses. The final multivariate model included sex, age at diagnosis, tumor location, tumor stage, and adjuvant chemotherapy. These covariates were also included in multivariate analyses for single gene promoter hypermethylation, MSI screening status, and the *BRAF* V600E mutation. Other factors tested, but not meeting the criteria for inclusion in the multivariate analyses, were aberrant p53 protein expression and mucinous histologic type. Preoperative radiotherapy met the criteria for inclusion in the multivariate model for rectal cancer cases, but subgroup analyses for rectal cancer alone were not presented (for reasons of power). For CRC, preoperative radiotherapy was not included in the multivariate model because it was not a relevant potential confounder for the majority of cases. Twelve cases in CRUMS with missing values for MSI screening status were treated as a separate category in multivariate cancer-specific survival analyses. For variables with <12 missing values (tumor site, *BRAF* mutation, and adjuvant chemotherapy), patients with missing values were excluded from these analyses. All statistical tests were conducted using SPSS 14.0. Findings were considered statistically significant if $P < 0.05$.

Results

Clinical and molecular characteristics. CIMP status was successfully analyzed in 604 CRC tissue samples from two separate patient groups, including 190 patients from NSHDS and 414 patients from CRUMS. Tumors were classified into six subgroups based on a combination of CIMP

Published OnlineFirst March 2, 2010; DOI: 10.1158/1078-0432.CCR-09-2594

Table 1. Clinical and molecular characteristics of CRC cases according to combined CIMP and MSI screening status

A. NSHDS cases

	Total*	MSS†			P‡	MSI†			P‡
		CIMP-negative§	CIMP-low§	CIMP-high§		CIMP-negative§	CIMP-low§	CIMP-high§	
Frequency (%)	190	91 (47.9)	61 (32.1)	14 (7.4)		4 (2.1)	7 (3.7)	13 (6.8)	
Age at diagnosis (y)‖	63 (58-67)	63 (57-67)	63 (59-66)	65 (63-68)	0.219	58 (57-60)	68 (59-69)	63 (61-68)	0.105
Sex, *n* (%)									
Men	82 (43.2)	42 (46.2)	26 (42.6)	5 (35.7)		4 (100.0)	4 (57.1)	1 (7.7)	
Women	108 (56.8)	49 (53.8)	35 (57.4)	9 (64.3)	0.738	0 (0.0)	3 (42.9)	12 (92.3)	<0.001
Tumor site, *n* (%)									
Right-sided colon	61 (32.1)	15 (16.5)	19 (31.1)	8 (57.1)		2 (50.0)	4 (57.1)	13 (100.0)	
Left-sided colon	56 (29.5)	29 (31.9)	22 (36.1)	2 (14.3)		1 (25.0)	2 (28.6)	0 (0.0)	
Rectum	73 (38.4)	47 (51.6)	20 (32.8)	4 (28.6)	0.007	1 (25.0)	1 (14.3)	0 (0.0)	0.023
Stage, *n* (%)									
I	33 (17.4)	23 (25.3)	6 (9.8)	2 (14.3)		0 (0.0)	1 (14.3)	1 (7.7)	
II	68 (35.8)	29 (31.9)	21 (34.4)	3 (21.4)		3 (75.0)	3 (42.9)	9 (69.2)	
III	46 (24.2)	25 (27.5)	16 (26.2)	1 (7.1)		1 (25.0)	0 (0.0)	3 (23.1)	
IV	43 (22.6)	14 (15.4)	18 (29.5)	8 (57.1)	0.010	0 (0.0)	3 (42.9)	0 (0.0)	0.108
Tumor grade									
Well to moderately differentiated	—	—	—	—		—	—	—	
Moderately to poorly differentiated	—	—	—	—	—	—	—	—	—
Histology type, *n* (%)									
Nonmucinous	151 (79.9)	83 (91.2)	46 (76.7)	10 (71.4)		3 (75.0)	2 (28.6)	7 (53.8)	
Mucinous	38 (20.1)	8 (8.8)	14 (23.3)	4 (28.6)	0.014	1 (25.0)	5 (71.4)	6 (46.2)	0.303
BRAF V600E, *n* (%)									
Wild-type	154 (81.5)	87 (96.7)	50 (82.0)	4 (28.6)		4 (100.0)	5 (71.4)	4 (30.8)	
Mutated	35 (18.5)	3 (3.3)	11 (18.0)	10 (71.4)	<0.001	0 (0.0)	2 (28.6)	9 (69.2)	0.032
p53 screening status¶									
Normal	75 (40.1)	33 (36.7)	20 (33.3)	7 (50.0)		4 (100.0)	2 (28.6)	9 (75.0)	
Aberrant	112 (59.9)	57 (63.3)	40 (66.7)	7 (50.0)	0.507	0 (0.0)	5 (71.4)	3 (25.0)	0.040
Adjuvant chemotherapy									
No	147 (82.1)	72 (82.8)	48 (82.8)	12 (85.7)		3 (100.0)	5 (71.4)	7 (70.0)	
Yes	32 (17.9)	15 (17.2)	10 (17.2)	2 (14.3)	1.000	0 (0.0)	2 (28.6)	3 (30.0)	0.817
Preoperative radiotherapy**									
No	26 (35.6)	16 (34.0)	9 (45.0)	1 (25.0)		0 (0.0)	0 (0.0)	0 (0.0)	
Yes	47 (64.4)	31 (66.0)	11 (55.0)	3 (75.0)	0.583	1 (100.0)	1 (100.0)	0 (0.0)	—

B. CRUMS cases

	Total††	MSS†			P‡	MSI†			P‡
		CIMP-negative§	CIMP-low§	CIMP-high§		CIMP-negative§	CIMP-low§	CIMP-high§	
Frequency (%)	414	194 (48.5)	132 (33.0)	12 (3.0)		12 (3.0)	15 (3.8)	35 (8.8)	
Age at diagnosis (y)‖	73 (65-79)	72 (64-79)	73 (66-81)	74 (69-78)	0.644	72 (61-77)	75 (61-83)	73 (69-77)	0.728
Sex, *n* (%)									
Men	233 (56.3)	113 (58.2)	79 (59.8)	3 (25.0)		5 (41.7)	7 (46.7)	19 (54.3)	
Women	181 (43.7)	81 (41.8)	53 (40.2)	9 (75.0)	0.067	7 (58.3)	8 (53.3)	16 (45.7)	0.720

(*Continued on the following page*)

Published OnlineFirst March 2, 2010; DOI: 10.1158/1078-0432.CCR-09-2594

Table 1. Clinical and molecular characteristics of CRC cases according to combined CIMP and MSI screening status (Cont'd)

B. CRUMS cases

	Total††	MSS†			P‡	MSI†			P‡
		CIMP-negative§	CIMP-low§	CIMP-high§		CIMP-negative§	CIMP-low§	CIMP-high§	
Tumor site, *n* (%)									
Right-sided colon	132 (32.0)	26 (13.5)	46 (34.8)	9 (75.0)			4 (33.3)	10 (66.7)	32 (91.4)
Left-sided colon	127 (30.8)	79 (41.1)	37 (28.0)	2 (16.7)		2 (16.7)	3 (20.0)	2 (5.7)	
Rectum	153 (37.1)	87 (45.3)	49 (37.1)	1 (8.3)	<0.001	6 (50.0)	2 (13.3)	1 (2.9)	<0.001
Stage, *n* (%)									
I	63 (15.5)	38 (19.8)	15 (11.7)	0 (0.0)		1 (8.3)	2 (13.3)	4 (11.4)	
II	163 (40.0)	72 (37.5)	52 (40.6)	2 (16.7)		6 (50.0)	6 (40.0)	20 (57.1)	
III	88 (21.6)	38 (19.8)	30 (23.4)	5 (41.7)		2 (16.7)	5 (33.3)	6 (17.1)	
IV	93 (22.9)	44 (22.9)	31 (24.2)	5 (41.7)	0.083	3 (25.0)	2 (13.3)	5 (14.3)	0.825
Tumor grade									
Well to moderately differentiated	203 (49.6)	102 (53.1)	56 (43.4)	7 (58.3)		10 (83.3)	6 (40.0)	14 (40.0)	
Moderately to poorly differentiated	206 (50.4)	90 (46.9)	73 (56.6)	5 (41.7)	0.192	2 (16.7)	9 (60.0)	21 (60.0)	0.024
Histology type, *n* (%)									
Nonmucinous	347 (85.0)	175 (91.1)	111 (86.0)	8 (66.7)		8 (72.7)	12 (80.0)	22 (62.9)	
Mucinous	61 (15.0)	17 (8.9)	18 (14.0)	4 (33.3)	0.027	3 (27.3)	3 (20.0)	13 (37.1)	0.486
BRAF V600E, *n* (%)									
Wild-type	356 (86.6)	190 (98.4)	123 (93.9)	4 (33.3)		12 (100.0)	12 (80.0)	3 (8.8)	
Mutated	55 (13.4)	3 (1.6)	8 (6.1)	8 (66.7)	<0.001	0 (0.0)	3 (20.0)	31 (91.2)	<0.001
p53 screening status¶									
Normal	161 (39.7)	68 (35.6)	44 (34.1)	7 (58.3)		6 (50.0)	6 (40.0)	22 (66.7)	
Aberrant	245 (60.3)	123 (64.4)	85 (65.9)	5 (41.7)	0.252	6 (50.0)	9 (60.0)	11 (33.3)	0.196
Adjuvant chemotherapy									
No	358 (87.5)	172 (88.7)	113 (88.3)	10 (83.3)		10 (83.3)	11 (73.3)	30 (85.7)	
Yes	51 (12.5)	22 (11.3)	15 (11.7)	2 (16.7)	0.775	2 (16.7)	4 (26.7)	5 (14.3)	0.544
Preoperative radiotherapy**									
No	67 (43.8)	35 (40.2)	23 (46.9)	0 (0.0)		2 (33.3)	1 (50.0)	0 (0.0)	
Yes	86 (56.2)	52 (59.8)	26 (53.1)	1 (100.0)	0.697	4 (66.7)	1 (50.0)	1 (100.0)	1.000

*The following numbers of missing cases were present in NSHDS: tumor grade, all; mucinous histologic type, 1; *BRAF* mutation status, 1; p53 screening status, 3; adjuvant chemotherapy, 11.

†Cases lacking nuclear staining of tumor cells for at least one of MLH1, MSH2, MSH6, or PMS2 were considered to have a positive MSI screening status.

‡Kruskall-Wallis test for continuous variables and χ^2 test or Fisher's exact test for categorical variables.

§CIMP-negative, 0 genes hypermethylated; CIMP-low, 1 to 5 genes hypermethylated; CIMP-high, 6 to 8 genes hypermethylated.

‖Median (25th-75th percentile).

¶Cases were considered to have overexpression of p53 if ≥25% of tumor cells (by semiquantitative assessment) showed nuclear staining for p53 protein.

**Only rectal cancers were considered. The percentages presented therefore reflect the proportion of rectal cancer cases that received preoperative radiotherapy.

††The following numbers of missing cases were present in CRUMS: MSI screening status, 14; tumor site, 2; tumor stage, 7; tumor grade, 5; mucinous histologic type, 6; *BRAF* mutation status, 3; p53 screening status, 8; adjuvant chemotherapy, 5.

status (CIMP-negative, CIMP-low, or CIMP-high; based on the CIMP panel including *CDKN2A*, *MLH1*, *CACNA1G*, *NEUROG1*, *RUNX3*, *SOCS1*, *IGF2*, and *CRABP1*) and MSI screening status (MSS or MSI; based on immunohistochemistry). The frequencies of tumors in each subgroup, and their clinical and molecular characteristics, are presented separately for NSHDS and CRUMS in Table 1A and B, respectively. In both patient groups, CIMP-high

Published OnlineFirst March 2, 2010; DOI: 10.1158/1078-0432.CCR-09-2594

tumors were associated with MSI, a proximal tumor site, a mucinous histologic type, the *BRAF* V600E mutation, and normal expression of p53 (data not shown). For proximal location and the *BRAF* V600E mutation, this was true regardless of MSI screening status. In CRUMS, both MSI and MSS CIMP-high tumors were also more often of mucinous histologic type, and more often had a normal expression of p53, whereas a different pattern was seen in NSHDS. In NSHDS, CIMP-high patients were more often females, especially in combination with MSI. Stage IV seemed to be overrepresented in MSS CIMP-high tumors in both patient groups, although the result was only statistically significant in NSHDS.

Due to the recruitment protocol of NSHDS, the patients were younger in NSHDS (median age, 63; range, 41-74 years) than in CRUMS (median age, 73; range, 26-96 years; $P < 0.001$). Women were also overrepresented in NSHDS due to the inclusion of the all-female MSP cohort (56.8% and 43.7% in NSHDS and CRUMS, respectively; $P = 0.003$).

CIMP status and cancer-specific survival. In the 574 CRC patients with complete cancer-specific survival data (190 from NSHDS and 384 from CRUMS), patients with CIMP-low had a poorer prognosis compared with CIMP-negative in NSHDS (log-rank $P = 0.008$; Fig. 1A). This finding was consistent but not statistically significant in CRUMS patients (Fig. 1B). For CIMP-high, Kaplan-Meier curves showed a nonsignificant decreased cancer-specific survival compared with CIMP-negative patients in both NSHDS and CRUMS (Fig. 1A and B). In multivariate

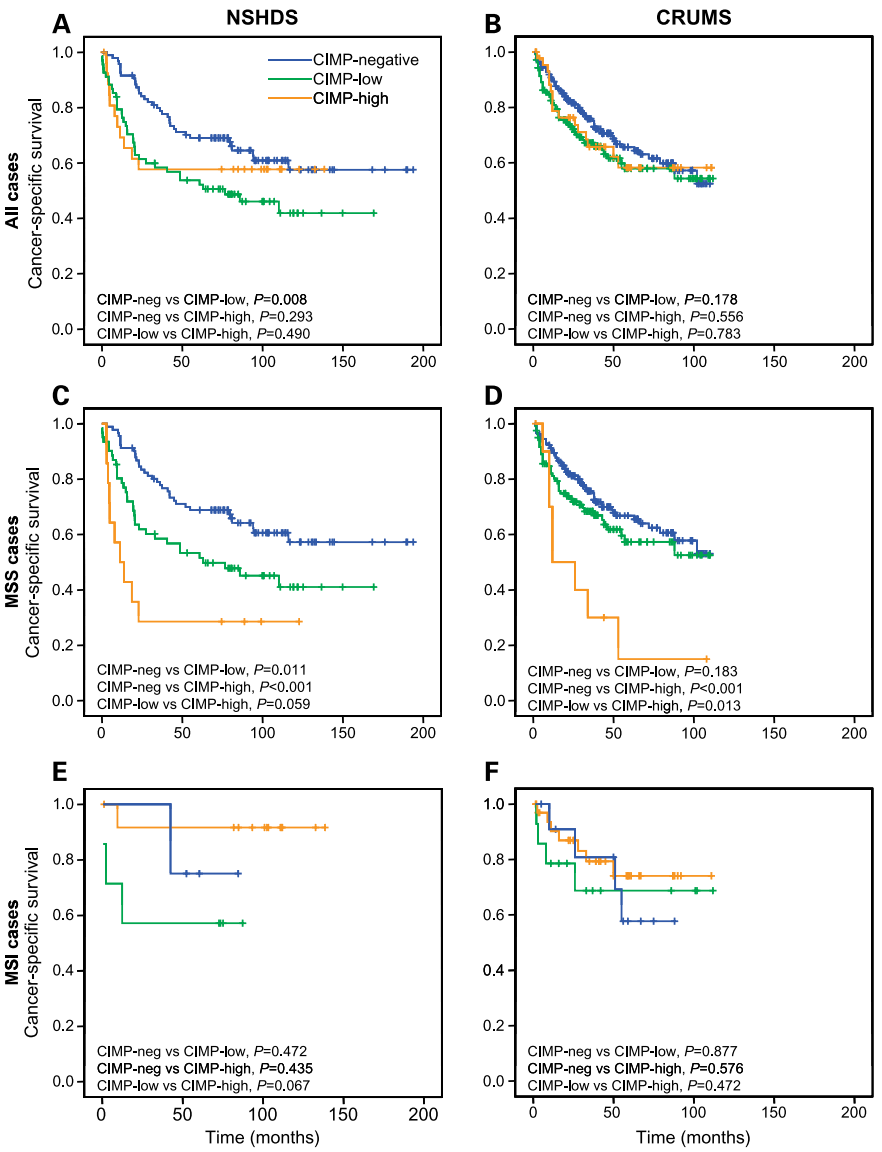

Fig. 1. Cumulative cancer-specific survival of CRC cases (stages I-IV) in two separate patient groups: the population-based NSHDS and CRUMS. Kaplan-Meier plots by the CIMP subgroups (CIMP-negative, CIMP-low, and CIMP-high) are shown for all cases in NSHDS (A) and CRUMS (B), as well as separately for each patient group according to MSI screening status of the tumors: MSS (C and D) and MSI (E and F). Log-rank tests were used to calculate *P* values for differences between groups.

Published OnlineFirst March 2, 2010; DOI: 10.1158/1078-0432.CCR-09-2594

Table 2. HRs and 95% CIs for cancer-specific death in CRC subgroups based on CIMP and MSI screening status

	NSHDS			CRUMS		
	n (%)*	HR (95% CI)		*n* (%)*	HR (95% CI)	
		Univariate	Multivariate†		Univariate	Multivariate†
All cases						
CIMP-negative‡	95 (50.0)	1	1	198 (51.6)	1	1
CIMP-low‡	68 (35.8)	1.83 (1.15-2.92)	2.01 (1.20-3.37)	139 (36.2)	1.29 (0.88-1.88)	1.48 (1.00-2.22)
CIMP-high‡	27 (14.2)	1.41 (0.71-2.79)	1.84 (0.87-3.89)	47 (12.2)	1.17 (0.68-2.04)	1.10 (0.59-2.03)
MSS§						
CIMP-negative‡	91 (54.8)	1	1	182 (58.3)	1	1
CIMP-low‡	61 (36.7)	1.83 (1.13-2.96)	1.89 (1.12-3.21)	119 (38.1)	1.31 (0.87-1.96)	1.45 (0.95-2.23)
CIMP-high‡	14 (8.4)	3.80 (1.87-7.74)	3.05 (1.40-6.63)	11 (3.5)	3.35 (1.60-7.06)	1.38 (0.62-3.07)
MSI§						
CIMP-negative‡	4 (16.7)	1	‖	12 (20.0)	1	1
CIMP-low‡	7 (29.2)	2.27 (0.23-22.01)	‖	14 (23.3)	1.10 (0.27-4.46)	3.87 (0.46-32.39)
CIMP-high‡	13 (54.2)	0.35 (0.02-5.46)	‖	34 (56.7)	0.70 (0.20-2.39)	1.23 (0.13-11.23)

*Number of cases included in the survival analyses.

†Adjusted for sex, age at diagnosis, tumor location, tumor stage, and adjuvant chemotherapy. p53 screening status and mucinous histology were also tested but did not meet the criteria for inclusion.

‡CIMP-negative, 0 genes hypermethylated; CIMP-low, 1 to 5 genes hypermethylated; CIMP-high, 6 to 8 genes hypermethylated.

§Cases lacking nuclear staining of tumor cells for at least one of MLH1, MSH2, MSH6, or PMS2 were considered to have a positive MSI screening status.

‖HRs are not shown for multivariate analyses of NSHDS cases with a positive MSI screening status because the low number of cases in each subgroup produced noninterpretable results.

analysis, the HRs for CIMP-low remained statistically significant in NSHDS [HR, 2.01; 95% confidence interval (95% CI), 1.20-3.37] and were of borderline significance in CRUMS (HR, 1.48; 95% CI, 1.00-2.22), whereas the results for CIMP-high were essentially unaltered compared with univariate analyses (Table 2).

The *BRAF* V600E mutation was associated with a poor prognosis compared with wild-type in NSHDS (multivariate HR, 2.51; 95% CI, 1.42-4.45) but not in CRUMS (multivariate HR, 1.03; 95% CI, 0.59-1.80). Adding *BRAF* mutation status to the multivariate model in Table 2 attenuated the HRs for CIMP-low (HR, 1.67; 95% CI, 0.96-2.93) and CIMP-high in NSHDS (HR, 1.08; 95% CI, 0.43-2.70), whereas the results in CRUMS were largely unchanged (data not shown).

A nonstatistically significant trend of a longer cancer-specific survival was seen for patients with MSI versus MSS tumors in both patient groups (multivariate HR for NSHDS, 0.53; 95% CI, 0.17-1.58; multivariate HR for CRUMS, 0.84; 95% CI, 0.47-1.49). Adding MSI screening status to the multivariate model in Table 2 increased the HR for CIMP-high in NSHDS (HR, 2.34; 95% CI, 1.08-5.09), whereas the results for CIMP-high in CRUMS and for CIMP-low in both patient groups were largely unchanged (data not shown).

CIMP status and cancer-specific survival according to MSI screening status. The observation of a shorter cancer-specific survival in CIMP-low versus CIMP-negative patients was largely consistent in subgroups based on MSI screening status and statistically significant in the MSS subgroup of NSHDS (Fig. 1C-F). CIMP-high patients also had a shorter cancer-specific survival compared with CIMP-negative in the MSS subgroup (Fig. 1C and D). This finding was statistically significant in both patient groups. Although the magnitude and statistical significance of the associations were weakened in the multivariate analyses, particularly in CRUMS, the direction of the HRs was unchanged (Table 2). In contrast to MSS, Kaplan-Meier plots showed a trend of a better prognosis for CIMP-high patients in the MSI subgroup (Fig. 1E and F). However, this finding was not statistically significant, and the direction of the HRs was not consistent in multivariate models (Table 2).

Further adjusting the multivariate model for the *BRAF* V600E mutation did not affect the direction of HRs for subgroups based on MSI screening status, although the statistical significance of results for MSS in NSHDS was attenuated to borderline significance for CIMP-low (HR, 1.64; 95% CI, 0.94-2.89) and lost for CIMP-high (HR, 1.97; 95% CI, 0.76-5.11).

The main findings of a reduced cancer-specific survival in CIMP-low CRC patients, and in CIMP-high patients with MSS, were generally consistent in analyses based solely on colon cancer cases (i.e., excluding rectal cancer; data not

Published OnlineFirst March 2, 2010; DOI: 10.1158/1078-0432.CCR-09-2594

Table 3. HRs and 95% CIs for cancer-specific death in CRC patients, for genes with versus without promoter hypermethylation

	NSHDS			CRUMS		
	n (%)*	HR (95% CI)		*n* (%)*	HR (95% CI)	
	Methylated†/ unmethylated	Univariate	Multivariate‡	Methylated†/ unmethylated	Univariate	Multivariate‡
CDKN2A	49 (25.8)/141	1.25 (0.76-2.04)	1.21 (0.72-2.03)	109 (28.4)/275	1.67 (1.16-2.42)	1.48 (1.00-2.19)
MLH1	18 (9.5)/172	0.46 (0.16-1.24)	0.65 (0.21-1.90)	42 (10.9)/342	0.45 (0.21-0.97)	0.53 (0.23-1.19)
CACNA1G	35 (18.4)/155	1.46 (0.85-2.51)	1.54 (0.85-2.77)	63 (16.4)/321	0.90 (0.54-1.46)	0.77 (0.44-1.32)
NEUROG1	61 (32.1)/129	1.37 (0.87-2.16)	1.59 (0.95-2.65)	109 (28.4)/275	1.33 (0.91-1.94)	1.30 (0.86-1.96)
RUNX3	43 (22.6)/147	1.53 (0.93-2.53)	1.65 (0.93-2.95)	76 (19.8)/308	1.09 (0.71-1.69)	0.94 (0.57-1.52)
SOCS1	25 (13.2)/165	1.23 (0.65-2.34)	1.24 (0.62-2.48)	49 (12.8)/335	1.32 (0.81-2.13)	1.35 (0.80-2.29)
IGF2	44 (23.2)/146	1.72 (1.05-2.81)	2.13 (1.19-3.82)	69 (18.0)/315	1.23 (0.79-1.92)	0.93 (0.57-1.51)
CRABP1	59 (31.1)/131	1.69 (1.08-2.67)	1.61 (0.97-2.69)	108 (28.1)/276	1.19 (0.81-1.75)	1.21 (0.79-1.84)

*Number of cases included in the survival analyses.
†Percent of methylated reference >10.
‡Adjusted for sex, age at diagnosis, tumor location, tumor stage, and adjuvant chemotherapy.

shown). In analyses restricted to patients who underwent surgery with a curative intent, results for CIMP-low were attenuated, whereas the results for CIMP-high patients with MSS were largely unchanged (Supplementary Fig. S1).

Gene-specific promoter hypermethylation and cancer-specific survival. Frequencies of promoter hypermethylation for the genes included in the CIMP panel are presented in Table 3 for patients with survival data available. In NSHDS and CRUMS, respectively, the frequencies of hypermethylation ranged from 9.5% and 10.9% (for *MLH1*) to 32.1 and 28.4% (for *NEUROG1*).

A statistically significant reduction in cancer-specific survival was seen in NSHDS cases with hypermethylation of *IGF2* (multivariate HR, 2.13; 95% CI, 1.19-3.82) and in CRUMS cases with hypermethylation of *CDKN2A* (multivariate HR, 1.48; 95% CI, 1.00-2.19). Hypermethylation of all other genes, with the exception of *MLH1*, was associated with a nonstatistically significant reduced cancer-specific survival in NSHDS, whereas the results in CRUMS were inconsistent (Table 3).

For promoter hypermethylation of *MLH1*, HRs were <1 in both patient groups, but statistical significance was lost in multivariate analyses (Table 3). The majority of tumors with *MLH1* hypermethylation were MSI (83.3% and 85.0% in NSHDS and CRUMS, respectively), which is a marker for better prognosis (28). Adjusting for MSI screening status attenuated the HR in NSHDS to null, whereas the HR in CRUMS was essentially unchanged (data not shown).

Discussion

In this study of archival CRC tissue samples, we analyzed the role of CIMP status in cancer-specific survival in 190 patients from a population-based cohort in northern Sweden and subsequently confirmed our findings, although not consistently statistically significantly, in a separate sample of 384 CRC patients from the university hospital in the same region. The main finding was a shorter cancer-specific survival in CIMP-low compared with CIMP-negative patients, regardless of MSI screening status, whereas for CIMP-high, a shorter cancer-specific survival was observed only in the subgroup of patients with MSS.

Observations of a reduced cancer-specific survival in CIMP-high or CIMP-low CRC patients may be confined to the subgroup showing MSS (6, 19, 20, 22, 26, 30). Our finding of a particularly poor prognosis in CIMP-high patients with MSS, as assessed by immunohistochemistry, supports this idea. Although this subgroup was small, the result was consistent in both patient groups and was largely independent of potential confounding factors. Some studies, however, have reported contradictive findings (20, 24, 27, 31).

We also observed a shorter cancer-specific survival among CIMP-low versus CIMP-negative patients that was independent of MSI screening status. Although not always statistically significant, this trend was apparent in both multivariate and subgroup analyses. In a previous study, patients with intermediate numbers of genes methylated also seemed to have a poorer prognosis in both the MSI and MSS subgroups (19). CIMP-low has also been found to be associated with a poorer cancer-specific survival in MSS but not in MSI patients (30), whereas another recent study reported similar findings in MSS patients but did not describe the survival of CIMP-low in MSI patients (26). Although present among the MSS subgroup in this study, a stepwise decrease in cancer-specific survival from CIMP-negative to CIMP-low to CIMP-high might

Published OnlineFirst March 2, 2010; DOI: 10.1158/1078-0432.CCR-09-2594

not necessarily be expected. The CIMP-low subgroup has been reported to be distinct from both CIMP-high and CIMP-negative rather than an intermediate between the two (8, 12, 13).

A reduced cancer-specific survival in CIMP-high or CIMP-low CRC patients with MSS might be due to the large fraction of *BRAF*-mutated tumors in this group (20, 22, 26, 27). Our results, as well as other recent findings (30), do not support such an effect. Adjusting for the *BRAF* V600E mutation altered the magnitude and statistical significance, but not the direction, of the multivariate risk estimates for CIMP-low and CIMP-high in the present study.

In the present study, and in several previous studies (6, 19, 20, 24, 26, 30, 37), CIMP-high tumors with MSI seemed to have a good prognosis. However, this subgroup tends to be small, and statistically significant results have rarely been reported, and contradictive results have recently been reported (25).

Discrepancies in results in studies of the role of CIMP in CRC prognosis may depend on methodologic issues. Several different laboratory methods, gene panels, and definitions for CIMP have been used over the years. Differences in background populations and thus cohort compositions might also explain some of the variation in results. In the present study, identical protocols were used in two nonoverlapping patient groups, with similar, although not identical, results.

Promoter hypermethylation in single genes, with the exception of *MLH1*, was generally nonsignificantly associated with a shorter cancer-specific survival, which is in line with the mixed results previously reported for these genes (17, 19, 29, 30, 37, 38). We found a longer cancer-specific survival in patients with *MLH1* hypermethylation, which was not statistically significant in multivariate analyses. Previous findings for *MLH1* have tended to be mixed (17, 19, 29, 37, 39, 40), with some results similar to ours (37, 39).

A main strength of this study was the two large, nonoverlapping, patient groups, which were from the same northern Swedish population but had different recruitment protocols, age ranges, and sex distributions. Other strengths included the validated eight-gene CIMP panel analyzed (11, 34) and the quantitative real-time PCR (MethyLight) methodology used, which has been developed and validated for use on archival tissue samples and which minimizes the detection of lower degrees of hypermethylation that might have little biological importance for gene expression (35). In addition, all analyses, including DNA extraction, bisulfate treatment, and MethyLight, were done at a single laboratory. The frequencies of CIMP-high and CIMP-low were also similar to those of a previous study, in which MethyLight was used to analyze the same eight-gene panel in a large, population-based patient group (11). However, studies of colon cancer exclusively, and studies using different gene panels or methodology, have generally noted somewhat higher CIMP-high frequencies (6, 9, 12, 18, 24, 30, 41).

Despite our relatively large sample size of 574 CRC patients with both CIMP and cancer-specific survival data (making this study among the larger studies to date), much larger numbers of patients would be needed for detailed subgroup analyses. For example, analyses within each tumor stage could help to define which patients with lower-stage tumors are likely to recur and might therefore benefit from adjuvant chemotherapy. However, the challenge of accumulating such a large cohort, and doing methylation analyses on all samples, will likely limit such investigations to future meta-analyses or pooled analyses.

The similarity of results in the two patient groups reduces the likelihood of chance findings. Statistical significance was, however, not reached for the larger patient group, which may depend on differences in the composition of the two groups. For example, the frequencies of MSI and female sex among CIMP-high tumors were different in the two patient groups, but we did not have sufficient power to evaluate this matter further.

The patients in the present study were generally diagnosed before the broad introduction of many novel therapies, including successful resection of liver metastases, into clinical practice. Treatment was thus fairly homogeneous within each tumor site and stage. Furthermore, the administration of adjuvant chemotherapy did not differ between CIMP subgroups, and adjusting for adjuvant chemotherapy in the multivariate analyses had only small effects on results. Residual confounding due to differences in treatment is therefore unlikely. Patients whose surgical treatment did not have a curative intent were included in the cancer-specific survival analyses. The CIMP-high MSS subgroup was overrepresented among these patients, who were virtually all stage IV and thus had a very poor prognosis. Stratifying the results by stage was not possible due to low power, but tumor stage was accounted for in the multivariate survival analyses. We also present results of analyses including only patients with potentially curative surgery in Supplementary Fig. S1. Although limited by low power, the main findings were generally consistent with the results for the full data set.

The patients were well characterized with respect to clinicopathologic factors, and we were thus able to take into consideration several potential confounders, including sex, age at diagnosis, tumor location, tumor stage, aberrant p53 expression, mucinous histologic type, the *BRAF* V600E mutation, MSI screening status, and adjuvant chemotherapy. However, mutations in *KRAS*, a defect of increasing interest in studies of CRC patient survival (42, 43), were not analyzed, which was a weakness of the study. Cases of hereditary nonpolyposis CRC, who are typically MSI, were not identified in the present study. However, as these together should not exceed 5% of the CRC cases studied (44), it is unlikely that their inclusion affected the main findings of the study. MSI screening status was determined by immunohistochemistry. Although MSI testing of standard markers by PCR is the gold standard for determination of MSI status, and allows

Published OnlineFirst March 2, 2010; DOI: 10.1158/1078-0432.CCR-09-2594

distinction between MSI-high and MSI-low (45), several studies have reported very high sensitivity and specificity for immunohistochemistry for the detection of MSI-high (46–48).

Despite numerous studies, the role of novel parameters in CRC, such as CIMP, MSI, and *BRAF* and *KRAS* mutations, is still unclear (49). However, their potential importance for refining the prediction of prognosis, as well as for the more accurate identification of CRC patients with a high risk of cancer-related death and who might therefore benefit from additional, and perhaps some day tailored, treatment, is increasingly being recognized. The results reported in the present study contribute to the growing knowledge, and highlight the increasing complexity, of this field.

In conclusion, in this study of CRC patients, CIMP-low was associated with a poor prognosis compared with CIMP-negative, regardless of MSI screening status, whereas for CIMP-high, the shorter cancer-specific survival was confined to the MSS subgroup. Although not consistently statistically significant, these results were similar in two separate patient groups and emphasize the potential importance of considering subgroups based on CIMP and MSI status in CRC research.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank all participants in NSHDS and CRUMS, as well as Kerstin Näslund (Department of Medical Biosciences, Umeå University) and Inger Cullman (Department of Chemistry, Umeå University) for excellent technical assistance.

Grant Support

Swedish Cancer Society (R. Palmqvist); Cancer Research Foundation in Northern Sweden (A.M. Dahlin, R. Palmqvist, M.L. Henriksson, V. Eklöf, J. Rutegård, and B.R. Van Guelpen); Swedish Research Council (R. Palmqvist); Faculty of Medicine, Umeå University, Umeå, Sweden (R. Palmqvist and J. Rutegård); J C Kempe Memorial Fund (A.M. Dahlin); and Cutting-Edge Research Grant from the County Council of Västerbotten, Sweden (R. Palmqvist).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 09/23/2009; revised 12/23/2009; accepted 01/12/2010; published OnlineFirst 03/02/2010.

References

1. Esteller M. Epigenetics in cancer. N Engl J Med 2008;358:1148–59.
2. Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP. CpG island methylator phenotype in colorectal cancer. Proc Natl Acad Sci U S A 1999;96:8681–6.
3. Ahuja N, Mohan AL, Li Q, et al. Association between CpG island methylation and microsatellite instability in colorectal cancer. Cancer Res 1997;57:3370–4.
4. Toyota M, Ohe-Toyota M, Ahuja N, Issa JP. Distinct genetic profiles in colorectal tumors with or without the CpG island methylator phenotype. Proc Natl Acad Sci U S A 2000;97:710–5.
5. Shannon BA, Iacopetta BJ. Methylation of the hMLH1, p16, and MDR1 genes in colorectal carcinoma: associations with clinicopathological features. Cancer Lett 2001;167:91–7.
6. Hawkins N, Norrie M, Cheong K, et al. CpG island methylation in sporadic colorectal cancers and its relationship to microsatellite instability. Gastroenterology 2002;122:1376–87.
7. Whitehall VL, Wynter CV, Walsh MD, et al. Morphological and molecular heterogeneity within nonmicrosatellite instability-high colorectal cancer. Cancer Res 2002;62:6011–4.
8. Kambara T, Simms LA, Whitehall VL, et al. BRAF mutation is associated with DNA methylation in serrated polyps and cancers of the colorectum. Gut 2004;53:1137–44.
9. Samowitz WS, Albertsen H, Herrick J, et al. Evaluation of a large, population-based sample supports a CpG island methylator phenotype in colon cancer. Gastroenterology 2005;129:837–45.
10. Ogino S, Odze RD, Kawasaki T, et al. Correlation of pathologic features with CpG island methylator phenotype (CIMP) by quantitative DNA methylation analysis in colorectal carcinoma. Am J Surg Pathol 2006;30:1175–83.
11. Ogino S, Kawasaki T, Kirkner GJ, Kraft P, Loda M, Fuchs CS. Evaluation of markers for CpG island methylator phenotype (CIMP) in colorectal cancer by a large population-based sample. J Mol Diagn 2007;9:305–14.
12. Ogino S, Kawasaki T, Kirkner GJ, Loda M, Fuchs CS. CpG island methylator phenotype-low (CIMP-low) in colorectal cancer: possible associations with male sex and KRAS mutations. J Mol Diagn 2006;8:582–8.
13. Shen L, Toyota M, Kondo Y, et al. Integrated genetic and epigenetic analysis identifies three different subclasses of colon cancer. Proc Natl Acad Sci U S A 2007;104:18654–9.
14. Jass JR. Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. Histopathology 2007;50:113–30.
15. Ogino S, Goel A. Molecular classification and correlates in colorectal cancer. J Mol Diagn 2008;10:13–27.
16. Van Rijnsoever M, Elsaleh H, Joseph D, McCaul K, Iacopetta B. CpG island methylator phenotype is an independent predictor of survival benefit from 5-fluorouracil in stage III colorectal cancer. Clin Cancer Res 2003;9:2898–903.
17. Shen L, Catalano PJ, Benson AB III, O'Dwyer P, Hamilton SR, Issa JP. Association between DNA methylation and shortened survival in patients with advanced colorectal cancer treated with 5-fluorouracil based chemotherapy. Clin Cancer Res 2007;13:6093–8.
18. van Rijnsoever M, Grieu F, Elsaleh H, Joseph D, Iacopetta B. Characterisation of colorectal cancers showing hypermethylation at multiple CpG islands. Gut 2002;51:797–802.
19. Ward RL, Cheong K, Ku SL, Meagher A, O'Connor T, Hawkins NJ. Adverse prognostic effect of methylation in colorectal cancer is reversed by microsatellite instability. J Clin Oncol 2003;21:3729–36.
20. Samowitz WS, Sweeney C, Herrick J, et al. Poor survival associated with the BRAF V600E mutation in microsatellite-stable colon cancers. Cancer Res 2005;65:6063–9.
21. Krtolica K, Krajnovic M, Usaj-Knezevic S, Babic D, Jovanovic D, Dimitrijevic B. Comethylation of p16 and MGMT genes in colorectal carcinoma: correlation with clinicopathological features and prognostic value. World J Gastroenterol 2007;13:1187–94.
22. Lee S, Cho NY, Choi M, Yoo EJ, Kim JH, Kang GH. Clinicopathological features of CpG island methylator phenotype-positive colorectal cancer and its adverse prognosis in relation to KRAS/BRAF mutation. Pathol Int 2008;58:104–13.
23. Deng G, Kakar S, Tanaka H, et al. Proximal and distal colorectal cancers show distinct gene-specific methylation profiles and clinical and molecular characteristics. Eur J Cancer 2008;44:1290–301.
24. Ogino S, Nosho K, Kirkner GJ, et al. CpG island methylator phenotype, microsatellite instability, BRAF mutation and clinical outcome in colon cancer. Gut 2009;58:90–6.

Published OnlineFirst March 2, 2010; DOI: 10.1158/1078-0432.CCR-09-2594

25. Sanchez JA, Krumroy L, Plummer S, et al. Genetic and epigenetic classifications define clinical phenotypes and determine patient outcomes in colorectal cancer. Br J Surg 2009;96:1196–204.
26. Kim JH, Shin SH, Kwon HJ, Cho NY, Kang GH. Prognostic implications of CpG island hypermethylator phenotype in colorectal cancers. Virchows Arch 2009;455:485–94.
27. Ferracin M, Gafa R, Miotto E, et al. The methylator phenotype in microsatellite stable colorectal cancers is characterized by a distinct gene expression profile. J Pathol 2008;214:594–602.
28. Popat S, Hubner R, Houlston RS. Systematic review of microsatellite instability and colorectal cancer prognosis. J Clin Oncol 2005;23:609–18.
29. Ogino S, Meyerhardt JA, Kawasaki T, et al. CpG island methylation, response to combination chemotherapy, and patient survival in advanced microsatellite stable colorectal carcinoma. Virchows Arch 2007;450:529–37.
30. Barault L, Charon-Barra C, Jooste V, et al. Hypermethylator phenotype in sporadic colon cancer: study on a population-based series of 582 cases. Cancer Res 2008;68:8541–6.
31. Kakar S, Deng G, Sahai V, et al. Clinicopathologic characteristics, CpG island methylator phenotype, and BRAF mutations in microsatellite-stable colorectal cancers without chromosomal instability. Arch Pathol Lab Med 2008;132:958–64.
32. Van Guelpen B, Hultdin J, Johansson I, et al. Low folate levels may protect against colorectal cancer. Gut 2006;55:1461–6.
33. Benlloch S, Paya A, Alenda C, et al. Detection of BRAF V600E mutation in colorectal cancer: comparison of automatic sequencing and real-time chemistry methodology. J Mol Diagn 2006;8:540–3.
34. Weisenberger DJ, Siegmund KD, Campan M, et al. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. Nat Genet 2006;38:787–93.
35. Ogino S, Kawasaki T, Brahmandam M, et al. Precision and performance characteristics of bisulfite conversion and real-time PCR (MethyLight) for quantitative DNA methylation analysis. J Mol Diagn 2006;8:209–17.
36. Baas IO, Mulder JW, Offerhaus GJ, Vogelstein B, Hamilton SR. An evaluation of six antibodies for immunohistochemistry of mutant p53 gene product in archival colorectal neoplasms. J Pathol 1994;172:5–12.
37. Lee S, Cho NY, Yoo EJ, Kim JH, Kang GH. CpG island methylator phenotype in colorectal cancers: comparison of the new and classic CpG island methylator phenotype marker panels. Arch Pathol Lab Med 2008;132:1657–65.
38. Liang JT, Chang KJ, Chen JC, et al. Hypermethylation of the p16 gene in sporadic T3N0M0 stage colorectal cancers: association with DNA replication error and shorter survival. Oncology 1999;57:149–56.
39. Maestro ML, Vidaurreta M, Sanz-Casla MT, et al. Role of the BRAF mutations in the microsatellite instability genetic pathway in sporadic colorectal cancer. Ann Surg Oncol 2007;14:1229–36.
40. Ide T, Kitajima Y, Ohtaka K, Mitsuno M, Nakafusa Y, Miyazaki K. Expression of the hMLH1 gene is a possible predictor for the clinical response to 5-fluorouracil after a surgical resection in colorectal cancer. Oncol Rep 2008;19:1571–6.
41. Ogino S, Cantor M, Kawasaki T, et al. CpG island methylator phenotype (CIMP) of colorectal cancer is best characterised by quantitative DNA methylation analysis and prospective cohort studies. Gut 2006;55:1000–6.
42. Andreyev HJ, Norman AR, Cunningham D, Oates JR, Clarke PA. Kirsten ras mutations in patients with colorectal cancer: the multicenter "RASCAL" study. J Natl Cancer Inst 1998;90:675–84.
43. Andreyev HJ, Norman AR, Cunningham D, et al. Kirsten ras mutations in patients with colorectal cancer: the 'RASCAL II' study. Br J Cancer 2001;85:692–6.
44. de la Chapelle A. Genetic predisposition to colorectal cancer. Nat Rev Cancer 2004;4:769–80.
45. Boland CR, Thibodeau SN, Hamilton SR, et al. A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res 1998;58:5248–57.
46. Lindor NM, Burgart LJ, Leontovich O, et al. Immunohistochemistry versus microsatellite instability testing in phenotyping colorectal tumors. J Clin Oncol 2002;20:1043–8.
47. Ruszkiewicz A, Bennett G, Moore J, et al. Correlation of mismatch repair genes immunohistochemistry and microsatellite instability status in HNPCC-associated tumours. Pathology (Phila) 2002;34:541–7.
48. Bertagnolli MM, Niedzwiecki D, Compton CC, et al. Microsatellite instability predicts improved response to adjuvant therapy with irinotecan, fluorouracil, and leucovorin in stage III colon cancer: Cancer and Leukemia Group B Protocol 89803. J Clin Oncol 2009;27:1814–21.
49. Walther A, Johnstone E, Swanton C, Midgley R, Tomlinson I, Kerr D. Genetic prognostic and predictive markers in colorectal cancer. Nat Rev Cancer 2009;9:489–99.

Published OnlineFirst March 2, 2010; DOI: 10.1158/1078-0432.CCR-09-2594

Clinical Cancer Research

The Role of the CpG Island Methylator Phenotype in Colorectal Cancer Prognosis Depends on Microsatellite Instability Screening Status

Anna M. Dahlin, Richard Palmqvist, Maria L. Henriksson, et al.

Clin Cancer Res 2010;16:1845-1855. Published OnlineFirst March 2, 2010.