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Association Between Molecular Subtypes of Colorectal Cancer and Patient Survival

Short Title: Colorectal cancer subtypes and survival

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Abbreviations: CRC = colorectal cancer, MSI = microsatellite instability, MSS = microsatellite stable, CIMP = CpG island methylator phenotype, HR = hazard ratio, CI = confidence interval, SCCFR = Seattle Colon Cancer Family Registry, SEER = Surveillance, Epidemiology, and End Results, PMR = percentage of methylated reference, BMI = body mass index, IWHS = Iowa Women's Health Study

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ABSTRACT

Background and Aims. Colorectal cancer (CRC) is a heterogeneous disease that can develop via several pathways. Different CRC subtypes, identified based on tumor markers, have been proposed to reflect these pathways. We evaluated the significance of these previously proposed classifications to survival.

Methods. Participants in the population-based Seattle Colon Cancer Family Registry were diagnosed with invasive CRC from 1998 through 2007 in western Washington State (n=2706), and followed for survival through 2012. Tumor samples were collected from 2050 participants and classified into 5 subtypes based on combinations of tumor markers: type 1 (microsatellite instability [MSI] high, CpG island methylator phenotype [CIMP] positive, positive for *BRAF* mutation, negative for *KRAS* mutation); type 2 (microsatellite stable [MSS] or MSI-low, CIMP-positive, positive for *BRAF* mutation, negative for *KRAS* mutation); type 3 (MSS or MSI-low, non-CIMP, negative for *BRAF* mutation, positive for *KRAS* mutation); type 4 (MSS or MSI-low, non-CIMP, negative for mutations in *BRAF* and *KRAS*); and type 5 (MSI-high, non-CIMP, negative for mutations in *BRAF* and *KRAS*). Multiple imputation was used to impute tumor markers for those missing data on 1-3 markers. We used Cox regression to estimate hazard ratios (HR) and 95% confidence intervals (CI) for associations of subtypes with disease-specific and overall mortality, adjusting for age, sex, body mass, diagnosis year, and smoking history.

Results. Compared to participants with type 4 tumors (the most predominant), participants with type 2 tumors had the highest disease-specific mortality (HR=2.20, 95% CI: 1.47-3.31); subjects with type 3 tumors also had higher disease-specific mortality (HR=1.32, 95% CI: 1.07-1.63). Subjects with type 5 tumors had the lowest disease-specific mortality (HR=0.30, 95% CI: 0.14-0.66). Associations with overall mortality were similar to those with disease-specific mortality.

Conclusions. Based on a large, population-based study, CRC subtypes, defined by proposed etiologic pathways, are associated with marked differences in survival. These findings indicate the clinical importance of studies into the molecular heterogeneity of CRC.

Keywords: oncogene, methylation, serrated colorectal cancer, prognostic factor

INTRODUCTION

Increasing evidence indicates that colorectal cancer (CRC) is a biologically heterogeneous disease that can develop via a number of distinct pathways involving different combinations of genetic and epigenetic changes.[1,2] Proposed subtype classifications for CRC, based on the presence of microsatellite instability (MSI), the CpG island methylator phenotype (CIMP), and somatic mutations in *BRAF* and *KRAS*, are thought to approximate these distinct pathways.[1,2] In particular, CRC reflective of the "traditional" adenoma-carcinoma pathway has been described as typically demonstrating absent (microsatellite stable, MSS) to low-level MSI (MSI-low) without CIMP and without somatic *BRAF* or *KRAS* mutations; CRC resulting from a "serrated" pathway has been described as frequently *BRAF*-mutated and CIMP-positive; and an additional pathway has been suggested for *KRAS*-mutated CRC that is MSS/MSI-low and CIMP-low.[2,3]

The biologic distinctions between CRC subtypes resulting from different etiologic pathways may plausibly translate to differences in survival. As tumor markers that may reflect such different pathways, MSI, CIMP, *BRAF*-mutation, and *KRAS*-mutation status have each been studied extensively, with evidence of differences in the distribution of tumor site, sex, age and stage at diagnosis, and survival.[4-22] However, the significance of subtype classifications based on combinations of these four tumor markers with respect to survival has been minimally described.[3,23] In the only prior study to evaluate differences in survival across CRC subtypes defined by these four tumor markers in combination, Samadder et al. suggested that CRC with a *BRAF*-mutated/CIMP-high phenotype, suggestive of the serrated pathway, was associated with modestly worse survival than CRC with a MSS/CIMP-negative/*BRAF*-mutation negative/*KRAS*-mutation negative phenotype, suggestive of the traditional pathway.[3]

Using data from the population-based Seattle Colon Cancer Family Registry (SCCFR) and the Postmenopausal Hormones Supplemental Study to the SCCFR (PMH-SCCFR),[24,25] we

further explored the relationship between CRC molecular subtypes, defined by common tumor marker combinations, and survival.

METHODS

Study population

A description of the study populations has been published elsewhere.[24,25] Briefly, SCCFR study participants included persons diagnosed with incident invasive CRC between January 1998 and June 2002 who, at the time of diagnosis, were aged 20-74 years and resided in King, Pierce, or Snohomish counties of Washington State (Supplementary Table 1). Over this same period, women aged 50-74 at CRC diagnosis and residing in 10 surrounding counties were also recruited for participation in the PMH-SCCFR. During a second SCCFR recruitment phase (diagnosis dates April 2002 to July 2007), eligible participants were identified as individuals diagnosed at ages 18-49 with invasive CRC within the combined 13-county region. All cases were identified through the population-based Surveillance, Epidemiology, and End Results (SEER) cancer registry serving western Washington State. Eligibility was limited to English speakers with publicly-available telephone numbers. Of 3,525 eligible individuals contacted, 302 (9%) were deceased, 401 (11%) refused participation, 92 (3%) were lost to follow-up prior to interview, and 24 (1%) completed only a partial interview. Among participants who completed the interview (N=2,706), adequate tumor specimens were available for 77% (N=2,080). Participants for whom tumor specimens were not obtained were excluded from this analysis.

This study was approved by the Institutional Review Board of the Fred Hutchinson Cancer Research Center in accordance with assurances filed with and approved by the U.S. Department of Health and Human Services.

Tumor characteristics

DNA extracted from paraffin-embedded formalin-fixed diagnostic tumor tissue specimens was used in tumor marker testing. Testing for MSI was based on a 10-gene panel in DNA from tumor and normal surrounding tissue (BAT25, BAT26, BAT40, MYCL, D5S346, D17S250, ACTC, D18S55, D10S197, BAT34C4) for the majority of cases (N=1,430):[24,26] tumors were classified as MSI-high if instability was observed for ≥30% of markers, and MSS/MSI-low if instability was observed in <30% of markers. For other cases (N=534), MSI status was based on immunohistochemistry testing of four markers (MLH1, MSH2, MSH6, PMS2): cases whose tissue exhibited positive staining for all markers were considered MSS/MSI-low, whereas cases negative for the expression of at least one marker were considered MSI-high.[27,28] Tumor DNA was tested for the p.V600E *BRAF* mutation (N=1,948) using a fluorescent allele-specific PCR assay as described previously;[29] this mutation accounts for ~90% of *BRAF* mutations in CRC.[30] Mutations in *KRAS* codons 12 and 13 were identified through forward and reverse sequencing of amplified tumor DNA (N=1,894);[8,31] mutations in this hotspot region account for ~80% of *KRAS* mutations in CRC.[32,33] CIMP testing was completed for a large subset of cases (N=1,508) based on a validated quantitative DNA methylation assay using a five-gene panel (*CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3*, *SOCS1*).[34-36] As described elsewhere,[34] tumors were classified as CIMP-positive if the percentage of methylated reference (PMR) ratio was ≥10 for at least three of five markers and as non-CIMP if the PMR ratio was ≥10 for fewer than three markers; PMR is calculated as the amount of methylated tumor DNA at a specific locus (normalized to input bisulfite DNA amount measured at *ALU* repetitive elements) divided by the *ALU*-normalized amount in a methylated reference sample, multiplied by 100. Tumor site and stage information was available from SEER.

Subtype classifications

Tumor subtypes were defined as follows, consistent with previously-suggested classifications:[1,2] 1) “type 1” (i.e., MSI-high, CIMP-positive, *BRAF*-mutated, *KRAS*-mutation negative); 2) “type 2” (i.e., MSS/MSI-low, CIMP-positive, *BRAF*-mutated, *KRAS*-mutation

negative); 3) “type 3” (i.e., MSS/MSI-low, non-CIMP, *BRAF*-mutation negative, *KRAS*-mutated); 4) “type 4” (i.e., MSS/MSI-low, non-CIMP, *BRAF*-mutation negative, *KRAS*-mutation negative); and 5) “type 5” (i.e., MSI-high, non-CIMP, *BRAF* mutation-negative, *KRAS*-mutation negative). Other marker combinations were grouped together as an “other” category for tabulations. In sensitivity analyses, we explored changes to the type 3 subtype classification for comparison to previous reports,[3] removing cases for whom all methylation markers had a PMR ratio <10 from this subgroup.

Of the N=2080 cases for whom tumor tissue was available, N=30 were excluded due to insufficient tissue or uninformative assays. Multiple imputation was used to approximate tumor marker status for cases with one (N=564), two (N=104), or three missing markers (N=38):[37,38] the imputation model included variables for MSI, *BRAF*- and *KRAS*-mutation status, methylation status for the five genes used in classifying CIMP, stage, histology, sex, age at diagnosis, diagnosis year, body mass index (BMI), height, smoking history, use of non-steroidal anti-inflammatory drugs at diagnosis, history of endoscopic screening prior to diagnosis, education, race, first line of therapy, time from diagnosis to interview, censoring indicators, and analysis time. Iterative rounds of imputation (N=25) were performed using the *mi* command in STATA SE version 13.1 (College Station, Texas). Tumor subtype classifications were thus determined on the basis of assayed and, as necessary, imputed tumor markers. In addition to analyses utilizing these imputed data, we conducted sensitivity analyses using a complete-case approach, wherein only cases with complete tumor marker data were included.

Outcome information

Vital status, death date, and cause of death were determined through linkage to SEER and the National Death Index. CRC-specific deaths included those with an underlying cause attributed to ICD-10 codes C18.0-C20.0 or C26.0.[39] Vital status linkage was performed periodically, with the most recent linkage capturing deaths occurring through December, 2012.

Statistical analysis

We used Cox proportional hazards regression to evaluate relative differences in survival after diagnosis by tumor subtype, using the type 4 subtype as the referent category. The time axis was defined as days since diagnosis, with left truncation to account for time between diagnosis and enrollment (mean=8.6 months). We conducted separate analyses for CRC-specific and overall survival. Participants alive at their last vital status assessment were censored at that date; in analyses of CRC-specific survival, persons who died due to causes other than CRC were censored at the time of death. Proportional hazards assumptions were assessed by testing for a non-zero slope of the scaled Schoenfeld residuals on ranked failure times.[40]

Regression models included adjustment terms for age (continuous and ten-year categories), sex, BMI (continuous), diagnosis year, and cigarette smoking history (never, former, current smoker). In secondary analyses, we further adjusted for stage via stratification of the baseline hazards. We also assessed potential confounding by several additional characteristics: tumor site, family history of CRC, race, education, history of endoscopy screening prior to diagnosis, non-steroidal anti-inflammatory drug use at the time of diagnosis, and receipt of chemotherapy as first course of treatment. However, these latter factors were not retained in our analytic models as adjustment for each variable had minimal impact on point estimates (i.e., <5% change). In sensitivity analyses, we also evaluated associations separately by sex and study phase [first (1998-2002), second (2002-2007)]. To account for multiple comparisons we used Hochberg's step-up method to control for the family-wise error rate of 0.05 across each family of pairwise comparisons across subtypes (i.e., 5 tests per family).[41]

RESULTS

Among the N=2,080 cases with available tumor tissue, 99% (N=2,050) had information on at least one tumor marker and were included in the analysis; 65% (N=1,344) had complete data on all tumor markers. Approximately 16% of cases had tumors that were MSI-high, 13%

had tumors that were *BRAF*-mutated, 31% had *KRAS*-mutated tumors, and 18% had CIMP-positive tumors. Among those with complete tumor marker data, 7% (N=100) were classified as having the type 1 subtype, 4% (N=55) had type 2 CRC, 26% (N=353) had type 3 CRC, 47% (N=631) had type 4 CRC, and 4% (N=50) were classified as having type 5 CRC; approximately 12% exhibited other tumor marker combinations (Supplementary Figure 1). Cases with types 1 or 2 CRC, particularly those with type 2 CRC, had the highest mean age at diagnosis and were most likely to be female (Table 1). Type 1, 2, and 5 tumors were rarely located outside the proximal colon (≤20%). Cases with type 2 CRC were least likely to have been diagnosed with stage I disease and had the lowest 5-year survival (46%). Cases with missing data on one to three tumor markers were younger at diagnosis and more likely to have stage IV CRC relative to other case groups.

Kaplan-Meier curves illustrate unadjusted differences in CRC-specific (Figure 1) and overall survival (Figure 2) across subtypes. Observed patterns of survival differences were maintained in multivariable-adjusted analyses (Table 2). With respect to both outcomes, mortality rates were highest for type 2 CRC (HR=2.20, 95% CI: 1.47-3.31, and HR=1.55, 95% CI: 1.08-2.22 for CRC-specific and overall mortality, respectively) and lowest for type 5 CRC (HR=0.30, 95% CI: 0.14-0.66, and HR=0.61, 95% CI: 0.39-0.96); however, after accounting for multiple comparisons, associations with overall mortality were not statistically significant for these subgroups. CRC-specific survival was similarly favorable for both MSI-high subtypes (i.e., types 1 and 5). CRC-specific mortality was statistically significantly higher in the type 3 versus type 4 subgroup (HR=1.32, 95% CI: 1.07-1.63); a similar association was noted with respect to overall mortality.

Adjustment for stage at diagnosis had a modest impact on observed associations. Most point estimates were slightly attenuated with stage-adjustment, but patterns of survival differences across subtypes persisted (Table 2). Sensitivity analyses restricted to cases with complete tumor marker data showed more pronounced survival differences across subtypes

(Table 2). In sensitivity analyses excluding cases with a PMR ratio <10 on all CIMP markers from the type 3 subgroup, the poor survival profile of this group persisted (CRC-specific survival HR=1.44, 95% CI: 1.04-1.98, overall survival HR=1.35, 95% CI: 1.04-1.74, not shown). In other sensitivity analyses, patterns of survival differences by subtype were similar across strata defined by sex and study phase; in particular, in all strata, the type 2 case group was associated with the poorest survival (not shown).

DISCUSSION

In this large population-based cohort of individuals with incident invasive CRC, we found important differences in survival across CRC subtypes defined on the basis of pre-specified combinations of MSI, CIMP, *BRAF*-mutation, and *KRAS*-mutation status. Patients with MSI-high subtypes of disease (i.e., types 1 and 5) had the most favorable survival, whereas those with type 2 CRC (MSS/MSI-low, CIMP-positive, *BRAF*-mutated, *KRAS* mutation negative) had the highest mortality. Observed survival differences were consistent with differences in the distribution of stage across subtypes and stage-adjustment did diminish the strength and statistical significance of most findings; however, patterns of differences in survival were maintained after stage-adjustment. These findings contribute to a small but growing literature supporting the significance of CRC-subtype classifications defined by combinations of these tumor markers.

The subtypes evaluated in the present analysis are based on classifications first proposed by Jass in 2007.[1] Jass' types 1 and 2 correspond to the type 1 and 2 subtypes evaluated here, respectively, and were originally proposed as reflecting a serrated morphology, with origins in serrated polyps. Jass' type 3, similar to our type 3 subtype but restricted to CIMP-low tumors, was proposed as reflecting an alternate serrated pathway, with origins in *KRAS*-mutated adenomas, whereas Jass' type 4 subtype, consistent with our type 4 subtype, was proposed to reflect CRC arising from the traditional adenoma-carcinoma sequence. Jass' type 5

subtype, also consistent with our type 5 subtype classification, was suggested to be indicative of possible Lynch Syndrome as is reflected in the high prevalence of CRC family history in our type 5 case group.

To our knowledge, only one prior study has evaluated survival differences across CRC subtypes derived from the classifications proposed by Jass.[3] Samadder et al. noted differences in age at diagnosis, tumor site, and grade across three CRC subtypes defined by combinations of MSI, CIMP, *BRAF*, and *KRAS* status in the Iowa Women's Health Study (IWHS); however, no significant differences in subtype-specific survival were observed.[3] Noted limitations of the IWHS include restricted demographics and sample size. Also, the tumor subtypes of greatest significance in the present analysis were not distinguished by Samadder et al.: the authors combined type 1 and 2 case groups into a single serrated subtype classified without regard to MSI, and did not evaluate the type 5 subtype as a distinct case group.[3] Although we found type 1 and 2 CRC subtypes to be similar with the respect to their later age at diagnosis and proximal site distribution, we identified very different survival trajectories for these subtypes. This suggests that MSI status is a clinically-relevant marker of distinction in individuals with CRC suggestive of the serrated pathway. The observed favorable survival profile of the type 5 subtype further supports the need to distinguish MSI-high cases in CRC-subtype classification.

Most prior studies assessing the prognostic significance of MSI, CIMP, and *BRAF*- and *KRAS*-mutations in CRC have evaluated these markers individually.[4-15] MSI status is most consistently associated with survival:[15,42] in a recent meta-analysis, MSI-high CRC was associated with 40% better overall survival than MSS CRC (95% CI: 31-47%).[15] The *BRAF* V600E mutation has also consistently been associated with poor survival relative to CRC that is not *BRAF* mutated.[9-14,17-19] In contrast, studies of CRC survival in relation to CIMP[18-20] and *KRAS*-mutation status[4-8,10,11] have been inconsistent. Studies assessing associations between pairwise combinations of markers and CRC survival further support our findings of a complex interplay among these markers. In particular, previous studies have suggested that the

prognostic significance of *BRAF*-mutation status is more pronounced in, if not restricted to, patients with MSS/MSI-low CRC.[9,11,12,19,20,43] Other studies have reported higher mortality in MSS/CIMP-positive CRC relative to CRC with other MSI/CIMP combinations.[44,45]

The biologic basis for the observed differences in subtype-specific CRC survival remains an important topic for future research. Although the type 2 and 3 subtypes were diagnosed at an advanced stage, our finding that the higher mortality in these subtypes persisted after controlling for stage suggests that these are more inherently aggressive tumors and not simply tumors that were diagnosed late. Differences in response to available cancer therapies may also contribute to subtype-specific survival differences. Over the time period during which study participants were diagnosed with CRC, testing for the tumor markers in the present analysis was not clinically indicated for treatment decision-making. However, differential response to 5-fluorouracil-based chemotherapy by MSI[46,47] and CIMP status[48] has been reported, and differential response to newer anti-EGFR therapies (e.g., cetuximab) on the basis of *KRAS* and *BRAF* are well-documented.[10,49,50] Thus, the relationship of these subtype classifications to CRC treatment response merits further investigation.

The results of the present investigation should be interpreted in the context of study limitations. Information on the clinical management of CRC patients included in the analysis was limited; however, as described above, treatments were unlikely to differ across the evaluated CRC subtypes over the study period beyond any differences due to stage at diagnosis, diagnosis year, and tumor site. Tumor marker data were missing for a substantial proportion of cases. Participants for whom no tumor marker data was available were excluded from the analysis and were, on average, younger at diagnosis (mean age=53 versus 58 in included cases), more likely to be non-white (39% versus 17%), had lower five-year overall survival (63% versus 74%), and later stage at diagnosis (21% versus 11% distant stage). The prevalence of stage IV disease was lower in our study population than is reflected in SEER estimates for the study area,[51] further suggesting an exclusion of late-stage disease. Thus, it is plausible that the

distribution of tumor subtypes among excluded cases differed from that among included cases. We used multiple imputation to account for missing tumor marker data in cases with information on one to three tumor markers (N=706). Simulation studies comparing multiple imputation to complete-case analyses suggest that excluding observations with missing data can lead to considerable bias in regression coefficients and that such bias can be reduced via multiple imputation.[37,38] The fact that there were only modest differences in point estimates from multiple imputation versus complete-case analyses reflects the robustness of our conclusions to various analytic approaches. When tumor marker data were available, those data were based on single assays for each marker and, thus, do not capture information on intra-tumoral heterogeneity. Lastly, the tumor markers evaluated in the present analysis represent only a subset of those that might be relevant to CRC survival and subtype classification. It is likely that some etiologic and clinical heterogeneity remains within each of the evaluated CRC subtypes. Characterization of additional somatic mutations (e.g., in *KRAS* codon 61), gene amplifications (e.g., in *EGFR*), methylation sites (e.g., in *CDKN2A*), and other molecular alterations was beyond the scope of the present analysis, but could facilitate more refined and detailed classification of homogeneous CRC subtypes.

Important strengths of the present study include a long follow-up and large study population, which allowed for the evaluation of survival outcomes in less common CRC subtypes. The two smallest subtypes evaluated in the present analysis (i.e., types 2 and 5) demonstrated the most pronounced differences in survival. Further evaluation of these important CRC subtypes will require larger sample size.

Here we extend previous reports regarding the relevance of CRC subtypes defined jointly by MSI, CIMP, and *BRAF*- and *KRAS*-mutation status. Our findings suggest that the biologic distinctions between these subtypes translate to important differences in survival and highlight a poorer survival for CRC demonstrating the type 2, serrated-like phenotype. These

results support the value of considering these four markers in combination, in addition to their individual value as predictive and prognostic markers for CRC.

FIGURE LEGENDS.

Figure 1. Kaplan-Meier survival curves comparing disease-specific survival in colorectal cancer patients by tumor subtype: type 1 (dashed black), type 2 (dotted black), type 3 (solid gray), type 4 (solid black), type 5 (dashed gray), some other tumor marker combination (dotted gray). Subtypes are defined as follows: type 1 = MSI-high, *BRAF*-mutated, *KRAS*-mutation negative, CIMP+; type 2 = MSS/MSI-low, *BRAF*-mutated, *KRAS*-mutation negative, CIMP+; type 3 = MSS/MSI-low, *BRAF*-mutation negative, *KRAS*-mutated, non-CIMP; type 4 = MSS/MSI-low, *BRAF*-mutation negative, *KRAS*-mutation negative, non-CIMP; type 5 = MSI-high, *BRAF*-mutation negative, *KRAS*-mutation negative, non-CIMP

Figure 2. Kaplan-Meier survival curves comparing overall survival in colorectal cancer patients by tumor subtype: type 1 (dashed black), type 2 (dotted black), type 3 (solid gray), type 4 (solid black), type 5 (dashed gray), some other tumor marker combination (dotted gray). Subtypes are defined as follows: type 1 = MSI-high, *BRAF*-mutated, *KRAS*-mutation negative, CIMP+; type 2 = MSS/MSI-low, *BRAF*-mutated, *KRAS*-mutation negative, CIMP+; type 3 = MSS/MSI-low, *BRAF*-mutation negative, *KRAS*-mutated, non-CIMP; type 4 = MSS/MSI-low, *BRAF*-mutation negative, *KRAS*-mutation negative, non-CIMP; type 5 = MSI-high, *BRAF*-mutation negative, *KRAS*-mutation negative, non-CIMP

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**Author names in bold designate shared co-first authorship.*

Table 1. Demographic and clinical characteristics by colorectal cancer (CRC) case group*

	Type 1 MSI-high, *BRAF*-mutated, *KRAS*-mutation negative, CIMP-positive **(N=100, 7%)**	**Type 2** MSS/MSI-low, *BRAF*-mutated, *KRAS*-mutation negative, CIMP-positive **(N=55, 4%)**	**Type 3** MSS/MSI-low, *BRAF*-mutation negative, *KRAS*-mutated, non-CIMP **(N=353, 26%)**	**Type 4** MSS/MSI-low, *BRAF*- & *KRAS*-mutation negative, non-CIMP **(N=631, 47%)**	**Type 5** MSI-high, *BRAF*- & *KRAS*-mutation negative, non-CIMP **(N=50, 4%)**	**Other** **(N=155, 12%)**	**Unknown**† **(N=706)**	**X^2 p-value**
Age at diagnosis								
Mean (SD)	67.3 (5.3)	63.6 (8.4)	61.4 (9.0)	60.1 (9.8)	56.0 (12.2)	60.7 (10.9)	52.4 (12.1)	
<40	0 (0)	1 (2)	5 (1)	22 (3)	5 (10)	8 (5)	87 (12)	<0.01
40-49	1 (1)	2 (4)	37 (10)	59 (9)	10 (20)	13 (8)	292 (41)	
50-59	7 (7)	11 (20)	93 (26)	201 (32)	15 (30)	39 (25)	102 (14)	
60-69	51 (51)	27 (49)	140 (40)	222 (35)	10 (20)	58 (37)	137 (19)	
≥70	41 (41)	14 (25)	78 (22)	127 (20)	10 (20)	37 (24)	88 (12)	
Sex								
Male	17 (17)	16 (29)	149 (42)	333 (53)	21 (42)	71 (46)	318 (45)	<0.01
Female	83 (83)	39 (71)	204 (58)	298 (47)	29 (58)	84 (54)	388 (55)	
Race								
White	95 (95)	52 (95)	318 (90)	575 (91)	43 (86)	145 (94)	471 (67)	0.05
African-American	2 (2)	3 (5)	9 (3)	27 (4)	3 (6)	1 (1)	19 (3)	
Asian	2 (2)	0 (0)	16 (5)	11 (2)	2 (4)	3 (2)	18 (3)	
>1 race	1 (1)	0 (0)	6 (2)	2 (0.3)	0 (0)	2 (1)	9 (1)	
Other / Unknown	0 (0)	0 (0)	4 (1)	16 (3)	2 (4)	4 (3)	189 (27)	
CRC family history								
No	85 (85)	47 (85)	295 (84)	538 (85)	33 (66)	128 (83)	596 (84)	0.02
Yes	15 (15)	8 (15)	58 (16)	93 (15)	17 (34)	27 (17)	110 (16)	
Stage at diagnosis								
I	47 (47)	11 (20)	132 (38)	281 (45)	25 (50)	60 (39)	257 (37)	<0.01
II-III	52 (52)	37 (67)	174 (49)	282 (45)	24 (48)	85 (55)	338 (48)	
IV	1 (1)	7 (13)	46 (13)	66 (10)	1 (2)	10 (6)	107 (15)	
Unknown	0	0	1	2	0	0	4	
1st treatment course								
Received chemo	44 (46)	37 (71)	203 (59)	344 (67)	24 (50)	88 (59)	435 (63)	0.05
No chemo	52 (54)	15 (29)	142 (41)	271 (33)	24 (50)	62 (41)	256 (37)	
Unknown	4	3	8	16	2	5	15	

Table 1, cont.

	Type 1 MSI-high, *BRAF*-mutated, *KRAS*-mutation negative, CIMP-positive **(N=100, 7%)**	**Type 2** MSS/MSI-low, *BRAF*-mutated, *KRAS*-mutation negative, CIMP-positive **(N=55, 4%)**	**Type 3** MSS/MSI-low, *BRAF*-mutation negative, *KRAS*-mutated, non-CIMP **(N=353, 26%)**	**Type 4** MSS/MSI-low, *BRAF*- & *KRAS*-mutation negative, non-CIMP **(N=631, 47%)**	**Type 5** MSI-high, *BRAF*- & *KRAS*-mutation negative, non-CIMP **(N=50, 4%)**	**Other (N=155, 12%)**	**Unknown**† **(N=706)**	**X^2 p-value**
Tumor site								
Right colon:	93 (93)	43 (80)	136 (39)	132 (22)	42 (84)	111 (73)	250 (36)	<0.01
Cecum	36	17	72	54	20	38	81	
Ascending colon	33	17	33	30	11	37	82	
Hepatic flexure	11	2	9	10	3	11	25	
Transverse colon	11	6	19	31	8	19	41	
Splenic flexure	2	1	3	13	0	6	21	
Left colon:	6 (6)	9 (17)	102 (29)	218 (35)	4 (8)	18 (12)	187 (27)	
Descending colon	2	0	19	22	0	7	26	
Sigmoid colon	4	9	83	196	4	11	161	
Rectal:	1 (1)	2 (4)	112 (32)	268 (43)	4 (8)	24 (16)	258 (37)	
Rectosigmoid Junction	1	1	28	67	2	8	61	
Rectum	0	1	84	201	2	16	197	
Unknown	0	0	3	7	0	2	2	
MSI status								
MSS/MSI-L	0 (0)	55 (100)	353 (100)	631 (100)	0 (0)	84 (54)	535 (86)	--
MSI-H	100 (100)	0 (0)	0 (0)	0 (0)	50 (100)	71 (46)	85 (14)	
Missing	0	0	0	0	0	0	86	
***BRAF*-mutation status**								
Wildtype	0 (0)	0 (0)	353 (100)	631 (100)	50 (100)	118 (76)	533 (92)	--
Mutated	100 (100)	55 (100)	0 (0)	0 (0)	0 (0)	37 (24)	51 (8)	
Missing	0	0	0	0	0	0	102	
***KRAS*-mutation status**								
Wildtype	100 (100)	55 (100)	0 (0)	631 (100)	50 (100)	75 (48)	396 (72)	--
Mutated	0 (0)	0 (0)	353 (100)	0 (0)	0 (0)	80 (52)	154 (28)	
Missing	0	0	0	0	0	0	156	
CIMP status								
Non-CIMP	0 (0)	0 (0)	353 (100)	631 (100)	50 (100)	71 (46)	127 (77)	--
CIMP-positive	100 (100)	55 (100)	0 (0)	0 (0)	0 (0)	84 (54)	37 (23)	
Missing	0	0	0	0	0	0	542	
5-yr survival (%)								
Overall	80.5	46.2	67.8	78.0	84.1	71.8	75.3	
Disease-specific	89.5	49.2	72.4	82.5	93.1	79.7	78.7	

*Cases missing data on all 4 markers used in subtype classification are excluded from all analyses (N=616).
†Cases of "unknown" subtype have missing data on 1 to 3 markers used in subtype classification and are re-allocated to subtype groups through multiple imputation in analyses.

Table 2. Colorectal cancer (CRC)-specific and overall mortality by tumor subtype

Subtype*	Raw N Case Participants (col%)†		Raw N CRC-Specific Deaths (col%)†		CRC-Specific Mortality HR‡	(95% CI)	HR§	(95% CI)	Raw N Total Deaths (col%)†		Overall Mortality HR‡	(95% CI)	HR§	(95% CI)
Primary Analysis¶														
Type 1	100	(5)	9	(2)	0.41	(0.22-0.75)	0.54	(0.29-0.99)	42	(5)	0.88	(0.65-1.20)	1.05	(0.78-1.44)
Type 2	55	(3)	26	(4)	2.20	(1.47-3.31)	1.84	(1.21-2.78)	32	(3)	1.55	(1.08-2.22)	1.40	(0.98-2.01)
Type 3	353	(26)	112	(30)	1.32	(1.07-1.63)	1.25	(1.01-1.54)	173	(28)	1.26	(1.07-1.49)	1.23	(1.04-1.46)
Type 4	631	(49)	154	(52)	1.0	(ref)	1.0	(ref)	263	(49)	1.0	(ref)	1.0	(ref)
Type 5	50	(5)	4	(1)	0.30	(0.14-0.66)	0.42	(0.19-0.93)	14	(3)	0.61	(0.39-0.96)	0.74	(0.47-1.17)
Other	155	(12)	36	(11)	1.05	(0.76-1.44)	1.18	(0.87-1.62)	74	(13)	1.14	(0.90-1.43)	1.25	(0.99-1.57)
*Complete-Case Analyses***														
Type 1	100	(5)	9	(2)	0.43	(0.22-0.85)	0.56	(0.28-1.11)	42	(5)	0.94	(0.67-1.32)	1.12	(0.80-1.58)
Type 2	55	(3)	26	(4)	2.72	(1.78-4.17)	2.40	(1.56-3.70)	32	(3)	1.79	(1.23-2.59)	1.65	(1.14-2.41)
Type 3	353	(26)	112	(30)	1.54	(1.20-1.97)	1.44	(1.12-1.83)	173	(28)	1.40	(1.15-1.68)	1.36	(1.12-1.65)
Type 4	631	(49)	154	(52)	1.0	(ref)	1.0	(ref)	263	(49)	1.0	(ref)	1.0	(ref)
Type 5	50	(5)	4	(1)	0.31	(0.12-0.85)	0.46	(0.17-1.26)	14	(3)	0.70	(0.41-1.20)	0.84	(0.49-1.45)
Other	155	(12)	36	(11)	1.02	(0.71-1.47)	1.27	(0.87-1.84)	74	(13)	1.21	(0.94-1.57)	1.38	(1.06-1.79)

* Subtype classifications abbreviated as follows: Type 1 = MSI-high, *BRAF*-mutated, *KRAS*-mutation negative, CIMP-positive; Type 2 = MSS/MSI-low, *BRAF*-mutated, *KRAS*-mutation negative, CIMP-positive; Type 3 = MSS/MSI-low, *BRAF*-mutation negative, *KRAS*-mutated, non-CIMP; Type 4 = MSS/MSI-low, *BRAF*-mutation negative, *KRAS*-mutation negative, non-CIMP; Type 5 = MSI-high, *BRAF*-mutation negative, *KRAS*-mutation negative, non-CIMP

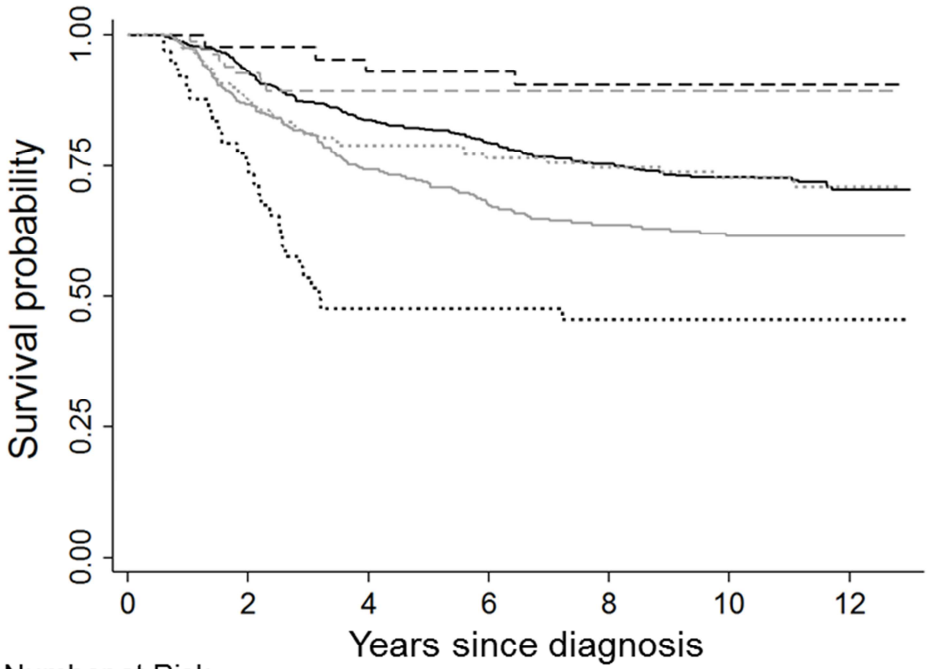
† Case counts and numbers of deaths by subtype are based on observed, non-imputed data. Column percents reflect imputed distributions.

‡ Adjusted for age at diagnosis, sex, BMI, diagnosis year, and smoking history.

§ Adjusted for stage at diagnosis is addition to age at diagnosis, sex, BMI, diagnosis year, and smoking history.

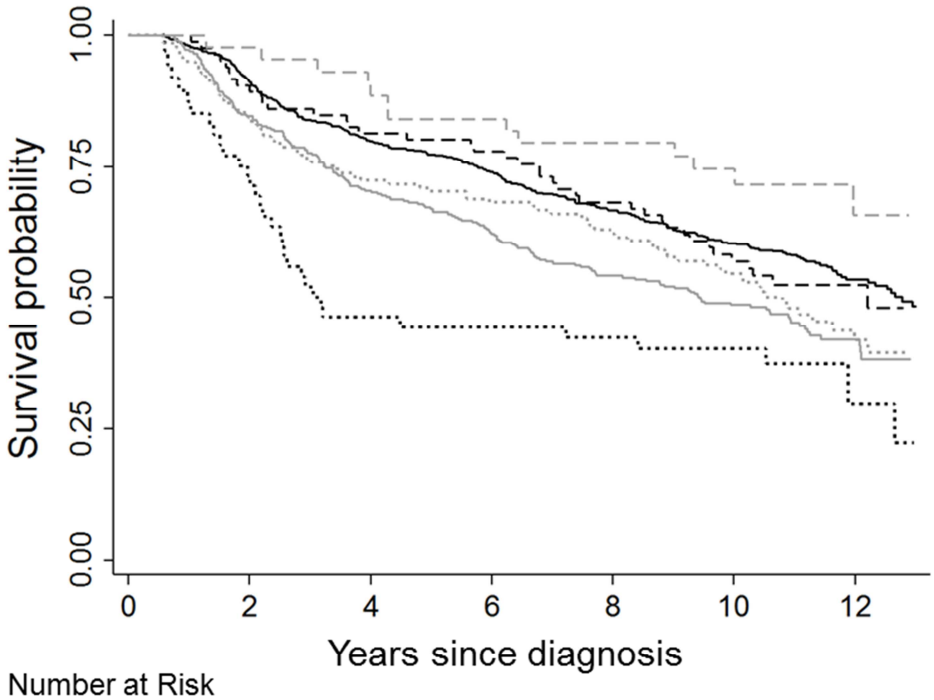
¶ Multiple imputation-based analysis in which missing tumor marker data was inferred based on known variables and then used to derive tumor subtype.

** Complete-case analysis. Cases missing data on any tumor markers are excluded.



Number at Risk

Type 1	100	78	71	66	56	43	14
Type 2	55	38	24	23	21	20	4
Type 3	353	268	223	191	160	130	37
Type 4	631	541	473	433	370	300	113
Type 5	50	43	39	37	32	26	10
Other	155	120	103	96	84	65	22



Number at Risk

Type 1	100	78	71	66	56	43	14
Type 2	55	38	24	23	21	20	4
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Type 4	631	541	473	433	370	300	113
Type 5	50	43	39	37	32	26	10
Other	155	120	103	96	84	65	22

Supplemental Table 1. Composition and design of included studies

	SCCFR – Phase I	PMH-SCCFR	SCCFR – Phase II	TOTAL
Years of diagnosis	1998-2002	1998-2002	2002-2007	1998-2007
Geographic area within Western Washington State	3 counties (King, Pierce, Snohomish)	10 counties (excluding King, Pierce, Snohomish)	13 counties	13 counties
Age at diagnosis (years):				
<40	79	0	121	200
40-49	218	0	417	635
50-59	487	104	0	591
60-69	629	144	0	733
≥70	362	105	0	467
% Female	45%	100%	50%	53%
N cases eligible	2359	439	727	3525
N cases completed interview	1813	353	540	2706
N cases with tumor tissue	1498	278	304	2080
Mean time from diagnosis to interview (months)	8.3	8.6	10.0	8.6

*SCCFR = Seattle Colon Cancer Family Registry; PMH-SCCFR = Postmenopausal Hormones Supplemental Study to the Seattle Colon Cancer Family Registry

Supplemental Figure 1. Distribution of tumor marker combinations and subtype classifications