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A novel classification of colorectal tumors based on microsatellite instability, the CpG island methylator phenotype and chromosomal instability: implications for prognosis

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Background: We studied the overlap between the major (epi)genomic events microsatellite instability (MSI), the CpG island methylator phenotype (CIMP) and chromosomal instability (CIN) in colorectal cancer (CRC), and whether specific (epi)genotypes were associated with CRC-related deaths.
Patients and methods: Molecular analyses using tumor DNA were successful in 509 CRC cases identified within the Netherlands Cohort Study in the period 1989–1993. Follow-up for the vital status until May 2005 was 100%.
Results: MSI (12.6%), CIMP-only (5.3%), CIMP + CIN (13.4%), CIN-only (58.2%) and triple-negative tumors (10.6%) differed significantly regarding tumor localization, differentiation grade, initial adjuvant therapy (AT) use and genetic characteristics ($P \leq 0.03$). CIMP-only, CIMP + CIN and triple-negative tumors, compared with CIN-only tumors, were significantly associated with a 3.67, 2.44 and 3.78-fold risk of CRC-related deaths after 2-year follow-up (95% confidence intervals, CIs, 1.70–7.91, 1.35–4.41 and 1.97–7.25, respectively), but not after late follow-up. MSI tumors were borderline significantly associated with a 0.40-fold risk of CRC-related deaths after late follow-up (95% CI 0.15–1.03).
Conclusion(s): This is the first study to show that specific (epi)genotypes may hold a differential prognostic value that may vary over time. Although no specific treatment data were available, an explanation for the differential findings over time might be that (epi)genotypes modify therapy response.
Key words: chromosomal instability, colorectal neoplasms, methylation, microsatellite instability, prognosis

introduction

In the pathogenesis of colorectal cancer (CRC), DNA mutations, especially due to genomic instability, chromosomal aberrations and DNA promoter hypermethylation significantly determine dysregulated gene expression contributing to tumorigenesis. Because the relative contributions of these mechanisms differ between CRCs, CRCs are classified as microsatellite unstable (MSI), CpG island methylator phenotype (CIMP) and chromosomally unstable (CIN). MSI is associated with changes in short microsatellite repeats, caused by defective mismatch repair, usually due to methylation of the MutL homolog 1 (*MLH1*) gene [1, 2]. CIMP results in transcriptional silencing of specific tumor suppressor and DNA repair genes, including *MLH1* [3–5]. CIN tumors show chromosomal gains and losses and structural rearrangements, possibly reflecting an increased mutation rate [6]. The mechanisms underlying CIMP and CIN remain elusive.

MSI, CIMP and CIN are not mutually exclusive, and although MSI and CIMP correlate well [7], the overlap between MSI, CIMP and CIN is unclear [8]. Consequently, it is unknown whether specific subgroups have clinical relevance, in addition to tumor staging [9]. Studies that analyzed MSI and CIMP [10] or MSI and CIN [11–16] have consistently shown good prognosis for MSI tumors [17], yet large CRC case series with concurrent information on MSI, CIMP, CIN and CRC-related deaths are lacking.

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Within the Netherlands Cohort Study (NLCS), we investigated the overlap between MSI, CIMP and CIN, and the association between specific (epi)genotypes and CRC-related deaths. Clinical information, *P53* overexpression status and mutation status in *APC*, *KRAS* and *BRAF V600E* were also available.

materials and methods

study population and design

CRC cases (ICD-O-1 153) were identified within the NLCS through record linkage to the population-based cancer registry and the national pathology database (PALGA) [18, 19]. The NLCS was designed to study associations between diet and cancer [20], and was approved by the institutional review boards of the TNO Nutrition and Food Research Institute and Maastricht University. The NLCS includes 120 852 participants who completed a self-administered questionnaire at baseline in 1986, when 55–69 years old. Participants who reported a history of cancer (excluding skin cancer) at baseline were excluded. The estimated completeness of cancer follow-up is >96% [21].

Figure 1 shows the collection of tumor material from CRC cases. Sufficient DNA, isolated from formalin-fixed, paraffin-embedded (FFPE) sections after macrodissection of tumor cells, was available for 733 cases [22]. Age, sex and family history of CRC were derived from the NLCS questionnaire. Information on tumor localization, incidence date, tumor node metastasis (TNM) stage and initial adjuvant therapy (AT) use was obtained from the cancer registry. Differentiation grade was derived from the PALGA reports. Follow-up for the vital status was carried out through linkage to the Central Bureau of Genealogy and the municipal population registries until 1 May 2005. Causes of death were retrieved from the Central Bureau for Statistics. Vital status was obtained for all cases. We excluded cases for which the cause of death was unknown ($n = 4$), cases diagnosed at autopsy ($n = 6$) and cases who died <30 days after diagnosis ($n = 19$).

(Epi)genetic instability analyses

MSI

MSI was determined by a pentaplex polymerase chain reaction (PCR) using five mononucleotide repeats: BAT-26, BAT-25, NR-21, NR-22 and NR-24. Allelic size variations in three or more repeats were a marker for MSI; other tumors were classified as microsatellite stable. This method was shown to have high sensitivity and specificity [23]. Analyses were successful in 90% of 733 cases.

CIMP

CIMP was defined by CpG island promoter hypermethylation of ≥3 out of five Weisenberger markers (*CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3* and *SOCS1*) [5]. As previously described, methylation was determined by a methylation-specific PCR (MSP) [24, 25] after bisulfite modification of 500 ng DNA (Zymo Research) [26]. MSP is a specific, qualitative method for which the results were shown in accordance with the results of other methods [27]. MSP analysis on FFPE tissue was facilitated by first amplifying the bisulfite-modified DNA (regardless of methylation status) using flanking PCR primers. All PCRs included controls for unmethylated alleles (DNA from normal lymphocytes), methylated alleles [normal lymphocyte DNA treated *in vitro* with SssI methyltransferase (New England Biolabs)] and a control without DNA. Analyses were successful in 81%, 79%, 79%, 90% and 83% of 733 cases for *CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3* and *SOCS1*, respectively.

CIN

CIN was determined by multiplex ligation-dependent probe amplification (SALSA® MLPA® reagents, MRC-Holland) [28–30], after DNA purification (QIAamp® micro DNA kit, Qiagen GmbH). Hundred nanograms of purified DNA were denatured at 98°C for 5 min, after which MLPA probes were added and allowed to hybridize for 16 h at 60°C in a thermocycler. Then, 1 U of ligase-65 enzyme was added and ligation was allowed to proceed for 15 min at 54°C. Primers, dNTPs and Taq polymerase were added after heat inactivation of the ligase-65 enzyme at 98°C. PCR amplification was done for 35 cycles (30 s at 95°C, 30 s at 60°C and 1 min at 72°C). Amplification products were quantified by capillary

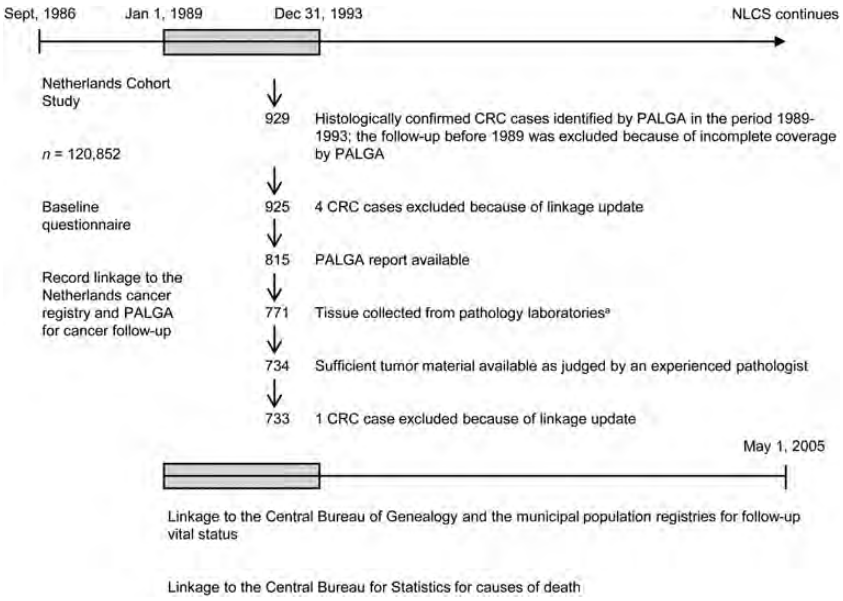

Figure 1. Flow chart of colorectal cancer (CRC) cases available for analyses in the Netherlands Cohort Study. [a]Tumor tissue was collected after approval by the ethical review boards of Maastricht University, the population-based cancer registry and PALGA; the pathology laboratories made available the tumor blocks between August 1999 and December 2001. CRC, colorectal cancer; PALGA, Netherlands pathology database; NLCS, Netherlands Cohort Study.

electrophoresis using the ABI 3730 DNA analyzer, with a LIZ-labeled internal size standard (LIZ-600, Genescan, Life Technologies Corporation, Applied Biosystems). A comparison of the sample peak pattern of case samples with that of control samples showed which sequences had copy number gains or losses.

We targeted gains in 8q23-qter, 13q14–31 and 20q13, and losses in 8p21-pter, 15q11-q21, 17p12–13 and 18q12–21 (custom-designed probe sets, MRC-Holland). Specific combinations of these abnormalities have been associated with progressed colorectal adenomas and CRC, indicating multiple CIN pathways [31]. For normalization purposes, analyses included reference probes and DNA from normal FFPE colon tissue. All samples were analyzed at least in duplo. Positive controls consisted of cell lines HT29 (when targeting gains) or COLO205 (when targeting losses); negative controls consisted of cell line LS174T. Sample probe ratios were averaged across runs, and, subsequently, for probes targeting the same regions. A ratio of ≥1.2 in 8q23-qter, 13q14–31 and 20q13 defined a gain; a ratio of ≤0.8 in 8p21-pter, 15q11-q21, 17p12–13 and 18q12–21 defined a loss. CIN was defined as the presence of two or more chromosomal changes [31, 32]. Analyses were successful in 87% of 733 cases.

gene mutation and expression analyses

APC and *KRAS* mutations were analyzed using a nested PCR approach, amplifying the mutation cluster region in *APC* and the exon 1 fragment in *KRAS*, followed by direct sequencing using the purified fragments [22, 33]. Immunohistochemical staining for *P53* expression was carried out according to the avidin–biotin–peroxidase complex method; positive staining of ≥20% of the tumor nuclei indicated overexpression [34]. The *BRAF V600E* mutation was analyzed by a semi-nested PCR and subsequent restriction fragment length polymorphism analysis [35] *APC*, *KRAS*, *P53* and *BRAF V600E* analyses were successful in 90%, 100%, 99% and 95% of 733 cases, respectively.

classification (epi)genotypes

MSI, CIMP and CIN status were available for 509 CRC cases. Our classification of (epi)genotypes was hypothesis-based, firstly differentiating MSI from MSS cases, because MSI cases are universally acknowledged as a distinct subgroup concerning biology and prognosis [1, 2, 17]. In the MSS group, we then differentiated CIMP-only, CIMP + CIN and CIN-only tumors, because CIMP is an early event in CRC development, distinct from CIN, although overlap is possible [36]. Finally, we differentiated triple-negative tumors, which have been recognized in the literature [8].

statistical analyses

Cause-specific survival was defined as the time from CRC diagnosis until CRC-related deaths or the end of follow-up. We estimated the influence of (epi)genotypes on cause-specific survival using Kaplan–Meier curves and Wilcoxon tests. Hazard ratios (HRs) and 95% confidence intervals (CIs) for CRC-related deaths were estimated using Cox regression. The proportional hazards assumption was tested using the scaled Schoenfeld residuals [37] and by inspecting −log-log transformed survival curves. Multivariable-adjusted models included the predefined potential prognostic factors age at diagnosis, sex, tumor localization, TNM stage, differentiation grade and initial AT use. Furthermore, analyses were stratified for TNM stage and we checked the influence of adjustment for *P53* overexpression status, and mutation status in *APC*, *KRAS* and *BRAF V600E*. All analyses were conducted using Stata (Stata Corp., College Station, TX). Statistical significance was indicated by a P value of <0.05 using two-sided tests.

results

prevalences (epi)genotypes

MSI, CIMP-only, CIMP + CIN, CIN-only and triple-negative (epi)genotypes comprised 12.6% ($n = 64$), 5.3% ($n = 27$), 13.4% ($n = 68$), 58.2% ($n = 296$) and 10.6% ($n = 54$) of cases, respectively (Figure 2). For descriptive purposes, we report that the MSI group contained 11 MSI-only, 3 MSI + CIN, 35 MSI + CIMP and 15 triple-positive cases.

clinical and genetic characteristics

(Epi)genotypes differed significantly regarding tumor localization, differentiation grade and initial AT use ($P \leq 0.03$) (Table 1). MSI and CIMP-only tumors were mostly proximal colon tumors (85.7% and 51.9%, respectively), whereas CIMP + CIN and CIN-only tumors were mostly distally located (distal colon to rectum: 52.3% and 82.1%, respectively). Triple-negative tumors were almost equally distributed across subsites. MSI, CIMP-only and CIMP + CIN tumors were more

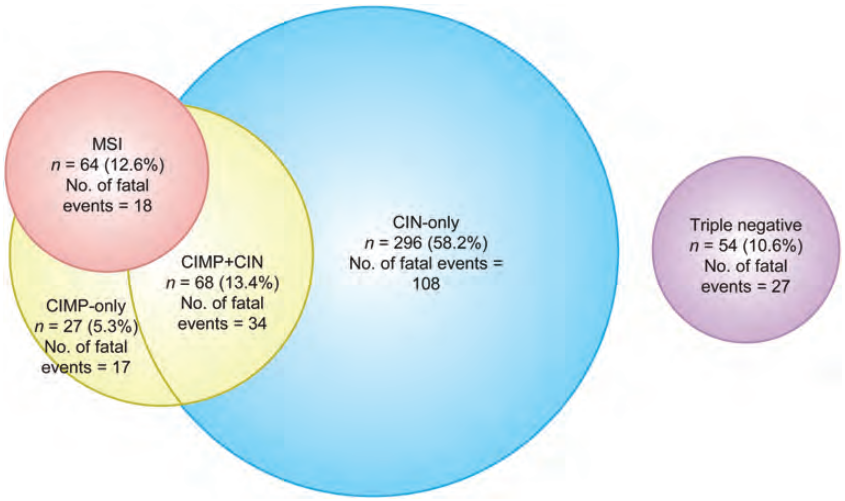

Figure 2. Venn diagram of (epi)genotypes based on MSI, CIMP and CIN status in colorectal cancer (CRC) cases from the Netherlands Cohort Study (total $n = 509$). CIMP, the CpG island methylator phenotype; CIN, chromosomal instability; MSI, microsatellite instability.

Table 1. Prevalence of (epi)genotypes in CRC cases from the Netherlands Cohort Study, by clinical and genetic characteristics (total $n = 509$)

Baseline characteristics	MSI	CIMP-only	CIMP + CIN	CIN-only	Triple negative	*P* value[a]
Total CRC cases, *n* (%)	64 (12.6)	27 (5.3)	68 (13.4)	296 (58.2)	54 (10.6)	
Clinical characteristics						
Age at diagnosis in years, mean (SD)	68.3 (4.7)	67.6 (3.3)	67.3 (3.8)	67.7 (4.2)	68.5 (3.8)	0.37[b]
Sex, *n* (%)						
Men	31 (48.4)	15 (55.6)	38 (55.9)	162 (54.7)	31 (57.4)	
Women	33 (51.6)	12 (44.4)	30 (44.1)	134 (45.3)	23 (42.6)	0.88
Tumor localization, *n* (%)[c]						
Proximal colon	54 (85.7)	14 (51.9)	32 (47.8)	52 (17.9)	15 (27.8)	
Distal colon	5 (7.9)	5 (18.5)	18 (26.9)	113 (38.8)	15 (27.8)	
Rectosigmoid	0 (0)	2 (7.4)	5 (7.5)	41 (14.1)	12 (22.2)	
Rectum	4 (6.3)	6 (22.2)	12 (17.9)	85 (29.2)	12 (22.2)	<0.001
TNM stage, *n* (%)[c]						
1	14 (23.0)	6 (23.1)	15 (22.7)	83 (29.4)	16 (30.8)	
2	27 (44.3)	8 (30.8)	18 (27.3)	97 (34.4)	16 (30.8)	
3	17 (27.9)	6 (23.1)	19 (28.8)	74 (26.2)	14 (26.9)	
4	3 (4.9)	6 (23.1)	14 (21.2)	28 (9.9)	6 (11.5)	0.18
Differentiation grade, *n* (%)[c]						
1	6 (10.5)	5 (23.8)	9 (13.8)	31 (11.9)	10 (22.7)	
2	30 (52.6)	11 (52.4)	43 (66.2)	199 (76.5)	28 (63.6)	
3	20 (35.1)	5 (23.8)	13 (20.0)	28 (10.8)	6 (13.6)	
4	1 (1.8)	0 (0)	0 (0)	2 (0.8)	0 (0)	0.002
Initial adjuvant therapy (AT) use, *n* (%)[c]						
Yes	6 (9.7)	1 (3.7)	14 (20.9)	59 (21.1)	6 (11.3)	
No	56 (90.3)	26 (96.3)	53 (79.1)	221 (78.9)	47 (88.7)	0.03
Family history of CRC, *n* (%)[c]						
Yes	5 (7.8)	2 (7.4)	8 (11.8)	35 (11.9)	5 (9.3)	
No	59 (92.2)	25 (92.6)	60 (88.2)	260 (88.1)	49 (90.7)	0.83
Genetic characteristics						
Truncating *APC* mutations, *n* (%)[c]						
Mutated	8 (13.3)	9 (34.6)	28 (41.8)	113 (41.1)	19 (38.0)	
Wild type	52 (86.7)	17 (65.4)	39 (58.2)	162 (58.9)	31 (62.0)	0.002
Activating *KRAS* mutations, *n* (%)						
Mutated	7 (10.9)	14 (51.9)	29 (42.6)	105 (35.5)	20 (37.0)	
Wild type	57 (89.1)	13 (48.1)	39 (57.4)	191 (64.5)	34 (63.0)	<0.001
P53 overexpression, *n* (%)[c]						
Yes	20 (31.3)	9 (33.3)	40 (58.8)	188 (64.4)	16 (29.6)	
No	44 (68.8)	18 (66.7)	28 (41.2)	104 (35.6)	38 (70.4)	<0.001
BRAF V600E mutation, *n* (%)[c]						
Mutated	37 (58.7)	5 (18.5)	18 (26.9)	14 (4.8)	6 (11.1)	
Wild type	26 (41.3)	22 (81.5)	49 (73.1)	280 (95.2)	48 (88.9)	<0.001

NOTE: percentages may not add up to 100% because of rounding off.

[a]*P* value for the χ^2 test, unless otherwise specified.

[b]*P* value for the Kruskal–Wallis test.

[c]Numbers do not add up to the total number of CRC cases because of missing data.

CIN, chromosomal instability; CIMP, the CpG island methylator phenotype; CRC, colorectal cancer; MSI, microsatellite instability; SD, standard deviation; TNM stage, tumor node metastasis stage.

often poorly differentiated than other tumors (20.0%–36.9% were grade 3 or 4 tumors), although the majority of tumors within all groups were classified as grade 2. CIMP-only tumors were rarely treated by initial AT (3.7%); treatment occurred slightly more often in CIMP + CIN and CIN-only tumor groups (20.9% and 21.1%, respectively). Groups did not significantly differ regarding the age at diagnosis, sex, TNM stage and family history of CRC.

Genetic characteristics differed significantly between (epi) genotypes ($P \leq 0.002$). *APC* and *KRAS* mutations occurred less often in MSI tumors (13.3% and 10.9%, respectively), but were prevalent in other groups (range: 34.6%–41.8% and 35.5%–51.9%, respectively). *P53* overexpression was present in all groups (range: 29.6%–64.4%), but most often occurred in CIN-only tumors. The *BRAF V600E* mutation most often occurred in MSI tumors (58.7%), and was particularly rare in CIN-only tumors (4.8%).

The distributions of clinical and genetic characteristics across MSI, non-MSI, CIMP, non-CIMP, CIN and non-CIN tumor groups are given in the supplementary Table S1, available at *Annals of Oncology* online for literature comparison.

survival

Information on potential confounders was complete for 422 CRC cases. In this group, 169 CRC-related deaths were identified. The median follow-up was 8.4 years; the maximum follow-up was 16.3 years. Kaplan–Meier curves show that survival significantly differed between (epi)genotypes ($P < 0.001$) (Figure 3). MSI cases showed the best survival of all groups. Increasingly worse survival was observed in cases with CIN-only, CIMP + CIN, triple-negative and CIMP-only tumors.

In Cox models, the proportional hazards assumption was violated. As the −log-log transformed survival curves seemed to converge at 2-year follow-up, we estimated HRs after 2-year follow-up and after late follow-up, by interacting the (epi) genotypes with the analysis time (Table 2). Unadjusted and multivariable-adjusted estimates were comparable. After early follow-up, CIMP-only, CIMP + CIN and triple-negative cases, compared with CIN-only cases, were at a significantly increased risk of CRC-related deaths. Multivariable-adjusted HRs were 3.67 (95% CI 1.70–7.91), 2.44 (95% CI 1.35–4.41) and 3.78 (95% CI 1.97–7.25), respectively. After late follow-up, the HRs were attenuated and no longer statistically significant. MSI cases, compared with CIN-only cases, did not differ in

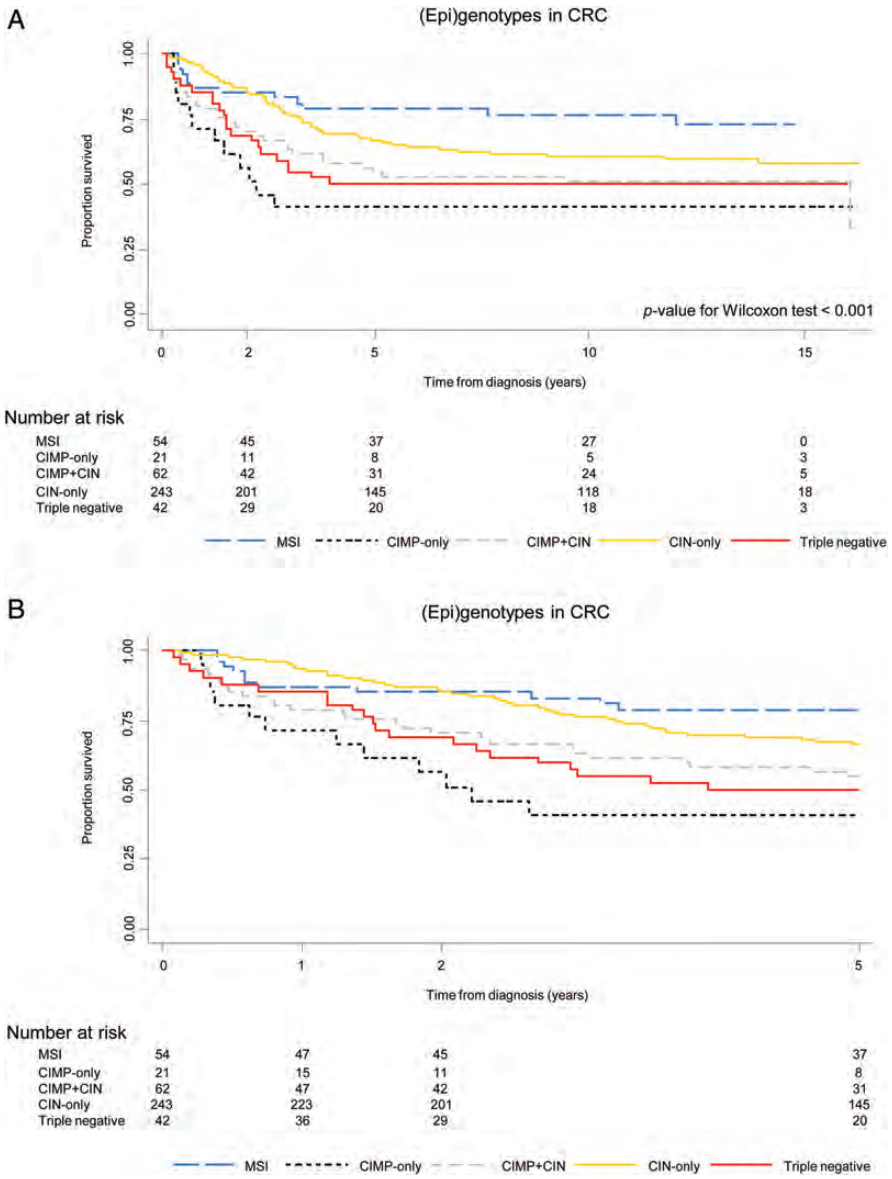

Figure 3. Cause-specific Kaplan–Meier curves according to (epi)genotypes in colorectal cancer (CRC) cases from the Netherlands Cohort Study (total $n = 422$), showing (A) complete follow-up and (B) 5-year follow-up. CRC, colorectal cancer; CIMP, the CpG island methylator phenotype; CIN, chromosomal instability; MSI, microsatellite instability.

Table 2. HRs for CRC-related deaths according to (epi)genotypes in CRC after early (≤2 year) and late follow-up (>2 years) ($n = 422$)

	≤2 years follow-up			>2 years follow-up		
	No. of fatal events	Survival time, years	HR (95% CI)	No. of fatal events	Survival time, years	HR (95% CI)
Unadjusted model						
MSI	8	96	1.06 (0.49–2.29)	5	365	0.37 (0.15–0.91)
CIMP-only	9	30	3.76 (1.81–7.83)	3	79	1.04 (0.33–3.34)
CIMP + CIN	18	99	2.32 (1.32–4.10)	12	344	0.96 (0.52–1.79)
CIN-only	35	447	1 (reference)	58	1,578	1 (reference)
Triple negative	13	70	2.38 (1.26–4.49)	8	217	1.02 (0.49–2.14)
Multivariable model[a]						
MSI	8	96	1.51 (0.66–3.47)	5	365	0.40 (0.15–1.03)
CIMP-only	9	30	3.67 (1.70–7.91)	3	79	1.41 (0.43–4.57)
CIMP + CIN	18	99	2.44 (1.35–4.41)	12	344	1.11 (0.58–2.12)
CIN-only	35	447	1 (reference)	58	1,578	1 (reference)
Triple negative	13	70	3.78 (1.97–7.25)	8	217	1.35 (0.64–2.86)
Multivariable model[b]						
MSI	8	96	1.60 (0.69–3.72)	5	365	0.41 (0.16–1.06)
CIMP-only	9	30	4.07 (1.86–8.91)	3	79	1.27 (0.38–4.23)
CIMP + CIN	18	99	2.61 (1.43–4.77)	12	344	1.13 (0.59–2.15)
CIN-only	35	447	1 (reference)	58	1,578	1 (reference)
Triple negative	13	70	4.10 (2.10–8.00)	8	217	1.34 (0.63–2.86)

[a]The model included age at diagnosis, sex, tumor localization, TNM stage, differentiation grade and initial adjuvant therapy (AT) use.
[b]The model included age at diagnosis, sex, tumor localization, differentiation grade and initial adjuvant therapy use; stratified estimation was carried out for TNM stage.
CI, confidence interval; CIN, chromosomal instability; CIMP, the CpG island methylator phenotype; CRC, colorectal cancer; HR, hazard ratio; MSI, microsatellite instability; TNM stage, tumor node metastasis stage.

their risk of CRC-related deaths after early follow-up (multivariable-adjusted HR = 1.51, 95% CI 0.66–3.47), but had a borderline significantly decreased risk after late follow-up (multivariable-adjusted HR = 0.40, 95% CI 0.15–1.03). This association became statistically significant after additional adjustment for *BRAF V600E* mutation status (HR = 0.33, 95% CI 0.12–0.88). Additional adjustment for *P53* overexpression status or mutation status in *APC*, *KRAS,* or *BRAF V600E*, showed no significant alterations (data not shown).

Kaplan–Meier curves show that survival significantly differed between (epi)genotypes within TNM stages 3 and 4 (Wilcoxon $P = 0.008$ and <0.001, respectively), but not within stages 1 and 2 (Figure 4). In stratified Cox analyses, the proportional hazards assumption was violated, but numbers did not allow interacting (epi)genotypes with analysis time. Instead, we repeated the overall analyses using the stratified estimation option (Table 2). This option allowed survival curves to be disproportional between tumor stage strata, while estimating HRs from a single model [38]. Our results were not essentially altered.

discussion

No prior studies analyzed the overlap between MSI, CIMP and CIN, or have classified tumors accordingly. The molecular classification of tumors is complicated by different definitions in the literature. With the exception of MSI, there is no gold standard regarding gene panels, marker thresholds and techniques to define CIMP [39] and CIN. We have used well-accepted methods to define MSI [23], CIMP [5, 24, 32] and CIN [28–32], and observed a prevalence for tumors characterized by MSI, CIMP-only, CIMP + CIN, CIN-only and triple negative of 12.6%, 5.3%, 13.4%, 58.2% and 10.6%, respectively. Comparably, prevalence rates of MSI and CIN in CRC were previously reported to be ∼15% and ∼65–70%, respectively [8]. The prevalence of CIMP ranges widely between studies (9%–90%), and consensus on the definition is called for [39].

Our classification, using MSI, CIMP and CIN, was hypothesis-based. A classification by Issa [36] incorporated mutation status in *APC*, *KRAS*, *P53* and *BRAF V600E*. We acknowledge that molecular, clinical and morphological features of CRCs displaying different instability types differ [40]. However, when applying Issa's classification to our population, numbers in groups were low (supplementary Figure S2, available at *Annals of Oncology* online), and triple-negative tumors were not distinguished. Triple-negative tumors might constitute covert CIN or CIMP tumors [8], but—unlike CIN or CIMP tumors—these did not show a predisposition for a particular subsite, nor did we observe distinct other features, e.g. a frequent family history of CRC. Therefore, the underlying biology of triple-negative tumors requires investigation. Novel genome-wide technologies appear to be promising for identifying the molecular alterations associated with this phenotype.

Next, we studied the association between (epi)genotypes and CRC-related deaths, and we observed CIMP-only, CIMP + CIN and triple-negative cases, compared with CIN-only cases, at an

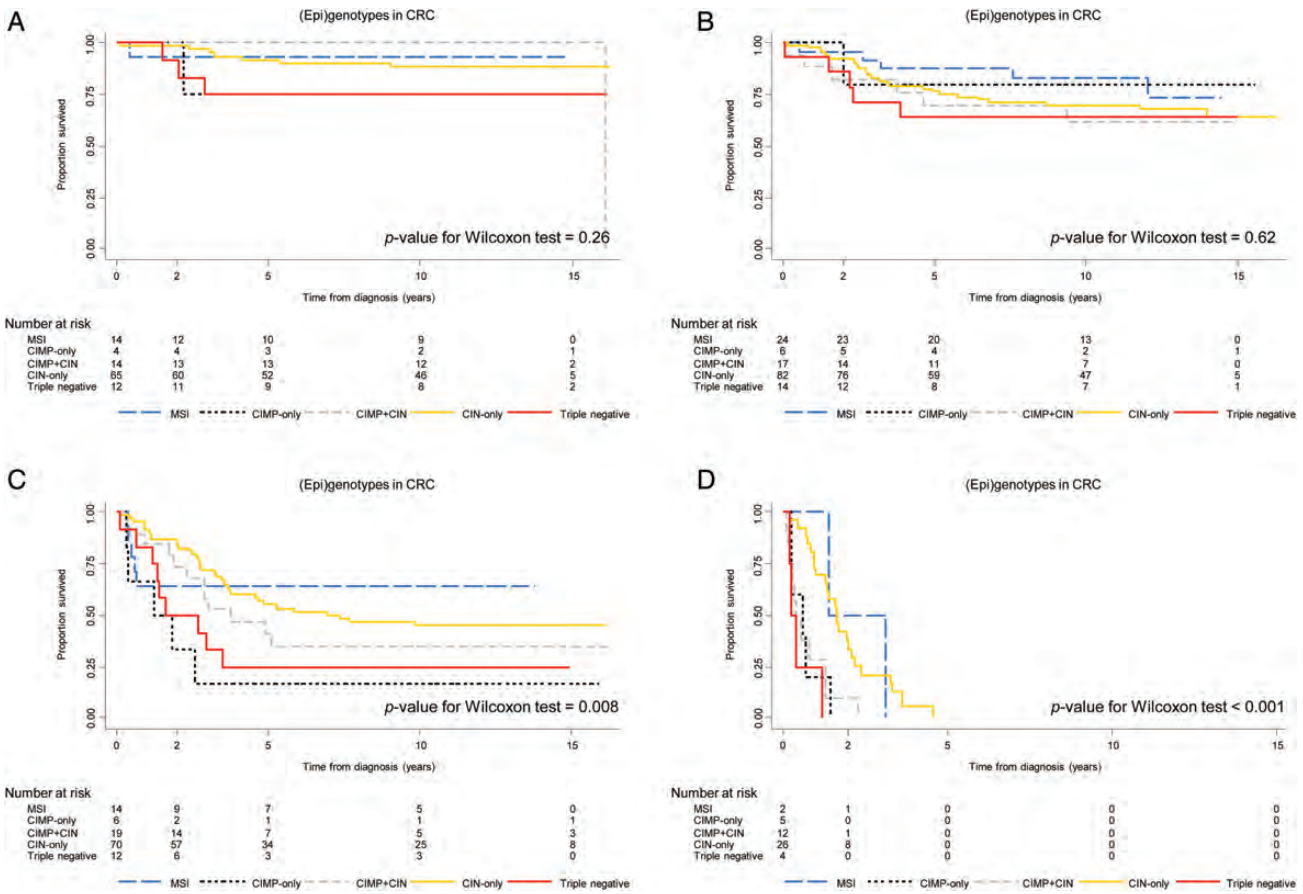

Figure 4. Cause-specific Kaplan–Meier curves according to (epi)genotypes in colorectal cancer (CRC) cases from the Netherlands Cohort Study (total $n = 422$) within (A) TNM stage 1, (B) TNM stage 2, (C) TNM stage 3 and (D) TNM stage 4. CRC, colorectal cancer; CIMP, the CpG island methylator phenotype; CIN, chromosomal instability; MSI, microsatellite instability; TNM, tumor node metastasis.

increased risk of CRC-related deaths after 2-year follow-up, whereas MSI cases were at a decreased risk after late follow-up. Although numbers in groups were small, these results are in accordance with the literature. Generally, poor prognosis has been associated with CIN tumors [8, 41, 42] and MSS CIMP tumors [10], whereas MSI cases show good prognosis [17]. Discordant results [43] may be due to heterogeneity in tumor groups, when groups are based on the overlap between only two instability types. Even when agnostically including MSI, CIMP and CIN in a prognostic model as separate variables, the triple-negative group is present in the reference groups and will influence results. Validation of our classification and prognostic results in an independent tumor series would help corroborate the prognostic value of specific (epi)genotypes.

Most heterogeneity may be present among CIMP tumors. We characterized a considerable group by CIMP + CIN (13.4%) and a smaller group by CIMP-only (5.3%). These groups could reflect CIMP-low tumors, as CIMP + CIN and CIMP-only tumors were frequently characterized by *KRAS* mutations and less often by the *BRAF V600E* mutation, which fits with the literature on CIMP-low tumors [40]. CIMP + CIN and CIMP-only cases showed poor short-term prognosis compared with CIN-only cases, whereas MSI tumors, of which the majority also had CIMP, showed good long-term prognosis. The CIMP-tumors in the MSI group could reflect CIMP-high tumors, as this group frequently exhibited the *BRAF V600E* mutation but not *KRAS* mutations [40]. An overruling beneficial effect of MSI on survival may be due to that MSI tumor cells are less fit to progress or metastasize, although this seems incompatible with the idea that instability drives tumor development [44]. Alternatively, a survival advantage of MSI cases could be related to immune response, as MSI tumors show strong infiltration with $CD8^+CD103^+$ lymphocytes, which have been shown less common in MSS tumors [44].

Complexity is added when considering the dimension of time. We observed that the prognostic value of (epi)genotypes varied over time. Partitioning of the time axis to model the potential effects of prognostic factors in the case of nonproportional hazards is an established method [45]. A nonproportional influence of prognostic factors on hazard rates was observed in several breast cancer studies, with common patterns being that of declining predictive strength or crossover [46]. Our results may have been influenced by our choice of reference group and by an influence of (epi) genotypes on therapy response. At the time of diagnosis of cases, common types of AT in the Netherlands may have been radiotherapy, especially in the case of rectal tumors, and 5-fluorouracil (5-FU) chemotherapy. We have no specific information on AT use, but as our reference group of CIN-

only tumors comprised the highest proportion of rectal tumors and initial AT use, this group may have derived a survival benefit from radiotherapy treatment. If this benefit faded over time and was not present in other groups, this could explain the attenuation of estimates that we observed after late follow-up. Future studies may be encouraged to carry out subsite-specific analyses, as rectal tumors have a distinct biology [47]; however, a sensitivity analysis confined to colon cancer cases did not alter conclusions at present. Response to 5-FU was previously investigated in relation to MSI and CIMP, but results were inconsistent [48–50]. CIN has been proposed to confer multidrug resistance [51]. Inconsistent findings may be explained by an interaction between specific (epi)genotypes and therapy type (influencing response) and the relationship between other factors and therapy response.

That adjustment for TNM stage and mutation status in key CRC genes did not essentially change the results, suggests that confounding was unlikely by these factors. As shown here and as reported previously [36, 40], the *BRAF V600E* mutation correlates with MSI and CIMP, and mutations in *KRAS*, *APC* and *P53* correlate with CIMP and CIN. However, none of these mutations have convincing prognostic relevance, although *KRAS* mutations may predict poor response to treatment with epidermal growth factor receptor inhibitors [8]. To confirm the independent prognostic effects of (epi)genotypes, future studies should stratify on the mutation status in key CRC genes and tumor stage, which would require large numbers or data pooling. Data pooling is complicated by differences in CIMP [39] and CIN measurements.

Strengths of our study include the population-based character, the nearly complete follow-up and the low overall prevalence of initial AT use. A limitation may be the inability to carry out stratified analyses for TNM stage because of low numbers. Still, this study is among the largest studies assessing molecular changes in CRC in relation to prognosis, and the first to shed light on the relative contributions of MSI, CIMP and CIN to survival.

In conclusion, our data on the interplay between MSI, CIMP and CIN showed that specific (epi)genotypes may hold differential prognostic value that may vary over time. Although no specific treatment data were available, an explanation for the differential findings over time might be that specific (epi) genotypes modify therapy response.

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disclosure

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