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Survival in stage II/III colorectal cancer is independently predicted by chromosomal and microsatellite instability, but not by specific driver mutations

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OBJECTIVES: **Microsatellite instability (MSI) is an established marker of good prognosis in colorectal cancer (CRC). Chromosomal instability (CIN) is strongly negatively associated with MSI and has been shown to be a marker of poor prognosis in a small number of studies. However, a substantial group of "double-negative" (MSI−/CIN−) CRCs exists. The prognosis of these patients is unclear. Furthermore, MSI and CIN are each associated with specific molecular changes, such as mutations in *KRAS* and *BRAF*, that have been associated with prognosis. It is not known which of MSI, CIN, and the specific gene mutations are primary predictors of survival.**

METHODS: **We evaluated the prognostic value (disease-free survival, DFS) of CIN, MSI, mutations in *KRAS*, *NRAS*, *BRAF*, *PIK3CA*, *FBXW7*, and *TP53*, and chromosome 18q loss-of-heterozygosity (LOH) in 822 patients from the VICTOR trial of stage II/III CRC. We followed up promising associations in an Australian community-based cohort (*N*=375).**

RESULTS: **In the VICTOR patients, no specific mutation was associated with DFS, but individually MSI and CIN showed significant associations after adjusting for stage, age, gender, tumor location, and therapy. A combined analysis of the VICTOR and community-based cohorts showed that MSI and CIN were independent predictors of DFS (for MSI, hazard ratio (HR)=0.58, 95% confidence interval (CI) 0.36–0.93, and *P*=0.021; for CIN, HR=1.54, 95% CI 1.14–2.08, and *P*=0.005), and joint CIN/MSI testing significantly improved the prognostic prediction of MSI alone (*P*=0.028). Higher levels of CIN were monotonically associated with progressively poorer DFS, and a semi-quantitative measure of CIN was a better predictor of outcome than a simple CIN+/− variable. All measures of CIN predicted DFS better than the recently described Watanabe LOH ratio.**

CONCLUSIONS: **MSI and CIN are independent predictors of DFS for stage II/III CRC. Prognostic molecular tests for CRC relapse should currently use MSI and a quantitative measure of CIN rather than specific gene mutations.**

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/ajg

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INTRODUCTION

Surgical resection for colorectal cancer (CRC) is often curative, but some patients with apparently localized disease at presentation have significant risk of relapse (1). These individuals are routinely offered systemic adjuvant chemotherapy based on 5-fluorouracil. Clinicopathological staging is the current standard for predicting the risk of relapse for resected primary CRC, but intra-stage variation remains (2).

The most frequently used molecular classifier of CRC is microsatellite instability (MSI), which is an increased tendency for insertion or deletion mutations at simple repeat sequences. MSI is found in ~10–15% of sporadic CRC and results from defective DNA mismatch repair (dMMR) (3). This phenotype can be assayed using either polymerase chain reaction polymerase chain reaction-based methods or immunohistochemistry for loss of MLH1 mismatch repair protein expression. Sporadic MSI+ tumors occur more frequently proximal to the splenic flexure and tend to show poor differentiation, mucinous histology and increased peritumoral lymphocytic infiltration (4). These tumors usually have near-diploid karyotypes and harbor a distinct set of driver mutations in genes such as *BRAF* and *TGFBR2* (5,6). MSI+ cancers also often exhibit a CpG island methylator phenotype (7). MSI has been reported by several studies to be a marker of good prognosis in CRC and a meta-analysis of 2,935 stage II or III CRCs found better overall survival for MSI or defective DNA mismatch repair (hazard ratio (HR) = 0.67, 95% CI 0.58–0.78) (8). In addition, it has been suggested that MSI predicts absence of benefit from 5-fluorouracil--based adjuvant chemotherapy (9,10).

Another molecular classifier of CRC is chromosomal instability (CIN), which is strongly negatively associated with MSI. CIN is broadly defined as the presence of multiple numerical and/or structural chromosomal abnormalities and is present in ~60% of CRCs (11,12). The causes of CIN are not known, but may include defects in mitosis, cell cycle checkpoints, double-strand break repair and telomeres. CIN+ tumors are more common in the distal colorectum, and tend to show non-mucinous histology, moderate differentiation, and fewer tumor-infiltrating lymphocytes (13,14). Such tumors appear to develop along the classic genetic pathway of colorectal tumorigenesis, with mutations in *APC, KRAS*, and *TP53* (15). CIN is more difficult to assess than MSI and hence is assayed less often. However, a meta-analysis of 3,094 patients with stage II or III CRC reported inferior overall survival associated with CIN (HR = 1.45, 95% CI 1.27–1.65) (16).

A small proportion (< 5%) of CRCs has both MSI and CIN, and their molecular features generally resemble those of MSI+/CIN− tumors (17). As many as 20% of CRCs are "double-negative" (MSI−/CIN−) (18–21). This type of tumor is relatively poorly characterized, although p53 mutations are less common than in MSI+/CIN+ lesions (17) and some double-negative CRCs may have a tendency to base substitution mutations (22). The prognosis of MSI+/CIN+ and MSI−/CIN− CRCs remains unclear. More generally, it is not known whether MSI and CIN are independent predictors of CRC relapse, with very few large studies having assayed both variables. Furthermore, as we have previously suggested that CIN+ CRCs can be sub-classified into CIN-low and CIN-high groups, it is possible that the extent of CIN provides additional prognostic information (15).

CRC driver mutations include somatic changes in *KRAS, BRAF, PIK3CA, TP53, FBXW7*, and *NRAS*. The first four of these mutations have been proposed and tested as indicators of CRC prognosis. *KRAS* in particular has been studied intensively, although analysis has been subject to publication bias, heterogeneity among studies, and controversy as to whether sub-group analysis by specific *KRAS* mutation is appropriate (23). For example, *KRAS* mutation has been associated with poor CRC prognosis in the QUASAR trial (1,583 patients) (24) and the RASCALII study (3,439 patients, stages II–IV) (25). In contrast, the PETACC-3 trial (1,564 patients, stages II and III) found no evidence for association of *KRAS* mutation with relapse-free survival or overall survival (26). Overall, the data are inconclusive as regards an association between *KRAS* and CRC prognosis, and a recent meta-analysis covering studies published between 1992 and 2011 showed no overall association (23).

There is relatively good evidence for *BRAF* as an indicator of poor outcome in CRC (27–32), but the association is complex, because *BRAF* mutation is paradoxically also strongly associated with MSI, an indicator of good prognosis. It has been suggested that *BRAF*'s indication of outcome is restricted to MSI− and/or late-stage disease. *PIK3CA* is a promising, but unproven prognostic indicator (27,33–35). There is inconsistent evidence to support p53 mutation as an indicator of outcome in CRC, an intriguing absence given the strong association between p53 and CIN, although p53 was shown some time ago to be associated with poorer survival in a large meta-analysis (36). In addition to the specific gene mutations, chromosome 18q deletion or loss-of-heterozygosity (LOH) has also been associated with poor prognosis, but this molecular marker is strongly associated with CIN (8).

Almost all of the specific CRC driver mutations are associated with CIN and/or MSI status. This raises the question as to which molecular changes are the primary indicators of prognosis. In order to address this question, we have analyzed MSI, CIN, mutations in *KRAS, NRAS, BRAF, PIK3CA, TP53*, and *FBXW7*, and chromosome 18q LOH in 822 resected stage II or III CRCs from the VICTOR clinical trial of rofecoxib post-primary therapy (37). We have also determined whether MSI and CIN are independent prognostic indicators, and tested the extent of CIN as a prognostic marker. We have followed up selected findings in an independent set of 375 community-based CRC patients.

METHODS

VICTOR study CRC patients

The VICTOR study recruited between April 2002 and September 2004, at 151 hospitals in the United Kingdom (37). Inclusion criteria were: histologically proven colorectal carcinoma

of stage III (T any, N1 or 2, M0) or stage II (T3 or 4, N0, M0) in patients who had undergone complete resection of the primary tumor without evidence of residual disease. Two thousand four hundred and thirty-four patients were randomly assigned to receive rofecoxib or placebo post-primary therapy, based on the potential utility of selective cyclooxygenase-2 inhibitors in promoting tumor cell apoptosis and inhibiting angiogenesis. Formalin-fixed paraffin-embedded tumor and matched normal specimens were requested from these cases and samples successfully retrieved from 822 patients. The median duration of follow-up of these patients was 58.5 months. After withdrawal of rofecoxib in September 2004, study treatment was stopped. Follow-up was continued at 3, 6, 12, 18, and 24 months after random assignment, and annually thereafter. Patients received a colonoscopy between 1 and 2 years after primary surgery and every 3 years thereafter, a computed tomography scan 1 year after surgery, and clinical examination and routine blood tests at outpatient visits. All recurrences were confirmed by computed tomography or magnetic resonance imaging scan, and patients were flagged for survival with the UK's Office of National Statistics. The clinicopathological characteristics and survival of the sampled patients (**Supplementary Table 1** online) were not significantly different from those of the entire trial set (details not shown). Of the patients with available tumor specimens, 130 of 402 stage II and 395 of 420 stage III cases had received standard neo-adjuvant or adjuvant 5-fluorouracil-based chemotherapy and/or concurrent chemoradiotherapy. Molecular analyses of anonymized patient samples and data were approved by Oxfordshire Research Ethics Committee B (Approval No. 05\Q1605\66).

Community series of CRC patients

A second, unselected, community-based series of 375 patients with stage II or III CRC was derived from the Royal Melbourne Hospital, Western Hospital Footscray and St Vincent's Hospital Sydney in Australia. Fresh-frozen tumor and matched normal specimens were retrieved from hospital tissue banks. Individuals with hereditary CRC syndromes were excluded. **Supplementary Table 1** shows patient characteristics. In all, 26 of 144 stage II and 176 of 231 stage III patients had received standard neo-adjuvant or adjuvant 5-fluorouracil-based chemotherapy or concurrent chemoradiotherapy. All patients were prospectively followed according to standard protocols, with 3-monthly clinic visits and testing for carcinoembryonic antigen levels, 12-monthly computed tomography scans of the chest, abdomen, and pelvis for 2 years after diagnosis, and then 6-monthly clinic visits and carcinoembryonic antigen testing until 5 years from diagnosis. Outcome data were collected at each clinic visit and entered into a comprehensive multi-site database. The method of detection of any recurrent disease, sites of relapse, and further treatment was also collected. Any missing data were retrieved by data-entry officers. The median duration of follow-up was 32.2 months. All patients gave informed consent, and this study was approved by the medical ethics committees of all sites.

MSI, CIN, LOH and mutation assessment

MSI and CIN were assessed using previously published methods (full details in **Supplementary Methods**). For CIN, the VICTOR tumors were assessed using image cytometry and the community cohort samples using single nucleotide polymorphism microarrays. These two methods were cross-validated for concordance using a panel of well-characterized CRC cell lines of known karyotype (see **Supplementary Methods**). Mutation screening of *KRAS, NRAS, BRAF, PIK3CA*, *TP53*, and *FBXW7* was performed using direct Sanger sequencing (details available from authors). The *TGFBR2* oligonucleotide repeat was not investigated owing to its strong association with MSI and the high prevalence of artifactual insertion–deletion mutations in PCR-based assays. Two microsatellite markers (D18S46 and D18S1110) close to *SMAD4* were used to assess 18q LOH. We also assessed LOH at 5q and 17p (see **Supplementary Data**). At each marker, LOH was considered present when a peak area in the tumor was reduced to 50% of the other allele, relative to the areas in the normal paired DNA. A locus was assigned as showing LOH if at least one informative (heterozygous, non-unstable) marker showed LOH.

Statistical analyses

Statistical analyses were conducted using R (R Development Core Team, 2011) and STATA (College Station, TX, USA). Outcome analyses for primary CRC were performed for disease-free survival (DFS) right truncated at 5 years, which was the primary endpoint of the VICTOR clinical trial. DFS was defined as time from surgery to the first confirmed relapse, with censoring done when a patient had recurrent disease or was alive without recurrence at last contact. Kaplan–Meier survival curves were generated and univariate survival distributions were compared using a log-rank test for MSI, CIN, measures of LOH, and specific gene mutations. For the last of these, we scored all cancers with previously described pathogenic changes in a gene (see http://www.sanger.ac.uk/genetics/CGP/cosmic/) as 'mutant' and all other cancers as "wildtype"; further details are available from the authors on request.

Cox proportional hazards models were used to estimate survival distributions and HRs in multivariate analyses. Test variables included one or more of MSI, CIN, measures of LOH, and specific gene mutations. In the multivariate analysis, we routinely included as co-variates patient age at diagnosis, gender, cancer location, tumor stage and grade, use of radio- or chemotherapy, and randomization to rofecoxib treatment. For analyses in which both the VICTOR and Community data sets were included, the study of origin of the patients was additionally included as a co-variate. The validity of the proportional hazards model was confirmed using Schoenfeld residuals in both data sets, while noting for the VICTOR samples some left truncation owing to the post-primary therapy randomization. Likelihood ratio tests and Aikake information criterion analyses were used to compare the nested and non-nested prognostic models that included different explanatory variables.

For pairwise associations between variables, typically between molecular variables and clinico-pathological features, we used

χ^2 or Fisher's exact tests for binary and their categorical variables, and the rank sum test for continuous variables such as patient's age. All statistical analyses were two-sided and considered significant if $P<0.05$.

RESULTS

Clinicopathological and molecular features of VICTOR patients and their cancers

In the cohort of 822 VICTOR patients, the median age at presentation was 64 years and nearly two-thirds were male (**Table 1**, **Supplementary Table 1**). The median length of rofecoxib treatment was 11.7 months for the 417 patients in this trial arm. The VICTOR cancers had clinico-pathological and molecular features that were typical of stage II/III CRCs. Cancers were distributed in expected proportions throughout the large bowel and were almost evenly split between stages II and III. Thirteen percent of tumors were MSI+ and 67% had CIN (**Tables 1** and **2**). MSI and CIN were almost mutually exclusive ($P<0.001$), only 2.7% of cancers being MSI+/CIN+. Twenty-two percent of cancers were "double-negative" (MSI−/CIN−). As expected, MSI+/CIN− tumors were more common in females,

Table 1. Clinical, pathological, and molecular features of CRC patients from the VICTOR and community-based cohorts according to MSI and CIN status

Characteristic	All	MSI+/CIN− *N* (%)	MSI−/CIN+ *N* (%)	MSI+/CIN+ *N* (%)	MSI−/CIN- *N* (%)	*P* value
VICTOR cohort						
Total *N*	822	88 (10.7)	533 (64.8)	22 (2.7)	179 (21.8)	
Age, years						
Mean±s.d.	64.2±9.9	64.4±11	64.1±9.6	66.8±10.3	64±9.9	0.683
Gender						
Male	532	43 (8.1)	353 (66.4)	11 (2.1)	125 (23.5)	0.002*
Female	290	45 (15.5)	180 (62.1)	11 (3.8)	54 (18.6)	
Site						
Proximal colon	281	69 (24.6)	143 (50.9)	12 (4.3)	57 (20.3)	<0.001*
Distal colon	339	12 (3.5)	245 (72.3)	5 (1.5)	77 (22.7)	
Rectum	202	7 (3.5)	145 (71.8)	5 (2.5)	45 (22.3)	
Stage						
II	402	58 (14.4)	232 (57.7)	11 (2.7)	101 (25.1)	<0.001*
III	420	30 (7.1)	301 (71.7)	11 (2.6)	78 (18.6)	
Community cohort						
Total *N*	375	54 (14.4)	262 (69.9)	8 (2.1)	51 (13.6)	
Age, years						
Mean±s.d.	68.0±12.5	71.8±11.3	67.6±12.4	64.5±16.4	66.5±13.1	0.128
Gender						
Male	205	18 (8.8)	153 (74.6)	1 (0.5)	33 (16.1)	<0.001*
Female	170	36 (21.2)	109 (64.1)	7 (4.1)	18 (10.6)	
Site						
Proximal colon	167	46 (27.5)	90 (53.9)	7 (4.2)	24 (14.4)	<0.001*
Distal colon	119	8 (6.7)	94 (79.0)	1 (0.8)	16 (13.4)	
Rectum	89	0 (0.0)	78 (87.6)	0 (0.0)	11 (12.4)	
Stage						
II	144	32 (22.2)	90 (62.5)	3 (2.1)	19 (13.2)	0.008*
III	231	22 (9.5)	172 (74.5)	5 (2.2)	32 (13.9)	

CIN, chromosomal instability; CRC, colorectal cancer; MSI, microsatellite instability.
P values are derived from ANOVA or χ^2 test across all groups. *$P<0.05$.

Table 2. Association between DFS and specific somatic mutations, including 18q LOH, in VICTOR (Cox proportional hazards single variable analysis, adjusted for clinicopathological variables)

Mutation/change	Frequency (%)	HR	SE	95% CIs	*P*
KRAS	35	1.18	0.18	0.88–1.59	0.273
BRAF	10	1.07	0.26	0.66–1.73	0.790
NRAS	4	0.82	0.34	0.36–1.85	0.631
PIK3CA	12	0.96	0.23	0.60–1.52	0.848
TP53	44	0.92	0.15	0.66–1.27	0.606
CDC4/FBXW7	5	0.96	0.37	0.45–2.06	0.914
LOH18q	48	1.21	0.18	0.90–1.62	0.200
MSI	13	0.49	0.14	0.28–0.86	0.013
CIN	68	1.63	0.30	1.15–2.33	0.007

CI, confidence interval; CIN, chromosomal instability; DFS, disease-free survival; HR, hazard ratio; LOH, loss-of-heterozygosity; MSI, microsatellite instability; SE, standard error. MSI and CIN are also shown, for comparison.

and tended to be proximally located and of stage II, whereas MSI − /CIN + tumors tended to be distally located and of stage III ($P < 0.05$ for all comparisons; **Table 1**). MSI − /CIN − cancers tended to be from males, distally located, and of stage II (**Table 1**).

The frequencies of mutations in *KRAS, NRAS, BRAF, PIK3CA, TP53*, and *FBXW7,* and of LOH at 5q, 17p, and 18q were close to those found in other studies (**Table 2**). In brief, proximal tumors tended to have *BRAF* mutations, and distal tumors to have *TP53* mutations and 17p LOH. Stage was not associated with any specific mutation, although higher grade (poorer differentiation) was associated with the presence of *BRAF* mutation and absence of *KRAS* mutation (details not shown). Overall, we confirmed that the previously described pairwise associations between MSI, CIN, and the specific gene mutations were present in VICTOR (full details reported in (17)). In summary, MSI showed a strong positive association with *BRAF* mutation and a strong negative association with *TP53* mutation. CIN showed the reverse associations, and was also associated with mutation at *NRAS*. *PIK3CA* and *KRAS* mutations were associated, but mutations at *KRAS, BRAF*, and *NRAS* were nearly mutually exclusive. *FBXW7* mutation was not associated with any other molecular variable.

No prognostic value of specific mutations in VICTOR

We focused on DFS, an appropriate measure for CRC treated in the adjuvant setting (38) and the primary endpoint of the VICTOR clinical trial, as the measure of outcome. We tested whether specific gene mutations (in *KRAS*, *NRAS, BRAF*, *PIK3CA*, *TP53*, and *FBXW7*) and 18q LOH predicted DFS in VICTOR. In a single-variable analysis adjusted for age at diagnosis, gender, tumor site, stage, and treatment, no mutation showed an association with survival (**Table 2**). Similarly, in forward and reverse multivariate stepwise analyses, no specific gene mutation was a significant independent predictor of DFS (details not shown).

MSI and CIN are independent predictors of prognosis in stage II/III CRC

We tested the molecular phenotypes MSI and CIN for association with prognosis in the VICTOR cases, adjusting as above for age at diagnosis, gender, tumor site, stage, and treatment. In single-variable analyses, both MSI (HR = 0.49 and $P = 0.013$) and CIN (HR = 1.63 and $P = 0.007$) were individually associated with DFS (**Table 2**). There was no association with randomization to rofecoxib (details not shown). In a multivariate analysis incorporating CIN and MSI, both of these variables showed borderline statistical significance for association with DFS (HR = 0.58, $P = 0.077$ and HR = 1.44, $P = 0.051$, respectively).

To clarify this finding, we combined the VICTOR cohort with an independent set of stage II/III CRC patients from an Australian population-based cohort (see Methods). This second cohort showed similar associations between MSI, CIN, and DFS in an adjusted, multivariate analysis (HR = 0.60, $P = 0.193$ and HR = 1.76, $P = 0.039$, respectively).

In a combined analysis of VICTOR and the population cohort adjusted for study, we found that both MSI and CIN were significant independent markers of DFS (for MSI, HR = 0.58, 95% CI 0.36–0.93, $P = 0.021$; for CIN, HR = 1.54, 95% CI 1.14–2.08, $P = 0.005$). Joint CIN/MSI testing significantly improved on the prognostic value of MSI alone ($P = 0.028$, likelihood ratio test).

We classified CRCs into four groups based on the MSI and CIN status, with the *caveat* that there were only 30 patients in the MSI + /CIN + group (**Table 1**). Compared with MSI − /CIN − cancers, MSI + /CIN − and MSI + /CIN + cases did not have significantly different DFS (MSI + /CIN − HR = 0.68, 95% CI 0.40–1.18, $P = 0.171$; MSI + /CIN + HR = 0.60, 95% CI 0.21–1.68, $P = 0.330$),

while MSI−/CIN+ cases showed significantly poorer outcomes (HR = 1.63, 95% CI, 1.18–2.27; $P = 0.003$; **Figure 1**, **Supplementary Figure 1** online).

We found no evidence that incorporating individual mutations into the model alongside MSI and CIN provided an improved prediction of DFS (details not shown).

Higher levels of CIN are associated with poorer prognosis

We wondered whether cancers with a high level of CIN had poorer outcomes than those with a low CIN level. For the population cohort, we converted autosome numbers to DNA index values using the strong correlation between these two measures in a set of samples assessed using both methods (DI = 0.0227*autosome number + 0.0592, $R^2 = 0.865$; **Supplementary Methods**). The median DI values did not differ significantly between VICTOR and the population cohort ($P = 0.83$, Wilcoxon test). In a study-adjusted analysis of only MSI− CIN+ cancers, there was suggestive evidence that DI provided additional prognostic information (HR = 1.42, 95% CI 0.97–2.08, $P = 0.071$) and an analysis by CIN quintile showed that DFS worsened monotonically with higher DI (details not shown).

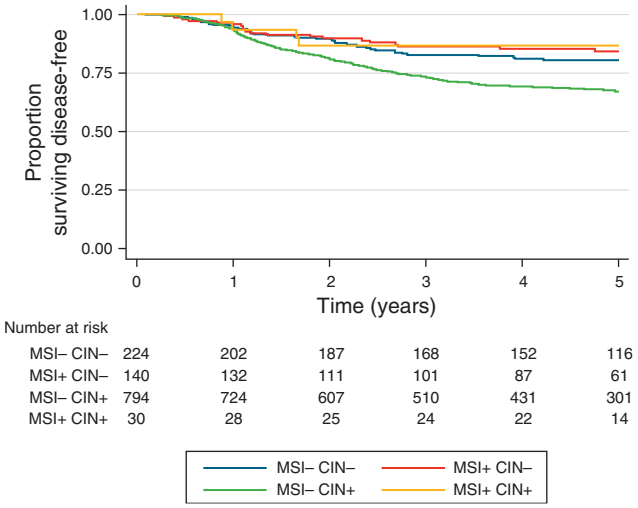

Figure 1. Kaplan–Meier plots for DFS for patients from VICTOR and the Community Cohort combined, according to MSI+ and CIN status. The equivalent plots for each study separately are shown in **Supplementary Figure 1** online.

We therefore undertook analysis of three alternative measures of the extent of CIN to refine the conventional CIN+ vs. CIN− classification (which uses a near-diploid vs. "other" cutoff). The three alternative CIN measures were: (i) CIN-low vs. CIN-high based on a DI of 1.6 (corresponding to 66 autosomes), (ii) DI as a quantitative trait, and (iii) a mixed binary-quantitative CIN/DI variable, in which CIN− cases were scored as 0 and CIN+ cases by their DI. These three alternative measures of CIN were assessed in a multivariate, adjusted analysis that additionally incorporated MSI, and each model was compared with the baseline CIN+ vs. CIN− model. Models were assessed by their likelihood ratio statistic and Aikake information criterion. Only the mixed binary-quantitative CIN/DI classification performed better than the baseline (for CIN/DI, HR = 1.34, $P = 0.00039$; for MSI, HR = 0.61, $P = 0.044$; **Figure 1**, **Supplementary Figure 1**, **Table 3**).

LOH is a good surrogate for CIN, but an inferior indicator of DFS

Recently, Watanabe *et al.* (39) studied survival in 1,103 patients, using LOH at chromosomes 2p, 5q, 17p, and 18q as a surrogate for CIN. They reported poor prognosis for MSI- tumors with high-frequency LOH, whereas MSI+ tumors and MSI− tumors with low-frequency LOH showed similarly favorable outcomes. We assessed the performance of the LOH-based measure of CIN proposed by Watanabe *et al.* Cancers were scored using the Watanabe LOH ratio and classified into "CIN-low", "CIN-high-mild" and "CIN-high-severe" bins as previously proposed

Table 3. Comparison of models in which the degree of CIN is measured as: (i) a binary trait, (ii) quantitative DNA index, (iii) mixed binary-quantitative CIN/DI, and (iv) Watanabe system ("CIN-low," "CIN-high-mild," and "CIN-high-severe"). Simple presence or absence of CIN is also shown as a baseline (v). In all models, MSI is also included as a predictive variable

	CIN				MSI				Overall	
Measure	**HR**	**SE**	**95% CIs**	***P***	**HR**	**SE**	**95% CIs**	**P**	**Ln likelihood**	**AIC**
CIN high vs. low	1.33	0.16	1.05–1.70	0.019	0.51	0.12	0.32–0.80	0.004	−1866.1	3750.1
DNA index	1.64	0.25	1.22–2.22	0.001	0.53	0.13	0.34–0.84	0.007	−1863.7	3745.4
Mixed CIN	1.37	0.12	1.15–1.64	0.0004	0.61	0.15	0.38–0.99	0.044	−1862.2	3742.3
Watanabe	1.34	0.14	1.09–1.65	0.006	0.58	0.14	0.36–0.94	0.027	−1864.8	3747.7
CIN+ vs. CIN−	1.68	0.28	1.22–2.32	0.002	0.64	0.16	0.39–1.04	0.070	−1863.3	3744.7

AIC, Akaike information criterion; CI, confidence interval; CIN, chromosomal instability; DI, DNA index; HR, hazard ratio; MSI, microsatellite instability; SE, standard error. The table shows the output of multivariate Cox proportional hazards analyses adjusted for clinico-pathological variables and study. In order to compare the model performance, analysis was restricted to the 1,076 samples for which all relevant data were available. The relative likelihood of two models is given by $\exp((AIC_1 - AIC_2)/2)$. Thus, the model in which the mixed CIN variable is used fits about five times better than the next-best model and 15 times better than the Watanabe variable.

(39). There was a strong association between LOH ratio and DI ($P < 0.001$, trend test). The Watanabe LOH ratio was a good independent predictor of DFS in an adjusted, study-adjusted analysis with MSI (for LOH ratio, OR = 1.34 and $P = 0.006$; for MSI, OR = 0.58 and $P = 0.027$; **Supplementary Table 2**). However, the LOH ratio was inferior to all other measures of CIN (**Table 3**) and provided a 15-fold worse fit than the mixed CIN/DI variables as a predictor of DFS in combination with MSI (**Table 3**).

DISCUSSION

We have used the VICTOR clinical trial and a population cohort to show that MSI and CIN are independent markers of DFS in stage II/III CRC: MSI indicates good prognosis and CIN, poor prognosis. It is therefore advisable to determine both of these molecular phenotypes if using molecular markers to assess prognosis in clinical practice. We have also shown that the degree of CIN—specifically, a mixed binary-quantitative variable in which CIN − cancers score as zero and other cancers score their DNA index—is a better prognostic indicator than simple presence or absence of CIN. In keeping with these findings, we have shown that patients with MSI − /CIN − (double-negative) CRCs have better prognosis than those with MSI − /CIN + CRCs. Furthermore, the outcome of MSI + /CIN + cancers in our data sets was similar to that of MSI + CIN − cancers, consistent with the similar mutation spectra of these tumor types (17).

In the VICTOR trial cohort, we found that no specific gene mutation (*KRAS, NRAS, BRAF, PIK3CA, TP53*, and *FBXW7*) was predictive of prognosis, either as a single-variable analysis or in a multivariate analysis with MSI and CIN. While *NRAS* and *FBXW7* have not been analyzed as prognostic markers previously, the other mutations have been considered, although some studies have not performed adjusted analyses and very few, if any, have incorporated MSI and CIN into their models. The most consistent evidence in the literature that single mutations can predict outcome relates to *BRAF*. However, this effect may principally be manifest in metastatic disease. The lack of *BRAF* predictive effect in our data is consistent with recent findings from Gavin *et al.* (40) who showed that in stage II/III CRC, *BRAF* mutation is principally an indicator of poor prognosis after relapse and is hence associated with overall, but not DFS, survival.

There exist two large studies that have investigated the prognostic values of both MSI and CIN in resected stage II/III CRCs. Sinicrope *et al.* (41) studied 528 patients and observed significantly poorer prognosis associated with the presence of CIN in MSI − tumors but not MSI + tumors. More recently, Watanabe *et al.* (39) studied survival in 1,103 patients and reported poor prognosis for MSI − tumors with high-level CIN, whereas MSI + tumors and MSI − tumors with low-level CIN showed similar favorable outcomes. However, neither of these studies showed that MSI and CIN were independent predictors of prognosis. In the case of Watanabe *et al.* (39), moreover, CIN was not measured directly, but assessed using a plausible, but non-validated, proxy measure of LOH on chromosomes 2p, 5q, 17p, and 18q. Not only may LOH at these sites often be copy-neutral (42), but the sites are the locations of CRC driver genes (such as *MSH2*, *APC*, *TP53*, and *SMAD4*), leaving it unclear to what extent the differential outcomes reflected CIN rather than the inactivation of these tumor suppressors. Moreover, the use of microsatellites means that LOH scores are difficult to assess in MSI + cancers. Our own analysis based on 5q, 17p, and 18q microsatellites—2p LOH is uncommon—showed that the Watanabe LOH score was an independent predictor of DFS, but performed less well than the direct measures of CIN.

It is necessary to add some caveats, many shared by other similar studies, to our conclusions. In order to achieve sufficient statistical power in some comparisons, we performed adjusted analyses of two different cohorts that had very similar patient profiles, but were non-identical in their recruitment and follow-up. For example, relapses in VICTOR were likely to have been detected earlier than in the community cohort. While such factors may introduce noise into an analysis of molecular markers, this does not of itself introduce bias into an adjusted analysis. Moreover, although we used two different methods for scoring CIN, these were cross-validated at a quantitative level (**Supplementary Methods**). Finally, our findings are not readily applicable to metastatic CRC.

In this study, we have demonstrated that MSI and CIN are independent predictors of the risk of relapse in stage II and III CRC after adjusting for standard clinicopathological variables. We have further demonstrated that higher level CIN is associated with poorer DFS. In joint analyses with mutation data from *KRAS, NRAS, BRAF, PIK3CA, FBXW7*, and *TP53*, MSI and the presence or extent of CIN remained the only independent prognostic predictors. Our finding of a monotonic association between CIN and poor outcome contrasts with reports in breast, ovarian, gastric, and lung cancers, in which CIN-intermediate tumors had the worst prognosis (43). In clinical diagnostic practice, we suggest that both MSI and CIN testing are useful for prognostic indication in CRC. The method used to detect CIN should be semi-quantitative or quantitative, and ideally should not use microsatellite markers. Currently, MSI testing, or mismatch repair protein immunohistochemistry, is performed more frequently than CIN analysis, but a much larger proportion of CRCs are CIN + than MSI +, and joint CIN/MSI testing significantly improves the prognostic value of MSI alone. Combined MSI and CIN typing has the potential to improve identification of stage II/III CRC patients who may benefit from more aggressive investigation and therapy. We anticipate that, as large-scale molecular profiling of CRCs becomes technically feasible and more affordable, increasingly refined prognostic classifiers will eventually be developed.

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CONFLICT OF INTEREST

Guarantor of the article: Oliver M. Sieber, PhD.

Specific author contributions: Data acquisition: D.M., E.D., S.L., P.Y.S., H.E.D., D.O., M.N., and P.M.; acquisition of samples and clinicopathological data: P.G., L.L., J.D., I.T.J., S.M., N.J.H., R.W., R.M., and D.K.; development of SNP microarray analysis algorithm: C.Y. and C.C.H.; data analysis: D.M., E.D., H.E.D., R.N.J., I.P.M.T., and O.M.S.; concept design and writing: I.P.M.T. and O.M.S.

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Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ Colorectal cancers (CRCs) acquire two major forms of genomic instability, microsatellite instability (MSI), and chromosomal instability (CIN), although some tumors have neither and a few have both.
- ✓ MSI is known to predict good prognosis in stage II/III colorectal cancer.
- ✓ Some evidence exists that CIN also predicts prognosis, and that this association may be independent of MSI.
- ✓ Individual mutations (e.g. KRAS, BRAF, p53, *et cetera*) have also been found to predict prognosis in some studies.

WHAT IS NEW HERE

- ✓ We have studied a large sample of stage II/III CRCs from a clinical trial and a second population-based set of CRCs.
- ✓ We show that MSI and CIN are independent predictors of prognosis in CRC after correction for known prognostic factors such as stage.
- ✓ We show that no individual mutation predicts prognosis independently.
- ✓ We show that the proposed CIN surrogate based on loss-of-heterozyosity by Watanabe *et al*. performs worse than CIN measured directly.
- ✓ We show that the more severe the degree of CIN, the worse the prognosis in CRC; this is different from some other cancer types.
- ✓ The findings have implications for those who use molecular testing to obtain prognostic information for stage II/III CRC.

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