Regulator of Chromosome Condensation 2 Identifies High-Risk Patients within Both Major Phenotypes of Colorectal Cancer

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Abstract

Purpose: Colorectal cancer has high incidence and mortality worldwide. Patients with microsatellite unstable (MSI) tumors have significantly better prognosis than patients with microsatellite stable (MSS) tumors. Considerable variation in disease outcome remains a challenge within each subgroup, and our purpose was to identify biomarkers that improve prediction of colorectal cancer prognosis.

Experimental Design: Mutation analyses of 42 MSI target genes were performed in two independent MSI tumor series (n = 209). Markers that were significantly associated with prognosis in the test series were assessed in the validation series, followed by functional and genetic explorations. The clinical potential was further investigated by immunohistochemistry in a population-based colorectal cancer series (n = 903).

Results: We identified the cell-cycle gene regulator of chromosome condensation 2 (RCC2) as a cancer biomarker. We found a mutation in the 5′ UTR region of RCC2 that in univariate and multivariate analyses was significantly associated with improved outcome in the MSI group. This mutation caused reduction of protein expression in dual luciferase gene reporter assays. siRNA knockdown in MSI colon cancer cells (HCT15) caused reduced cell proliferation, cell-cycle arrest, and increased apoptosis. Massive parallel sequencing revealed few RCC2 mutations in MSS tumors. However, weak RCC2 protein expression was significantly associated with poor prognosis, independent of clinical high-risk parameters, and stratifies clinically important patient subgroups with MSS tumors, including elderly patients (>75 years), stage II patients, and those with rectal cancer.

Conclusions: Impaired RCC2 affects functional and clinical endpoints of colorectal cancer. High-risk patients with either MSI or MSS tumors can be identified with cost-effective routine RCC2 assays. Clin Cancer Res; 1–12. ©2015 AACR.

Introduction

Each year, approximately 1.4 million patients are diagnosed with colorectal cancer, and only half of them survive 5 years (1). About 15% of the tumors have the microsatellite instability phenotype (MSI), which is caused by a malfunctioning DNA mismatch repair (MMR) system leading to accumulation of insertions and deletions (indels) in repetitive elements throughout the genome (2). Patients with nonhereditary MSI tumors have better prognosis than those with microsatellite stable (MSS) tumors (3–5), and MSI is currently implemented in clinical guidelines as a prognostic biomarker (6; www.nccn.org). However, considerable variation in disease outcome remains a challenge also within the MSI subgroup. For the other 85% of colorectal cancers, staging remains the main crude prognostic factor, which underlines the need for robust biomarkers that can stratify these patient groups and improve treatment strategies.

A number of target genes with indels in microsatellites have been reported in MMR-deficient colorectal cancer (7), and many studies have evaluated their prognostic impact, but typically only few genes have been investigated at a time. Their ability to predict disease outcome remains inconclusive.

The cell-cycle gene regulator of chromosome condensation 2 (RCC2) was identified as a potential target gene in MMR-deficient colorectal cancer.
colorectal cancer by a systematic database search in 2002 (8). 

RCC2 is a member of the RCC1 superfamily and is predicted to 

form a seven-bladed beta-propeller protein essential for the 

function of the chromosomal passenger complex (CPC; ref. 9). 

RCC2 has also been implicated as a negative dual regulator of 

RAC1 and ARF6, promoting directional cell migration (10). 

However, reports on the relation between 

RCC2 and cancer have been scarce, and to our knowledge, no direct links have been 

reported. 

Here, we investigated the mutation status for 42 reported 
target genes of MMR-deficient colorectal cancer in two inde-

pendent series to test the hypothesis that such mutations in 
cancer-critical genes provide a selective growth advantage 
and confer prognostic information. The functional, genetic and 
clinical relevance of the single gene, RCC2, that demonstrated 
prognostic relevance in the MSI group was further studied in 
cell line models as well as in a population-based consecutive 
series of colorectal cancer (MSI and MSS groups).

Materials and Methods

Patient samples

DNA was extracted for mutation analyses from 81 MSI 
tumors (referred to as the Scandinavian series) from colorectal 
cancer patients admitted to Swedish (n = 44; ref. 11) and 
Norwegian (n = 37; ref. 3) hospitals. Of these, clinical data 
and long-term patient follow-up data were available for the 37 
Norwegian samples (referred to as the test series; Supplementary 
Table S1).

Validation and further experiments were based on a popula-
tion-based consecutive series consisting of 1,290 colorectal cancer 
patients admitted to Oslo University Hospital (OUS; Aker, Nor-
way) in 1993–2003 (ref. 12; see Supplementary Fig. S1). Accor-
ding to national health policies, all patients with a newly diagnosed 
colorectal cancer and living within defined catchment areas should be referred to the local hospital for treatment. OUS-Aker served a population of more than 200,000 inhabitants in five 
districts within or neighboring the city of Oslo, the capital of Norway, and close to 100% of the colorectal cancer patients who were treated in this hospital were included in the study. Of the 

1,290 patients, 929 patients underwent major resection, and the formalin-fixed paraffin-embedded (FFPE) tissue was collected in a 
tissue microarray (TMA), including 670 colonic, 233 rectal, and 
26 synchronous carcinomas. From the same series, DNA was 
extracted for mutation analyses from 128 MSI tumors (referred to 
as the validation series, Supplementary Table S2). More than 
95% of patients were of Caucasian ethnicity (based on name 
orign). All patient samples were analyzed retrospectively.

The study was approved by the Regional Committee for Med-
ical and Health Research Ethics, South-Eastern Norway and the 
Norwegian Data Inspectorate. The research conformed to the 
Declaration of Helsinki and the research biobanks have been 
registered according to national legislation. The approved project 
and amendments (REK number 1.2005.1629) require that 
inform consent is obtained from patients being enrolled to 
the study.

Cell lines

HCT15, HT29, HCT116, LS1034, RKO, SW480, SW48, and 
HeLa CCL-2 cell lines were purchased from the ATCC and 
maintained according to the ATCC recommendations. Identi-
ties were verified by fingerprinting according to the AmpF/STR 
Identifiler PCR Amplification Kit (Life Technologies by Thermo 
Fisher Scientific), and matched to the profiles reported by 
ATCC. Cell lines were regularly tested for mycoplasma con-
tamination according to the MycoAlert Mycoplasma Detection 
Assay (Lonzza Cologne AG).

Determination of MSI

MSI status was determined using the consensus markers pro-
vided by the NCI as previously described (12).

Mutation analyses of gene targets downstream of MMR 
deficiency

Fragment analyses of the microsatellite containing regions of 
42 genes were performed (details in Supplementary Table S4). 
Electropherograms were visually examined for indels by 
two researchers independently (T.C. Ahlquist and E.C. Rayrvik), 
against corresponding fragments from DNA from four different 
disease-free individuals (Supplementary Fig. S6). Assays were 
duplicated in tandem runs using different PCR machines. Each 
gene was informative for at least 159 of the 209 samples in the test 
and validation series.

Mutation verification by sequencing

To validate the fragment analysis results, 18 of the 42 genes 
(ACVR2A, AIM2, ASTE1, AXIN2, BLM, EPHB2, GRK4, MBD4, 
PTHLH, RAD50, RBBP8, RCC2, SEMG1, SLC23A2, SYCP1, TAF1B, 
WISP3, and ZMYND8) were sequenced in a random selection of 
11 colorectal cancer tissue samples and 8 colorectal cancer MSI 
cell lines (details in Supplementary data).

Massive parallel sequencing

Whole-transcriptome paired-end sequencing was performed 
as previously described for seven cell lines (HCT15, HCT116, 
HT29, SW480, SW48, LS1034, and RKO; ref. 13). Whole-exome 
paired-end sequencing was carried out using the Illumina 
TruSeq DNA Sample Prep Kit, followed by the Illumina TruSeq 
Exome Enrichment Kit (Illumina Inc.), and sequencing was 
subsequently performed on an Illumina Genome Analyzer Ixl
for nine samples (tumor and normal tissue). Whole-genome paired-end sequencing was performed by BGI for the four colorectal cancer cell lines: HCT15, HCT116, HT29, and SW480. The sequence reads were aligned by BWA version 0.6.1 (14). Mutation data from all sequenced tumors by The Cancer Genome Atlas (TCGA) was accessed via the cbioPortal (http://www.cbioportal.org/public-portal/; ref. 15) on December 16, 2014.

Reporter gene assays
A dual luciferase reporter system (Promega) was used to determine reporter activity from vector constructs with mutated and wild-type RCC2 (Supplementary Fig. S3) in three cell lines: HCT15, HT29, and HeLa (details in Supplementary data).

Primary antibodies
For Western blotting, rabbit polyclonal anti-RCC2 antibodies were obtained from Novus Biologicals (Cat. No. NB110-40618), recognizing an N-terminal epitope between residue 1 and residue 50. Mouse monoclonal anti-β-actin antibodies were obtained from Sigma (Clone AC-47, Cat. No. A 5316), recognizing an N-terminal epitope of the β-isoform. Antibodies were employed at dilutions of 1:500 and 1:1,000, respectively. For immunocytochemistry and immunohistochemistry, rabbit polyclonal anti-RCC2 antibodies were obtained from Novus Biologicals (Cat. No. NB110-40619), recognizing a C-terminal epitope between residue 471 and residue 522, and employed at a 1:500 dilution. Alexa Fluor 594 phalloidin (Invitrogen, Cat. No. A12381) was used to visualize actin for immunocytochemistry according to the manufacturer’s instructions at a 1:40 dilution.

siRNA knockdown, Western blotting, and immunocytochemistry
Two siRNA oligonucleotides targeted against RCC2 were used to deplete endogenous RCC2 protein in HCT15 cells: Invitrogen Stealth Select RNAi RCC2HS125252 (I) and RCC2HS125253 (II). The first RCC2 siRNA (I) had the following sequence (sense strand): 5'-CCU GGU GAA GCU GUU UGA CUU CCC U-3'. The sequence (sense strand) of the second RCC2 siRNA (II) was: 5'-GGG CUU CCC AGA UCU AUG CUG GUU A-3'. Invitrogen Stealth RNAi Negative Control (Medium GC) served as negative control. The transfection reagent used was Lipofectamine 2000 (Invitrogen by Life Technologies) and the transfection procedure was carried out following the manufacturer’s instructions, at a final concentration of 80 nmol/L.

Western blotting and immunocytochemistry were employed to confirm knockdown of RCC2 protein expression and to visualize protein expression and cell morphology, respectively. Detailed protocols are reported elsewhere (16).

Flow cytometry
Flow cytometry analysis of cell-cycle distributions and levels of apoptosis was carried out using a terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay where treatment with terminal transferase (TdT) followed by staining with Streptavidin–PE and Hoechst 33258 was employed. A Becton Dickinson LSR II flow cytometer was used for the analysis. Data analysis was carried out with BD FACSDiva Software version 5.0.3 and ModFit LT Version 3.2.1 (Verity Software House). Detailed protocol is reported elsewhere (16).

Immunohistochemistry
The immunohistochemical (IHC) analysis was performed on FFPE tissue as described (ref. 17; details in Supplementary Data).

Statistical analysis
All statistics were performed using the SPSS 21.0 software (SPSS). Five-year overall survival (OS) and five-year time-to-recurrence (TTR) plots were generated using the Kaplan–Meier method. The log-rank test was used to compare survival curves. TTR and OS were defined according to the guidelines given by Punt and colleagues (18) where TTR is defined as the time from surgery to the first event of either death from the same cancer, local recurrence, or distant metastasis. Patients were censored at death from other cancer, noncancer death, and postoperative death (<3 months). OS was defined as the time from surgery to death from any cause. No patients were lost to follow-up in the study period.

Multivariate Cox proportional hazards regression modeling (forward selection) was employed to determine all available parameters with significant independent impact on patient survival. Age and gender were included as background variables. Tumor location was also included in the protein expression analyses for informative purposes, and it did not affect the multivariate models. Cases with missing data were excluded from analyses. Adjuvant treatment for patients with stage III colon cancer (<75 years of age) became standard treatment in 1997 and was considered in initial multivariate models. These patients were few and adjustment did not affect the models. Adjustment for pre- and postoperative radiotherapy for rectal cancer patients was also considered, but was pertinent to only a very limited number of patients and therefore not included in initial models. Tumor location was excluded as a covariate in the multivariate mutation analyses of patients with MSI tumors, as there are very few cases in the distant colon and the rectum category as compared with the proximal category. Also, for the covariate histopathologic grade, the G1 and the G2 category were combined to make larger and more robust groups for comparison. We believe this is reasonable as G1 and G2 are similar with regard to prognosis, and there is in addition considerable inter-pathologist variation in this assessment. Interaction tests were integrated in the Cox models to assess whether effects were different between subgroups, but must be interpreted carefully due to the low power of such tests. The proportional hazards assumptions were verified by graphical evaluation of plots of log(-log survival time) versus log time. Bonferroni correction was used to correct for multiple testing in the mutation analyses for the validation series.

For the analysis of RCC2 mutation and protein expression in relation to other clinicopathologic data, Wilcoxon rank-sum test (exact) was used to compare two groups with regard to an ordinal variable and Fisher exact test was used to compare categorical variables. Student t test for paired samples was used to estimate P values for comparisons in the dual luciferase experiments, siRNA knockdown experiments, cell proliferation experiments, and the flow cytometry experiments. For the dual luciferase experiments, ANOVA was also carried out of the
relative expression for the two vectors. Here, the cell lines were fixed factors and the experiments nested within each cell line as random factor.

All P-values were two-tailed, and considered statistically significant at P \( \leq 0.05 \) unless otherwise stated in the text.

**Results**

**Mutation status of multiple cancer-critical target genes in colorectal cancers with MSI**

The mutation frequencies of 42 known target genes in MMR-deficient colorectal cancer were assessed in two independent series, the Scandinavian series (n = 81) and the validation series (n = 122; Fig. 1). Thirty-seven genes were mutated in more than 12% of the samples in both series (Fig. 1A). The mutation frequency for each gene varied from 6% to 91% in the Scandinavian series and from 4% to 92% in the validation series, with a median of 17 (range 0–28) and 19 (range 0–29) mutated genes per sample, respectively.

Mutations in RCC2 were located in the mononucleotide (A)\( _{10} \) repeat within the 5’ UTR and were identified in 62% of all MSI tumors, 51% (41/81) and 69% (84/122) in the two series, respectively. Deletion of one or two bases (103/125 and 19/125, respectively) were far more common than any other alterations in both series.

The RCC2 5’ UTR mutation is an independent marker for improved outcome in patients with MSI

The mutation status of all 42 MSI target genes was analyzed for association with patient outcome in the test series (n = 37; 37 Norwegian patients from the 81 Scandinavian series) to identify the best candidate markers, which were then analyzed in the larger validation series (n = 122). From the six candidates identified by univariate 5-year TTR analysis (ACVR2A, AXIN2, EP300, MRE11A, OGT, and RCC2, Supplementary Table S5), only the RCC2 mutation was found to be significantly associated with outcome in both clinical series (Fig. 1B and C). Stratification by tumor stage in the validation series showed that for stage II patients after 5 years follow-up, only 12% of the patients with mutated RCC2 had experienced recurrence as compared with 39% for patients with wild-type RCC2 (Fig. 1E). The corresponding figures for stage III MSI patients where 44% and 55%, respectively. There were too few cases and/or events for robust analysis of stage I and IV patients with MSI.

Cox proportional hazards models were created (Table 1), and the RCC2 mutation status demonstrated significant prognostic impact, independently of clinical high-risk parameters (P = 0.0080). This result is significant according to standard Bonferroni correction for multiple testing (0.05/6 = 0.0083).

Cox models for the test series show a similar association (P = 0.054), although this series is too small to give robust estimates for the various covariates (Supplementary Table S6).

An additional more stringent analysis was performed post hoc in the validation series, restricted to patients with right-sided tumors who were alive 3 months after a complete resection (R0, no evidence of residual tumor), and who were followed for at least 36 months (n = 60). The association between RCC2 mutation status and TTR remained significant in univariate (P = 0.038, Fig. 1D) and multivariate analysis (P = 0.043).

For the test series and validation series, Wilcoxon and Fisher exact tests showed that mutations in RCC2 were not significantly associated (P < 0.05) with other clinicopathologic data, except surgical resection status (R-status, P = 0.039; Supplementary Tables S1–S3).

**Mutations in the complete gene sequence of RCC2**

To search for mutations elsewhere in the RCC2 gene, we used in-lab datasets from massive parallel sequencing of selected colorectal cancer samples and cell lines.

Three data sets were examined for RCC2 mutations in 16 samples: whole-genome paired-end sequencing data from the four colon cancer cell lines HCT15, HCT116, HT29, and SW480 (performed at BGI), paired-end RNA sequencing datasets from the same four, as well as the three additional colon cancer cell lines SW48, LS1034, and RKO (13), and exome sequencing data of 9 colorectal cancers (one MSI, 7 MSS and one unknown, unpublished data). Altogether, three deletions [one in the 5’UTR (A)\( _{10} \)] mononucleotide repeats (MNR) and two in the 3’ UTR], one missense and one synonymous mutation were found (Fig. 1F). Both the missense and the synonymous mutations were in the RKO cell line (MSI).

In addition, we used publicly available data: TCGA accessed through the cBioPortal (15). The provisional TCGA dataset comprises somatic mutation data from exome sequencing of 223 colorectal cancers. Here, RCC2 was mutated in 6 cases (2.7%), of which 5 were missense mutations (4 in MSI and 1 in MSS) and one was a frameshift deletion (in an MSS tumor; Fig. 1F). This frameshift mutation in codon 189 leads to a premature stop codon and truncation of the polypeptide sequence. The 5’ UTR and the 3’ UTR regions were not covered by the sequence capture protocol used by TCGA.

**The 5’ UTR RCC2 mutation affects mRNA structure**

Secondary structure models of a 184 nucleotide long RNA sequence comprising 122 nucleotides of the 5’UTR and 62 nucleotides of the coding sequence of wild-type and mutant RCC2 were constructed using Mfold (Supplementary Fig. S2).

The models revealed that a three-way RNA junction may be affected by the deletion of an A in the (A)\( _{10} \) repeat. Three-way RNA junctions are important RNA folds which are often involved in distant tertiary contacts, protein binding, and/or RNA binding (19). In the structural model, the wild-type sequence has a single unpaired nucleotide spanning the junction while the mutant has two unpaired nucleotides. This extra nucleotide may affect the flexibility and projection of the helices from this junction having adverse functional effects impinging on translation of the mRNA because either protein- or distant RNA contacts can be disturbed.

**The 5’ UTR RCC2 mutation causes reduced protein expression**

A dual luciferase gene reporter assay was carried out in three different cell lines (HCT15, HT29, and HeLa) to investigate the functional significance of the RCC2 5’UTR (A)\( _{10} \) mutation as compared to the wild-type (A)\( _{10} \). A wild-type or mutant 5’ UTR RCC2 fragment was inserted immediately upstream of the luciferase reporter gene in an SV40 promoter-based plasmid (Supplementary Fig. S3). Luciferase activity was determined following transient transfection. The mutant construct was found to exhibit significantly reduced luciferase activity across the cell lines compared with the wild-type (P = 0.0057, n = 17, two-tailed paired Student t test, Fig. 2). Statistical significance was also evident from an analysis of variance of the relative...
Figure 1. Microsatellite mutations in the 5’ UTR of RCC2 are associated with improved outcome for patients with colorectal cancers of the MSI phenotype. Microsatellite mutation frequencies for MSI target genes in the compiled Scandinavian series and Norwegian validation series (A). Kaplan-Meier TTR analysis (log-rank test) for colorectal cancer patients with MSI tumors stratified by the RCC2 5’ UTR mutation in the test series (B) and the validation series (C), and patients from the validation series with complete resection (R0, no evidence of residual tumor), right-sided tumors, and follow-up data of more than 36 months (D). Subgroup analysis for patients with stage II colorectal cancer (E). Nucleotide-level somatic mutation data in colorectal cancer from the complete length of RCC2 (F). Red pins represent mutations identified from own samples, and yellow pins represent mutations in the dataset from TCGA. Orange, UTRs; blue, coding sequence; green, protein domains. Abbreviations: RLD, RCC1-like domain; NLS, nuclear localization signal (putative); wt, wild type; mut, mutant.
expression for the two vectors, with the cell lines as fixed factors and the experiments nested within each cell line as random factor ($P = 0.024, n = 17$). A median 15% reduction of protein expression was observed.

Quantitative real-time PCR analysis (qPCR) of reporter transcript levels in the three cell lines showed no significant difference between wild-type and mutant, indicating that the observed reduction in mutant reporter activity occurs at the translational level ($P = 0.62, n = 15$, two-tailed paired Student t test).

RCC2 knockdown causes reduced cell growth and altered cell morphology

A colon cancer cell line with MSI, HCT15, was used to investigate the importance of RCC2 in regulation of cell growth and proliferation. Two different siRNA constructs were employed to knock down RCC2, both of which reduced the RCC2 protein level by more than 90% compared with cells transfected with negative control siRNA sequences (mock; Fig. 3). Depletion of RCC2 was associated with a significant 40% to 45% reduction in cell numbers (Fig. 3). Concurrently, RCC2-depleted cells exhibited a change in morphology, appearing larger and more distinctly epithelial-like (Fig. 3), indicating increased cell-spreading activity. Supporting evidence was provided by confocal microscopy analysis revealing that RCC2-depleted cells exhibited an altered, more elongated actin fiber pattern with predominant stress- and filopodia-like fibers (Fig. 3 and Supplementary Fig. S4).

Table 1. Univariate and multivariate analyses of RCC2 5′UTR mutation status in the validation series

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients n (%)</th>
<th>5-year TTR (%)</th>
<th>Univariate analysisa</th>
<th>Multivariate analysisb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>122 (100)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>92 (75)</td>
<td>67.0</td>
<td>0.29</td>
<td>0.64 (0.25–1.66)</td>
</tr>
<tr>
<td>Male</td>
<td>30 (25)</td>
<td>78.6</td>
<td>1</td>
<td>1.03 (0.00–1.06)</td>
</tr>
<tr>
<td>Agec</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RCC2 Wild-type</td>
<td>38 (31)</td>
<td>54.1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Mutated</td>
<td>84 (69)</td>
<td>77.4</td>
<td>0.0071</td>
<td>0.37 (0.18–0.77)</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I+II</td>
<td>83 (68)</td>
<td>83.3</td>
<td>1</td>
<td>6.5 (3.06–13.8)</td>
</tr>
<tr>
<td>III+IV</td>
<td>39 (32)</td>
<td>38.0</td>
<td>$1.8 \times 10^{-8}$</td>
<td>$1 \times 10^{-6}$</td>
</tr>
<tr>
<td>Histopathologic grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1 + G2</td>
<td>69 (57)</td>
<td>78.7</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>G3</td>
<td>47 (38)</td>
<td>60.5</td>
<td>0.063</td>
<td>1.8 (0.88–3.59)</td>
</tr>
<tr>
<td>Mucinousd</td>
<td>5 (4)</td>
<td></td>
<td></td>
<td>0.11</td>
</tr>
<tr>
<td>NDd</td>
<td>1 (1)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: G1, high differentiation; G2, moderate differentiation; G3, poor differentiation.

*aKaplan-Meier estimate (log-rank test).

bCox regression model (Wald test), all included parameters are displayed in the table.

cHRs are given per year of age.

dExcluded from the statistical analyses.

RCC2 knockdown causes G2–M arrest and increased apoptosis

Next, the effect of knockdown of RCC2 in HCT15 cells on cell-cycle progression and apoptosis was determined by flow cytometry. Depletion of RCC2 by siRNA resulted in a 45% decrease of the G0–G1 population ($P = 0.012, n = 3$), a 62% increase of the G2–M population ($P = 0.011, n = 3$) and a 17% increase of the S population ($P = 0.016, n = 3$), as compared with mock-transfected cells (Fig. 3F and G). In addition, the level of aneuploid (>4N) cells was elevated for RCC2-depleted
cells \((P = 0.028, n = 3)\). Interestingly, the level of apoptosis in RCC2-depleted cells was about three times higher than in control transfected cells \((0.6\% \text{ vs. } 1.7\%, P = 0.0077, n = 3)\), as detected by a TUNEL assay (Fig. 3H).

Weak RCC2 protein expression in situ is associated with poor outcome in patients with MSS tumors

We investigated the prognostic value of the in situ protein expression of RCC2 by immunohistochemistry in a consecutive
single-hospital series on a TMA of primary colorectal cancers (n = 903; 781 and 797 evaluable tissue cores for cytosolic and nuclear staining, respectively) tumors.

For cytosolic staining, both OS and TTR analyses showed significant associations with patient outcome (P = 5.2 × 10−7 and P = 8.3 × 10−6, respectively, Fig. 4I–J). The TTR analysis was restricted to R0 patients. Cox proportional hazards regression modeling including relevant background and clinical variables (Table 2) showed significant interaction between RCC2 cytosolic staining and gender, both for OS (P = 0.0059 for the interaction test and P = 0.0015 for RCC2) and for the TTR analyses (P = 0.0037 for the interaction test and P = 1.4 × 10−5 for RCC2). Subsequent subgroup analysis (TTR) for gender revealed significant associations confined to female patients (P = 6.2 × 10−6, Fig. 4K). The strong prognostic value of RCC2 cytosolic staining in females was confirmed in separate multivariate models for gender [P = 1.5 × 10−4; HR 0.37; confidence interval (CI), 0.24–0.58 for females and P = 0.65; HR 0.90; CI, 0.56–1.43 for men]. Interestingly, further stratification on MSS/MSI status showed that this relationship was confined to patients with MSS (Fig. 4L). MSI tumors had stronger cytoplasmic expression compared with MSS tumors (P = 0.012, Fisher exact test, n = 712); however, the number of patients with MSI and events was not sufficient to test for interaction with RCC2 cytosolic staining, nor carry out further subgroup analyses with regard to survival for the MSI group.

Interestingly, Wilcoxon rank-sum test (exact) revealed a significant association between RCC2 cytosolic staining and tumor stage (P = 2.1 × 10−4, n = 777). Weak cytosolic staining was more predominant among stage III and IV tumors than among stage I and II tumors.

In multivariate Cox proportional hazards regression modeling (TTR, including adjustment for gender, age, MSI/MSI-status, tumor location, differentiation, histopathologic grade, and stage) nuclear expression of RCC2 showed significant interaction with gender (P = 0.016; HR 3.0; CI, 1.2–7.3 for the interaction test and P = 0.001; HR 2.9; CI, 1.6–5.3 for RCC2). Nuclear staining stratified by gender and MSS/MSI-status showed significant associations for female patients (P = 0.045) and to MSS patients (P = 0.013, Fig. 4K and L).

Further exploratory subgroup analyses show interesting associations between reduced RCC2 expression and old age, rectal cancer, and early stage (I/II) disease (Supplementary Fig. S5).

Wilcoxon rank-sum test for the MSI cancers did not reveal any significant differences in the level of RCC2 staining between tumors with RCC2 5’UTR mutation as compared with those having wild-type 5’ UTR (cytosolic RCC2 staining: P = 0.47, n = 101; nuclear RCC2 staining: P = 0.50, n = 98).

Discussion

This study connects RCC2 directly to cancer showing that the mutation status of a MNR in the 5’UTR identifies high-risk patients in the MSI group with stage II colorectal cancer. We demonstrate that the mutation causes reduced protein expression in vitro, and that a reduced RCC2 protein level has functional impact on colon cancer cells. We also show that RCC2 protein expression carries prognostic information across all colorectal cancers, but particularly for female patients with MSS tumors, and that mutations in the RCC2 coding sequence are rare.

MRNs in UTRs are evolutionary conserved, indicating a functional role (20). Repeat sequences are prone to alteration in cancers with defect mismatch repair (MSI), and some of the alterations are involved in the regulation of gene and protein expression. UTRs are known to affect mRNA nuclear export, cytoplasmic localization, translational efficiency, and stability (21). The majority of translational control occurs at the level of initiation, thus implicating the 5’ UTR region as an important site for translational regulation (22). The (A)10 repeat in RCC2 is located in exon 1 in the 5’UTR of the gene, 77–86 bases upstream of the start codon. The luciferase reporters with mutant and wild-type RCC2 promoters showed that a single base deletion of this repeat leads to reduced protein expression. It should be noted that a recent study suggested RCC2 to be a downstream target for the known cancer related micro-RNA miR-29c, through its 3’ UTR miR-29c target sequence (23).

Global quantification of mammalian gene expression has shown that protein levels are predominantly controlled at the translational level (24). RCC2 mRNA and protein levels display average half-lives of 9 and 48 hours, respectively. However, the translation rate constant in NIH 3T3 cells is about 230 proteins per mRNA per hour, much higher than the average of 140 (24). This clearly suggests that translational regulation of RCC2, and thereby the effect of a 5’ UTR mutation reducing translation reported here, may have important consequences for the level of RCC2 protein.

Furthermore, we demonstrate that knockdown of RCC2 in colon cancer cells with MSI (HCT15) results in G2–M arrest, in accordance with previous observations in HeLa cells (9). As a protein fundamental to the integrity and proper function of the chromosomal passenger complex, RCC2 plays an important role in cell-cycle regulation, especially during mitosis and cell cleavage. Whether the deletions seen in RCC2 are sufficient to arrest the cells entering M-phase awaits further investigation, but the siRNA knockdown experiments, combined with the luciferase reporter analysis and the positive survival data for MSI tumors reported here, suggest that at least the cells are halted at the G2–M which is disadvantageous for the tumor cells and beneficial for the patient. Deregulation of the mitotic apparatus may cause some mitotic cells to enter apoptosis, but others to halt during mitosis, not completing cytokinesis, and thus creating multinuclear cells. This hypothesis is supported by the TUNEL analysis (Fig. 3H) that showed increased levels of apoptosis, as well as the cell-cycle analysis that demonstrated increased levels of >4N cells when RCC2 was knocked down. The occurrence of large multinuclear cells following RCC2 downregulation was also apparent from the confocal microscopy analysis. Together, these results provide an explanation for why the 5’ UTR mutation in RCC2 is associated with improved survival in MSI patients; tumors with a slightly reduced level of RCC2 are likely to grow and develop less efficiently as more cells will reach mitotic arrest. However, it is important to keep in mind that the anticipated 15% protein reduction resulting from the 5’UTR RCC2 mutation cannot be directly compared with the nearly complete knockdown of RCC2 protein in HCT15 cells, but it makes a functional connection probable. Deleterious mutations would generally not be selected for in a classical driver-oriented paradigm of cancer progression. However, recent evidence suggests that moderately deleterious mutations can evade negative selection.
Identification of High-Risk Patients in Colorectal Cancer

Figure 4.
Weak staining for the RCC2 protein is associated with poor patient outcome, particularly for female colorectal cancer patients with MSS tumors. A–H, differential IHC staining of RCC2 in colorectal carcinomas. Staining of tumor sections using an RCC2 selective antibody demonstrated a specific staining in both the cytosol and the nuclei of the cells. The staining was largely confined to epithelial cells, but some scattered staining was also observed in the tumor stroma. Representative staining of histospots (core diameter, 0.6 mm) is illustrated at 400× magnification. A–D, strong, moderate, weak, and negative nuclear staining, respectively. E, strong nuclear and cytosolic staining. F, predominant staining, respectively. E, strong nuclear staining. G, staining in undifferentiated tissue. H, staining in normal colon. I–L, survival analysis for RCC2 cytosolic (left) and nuclear (right) protein expression in a consecutive colorectal cancer series (n = 903). Green curves represent strong staining and blue curves represent weak staining. I, five-year OS. J–L, five-year TTR analysis for patients with complete resection (R0, no evidence of residual tumor). K, subgroup analysis for gender. L, subgroup analysis for gender and MSS. The Kaplan-Meier method (log-rank test) was used to draw the plots and determine statistical differences between patients with strong and weak RCC2 staining.
Table 2. Univariate and multivariate analyses of cytosolic RCC2 in situ protein expression in a consecutive colorectal cancer series

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients, n (%)</th>
<th>Univariate analysis(^a)</th>
<th>Multivariate analysis(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5-year OS/TTR (%)</td>
<td>P</td>
</tr>
<tr>
<td>Overall survival (OS)</td>
<td></td>
<td>903 (100)</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>474 (52)</td>
<td>49.6</td>
<td>0.40</td>
</tr>
<tr>
<td>Male</td>
<td>429 (48)</td>
<td>51.0</td>
<td></td>
</tr>
<tr>
<td>Age(^c)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RCC2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytosolic staining</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weak</td>
<td>319 (35)</td>
<td>39.5</td>
<td></td>
</tr>
<tr>
<td>Strong</td>
<td>462 (51)</td>
<td>58.4</td>
<td></td>
</tr>
<tr>
<td>ND(^d)</td>
<td>122 (14)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>133 (15)</td>
<td>77.4</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>363 (40)</td>
<td>62.3</td>
<td>1.61 (1.03–2.52)</td>
</tr>
<tr>
<td>III</td>
<td>237 (26)</td>
<td>47.3</td>
<td>2.49 (1.59–3.91)</td>
</tr>
<tr>
<td>IV</td>
<td>165 (18)</td>
<td>6.7</td>
<td>1.6 × 10⁻⁴</td>
</tr>
<tr>
<td>ND(^d)</td>
<td>5 (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor location</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proximal colon</td>
<td>367 (41)</td>
<td>48.2</td>
<td></td>
</tr>
<tr>
<td>Distal colon</td>
<td>302 (33)</td>
<td>46.4</td>
<td></td>
</tr>
<tr>
<td>Rectum</td>
<td>234 (26)</td>
<td>58.5</td>
<td>0.0061</td>
</tr>
<tr>
<td>Histopathologic grade</td>
<td></td>
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<tr>
<td>G1</td>
<td>84 (9)</td>
<td>63.1</td>
<td></td>
</tr>
<tr>
<td>G2</td>
<td>674 (75)</td>
<td>53.0</td>
<td>1.17 (0.76–1.79)</td>
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<tr>
<td>G3</td>
<td>108 (12)</td>
<td>33.3</td>
<td>2.64 (1.60–4.37)</td>
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<tr>
<td>Mucinous(^d)</td>
<td>12 (1)</td>
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<td></td>
</tr>
<tr>
<td>ND(^d)</td>
<td>25 (3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSI/MSS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSI</td>
<td>119 (13)</td>
<td>58.8</td>
<td>1.99 (1.33–2.98)</td>
</tr>
<tr>
<td>MSS</td>
<td>700 (78)</td>
<td>48.1</td>
<td>0.039</td>
</tr>
<tr>
<td>ND(^d)</td>
<td>84 (9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Residual tumor</td>
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<tr>
<td>R0</td>
<td>714 (79)</td>
<td>61.2</td>
<td></td>
</tr>
<tr>
<td>R1</td>
<td>19 (2)</td>
<td>42.1</td>
<td>1.73 (0.81–3.70)</td>
</tr>
<tr>
<td>R2</td>
<td>170 (19)</td>
<td>5.9</td>
<td>3.48 (2.23–5.42)</td>
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<td>Interaction parameter Gender × RCC2</td>
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<tr>
<td>Time to recurrence (TTR)</td>
<td></td>
<td>714 (100)</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>365 (53)</td>
<td>69.0</td>
<td>0.66 (0.42–1.05)</td>
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<tr>
<td>Male</td>
<td>349 (49)</td>
<td>68.3</td>
<td>1.03 (1.01–1.04)</td>
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<tr>
<td>Age(^c)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RCC2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytosolic staining</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weak</td>
<td>(238)</td>
<td>60.2</td>
<td>1.00 (0.67–1.49)</td>
</tr>
<tr>
<td>Strong</td>
<td>(381)</td>
<td>75.2</td>
<td>1.14 (0.75–1.75)</td>
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<tr>
<td>Stage</td>
<td></td>
<td></td>
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<tr>
<td>I</td>
<td>133 (19)</td>
<td>87.6</td>
<td>1.00 (0.67–1.49)</td>
</tr>
<tr>
<td>II</td>
<td>340 (48)</td>
<td>71.3</td>
<td>2.08 (2.17–3.70)</td>
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<tr>
<td>III</td>
<td>221 (31)</td>
<td>55.6</td>
<td>3.98 (2.25–7.05)</td>
</tr>
<tr>
<td>IV</td>
<td>15 (2)</td>
<td>28.6</td>
<td>3.48 (2.23–5.42)</td>
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<tr>
<td>ND(^d)</td>
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<tr>
<td>Tumor location</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Proximal colon</td>
<td>284 (40)</td>
<td>68.9</td>
<td>1.00 (0.67–1.49)</td>
</tr>
<tr>
<td>Distal colon</td>
<td>226 (32)</td>
<td>67.8</td>
<td>1.00 (0.67–1.49)</td>
</tr>
<tr>
<td>Rectum</td>
<td>204 (29)</td>
<td>69.2</td>
<td>1.14 (0.75–1.75)</td>
</tr>
<tr>
<td>Histopathologic grade</td>
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<tr>
<td>G1</td>
<td>79 (11)</td>
<td>76.6</td>
<td>1.00 (0.67–1.49)</td>
</tr>
<tr>
<td>G2</td>
<td>537 (75)</td>
<td>69.2</td>
<td>0.94 (0.54–1.62)</td>
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<tr>
<td>G3</td>
<td>70 (10)</td>
<td>52.9</td>
<td>2.06 (1.05–4.07)</td>
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<tr>
<td>Mucinous(^d)</td>
<td>9 (1)</td>
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<td>2.06 (1.05–4.07)</td>
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<tr>
<td>MSI/MSS</td>
<td></td>
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<tr>
<td>MSI</td>
<td>103 (14)</td>
<td>73.0</td>
<td>1.82 (1.05–3.18)</td>
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<tr>
<td>MSS</td>
<td>543 (76)</td>
<td>66.4</td>
<td>1.82 (1.05–3.18)</td>
</tr>
<tr>
<td>ND</td>
<td>68 (10)</td>
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<td></td>
</tr>
<tr>
<td>Interaction parameter Gender × RCC2</td>
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</tbody>
</table>

Abbreviations: G1, high differentiation; G2, moderate differentiation; G3, poor differentiation; R0, complete resection—no residual tumor; R1, microscopic residual cancer at the resection margin; R2, macroscopic or radiologic evidence of residual cancer, locally or distant.

\(^a\)Kaplan–Meier estimate (log-rank test).
\(^b\)Cox regression model (Wald test), all included parameters are displayed in the table.
\(^c\)HRs are given per year of age.
\(^d\)Excluded from the statistical analyses.
and accumulate alongside tumor progression in a balance with ‘classical’ driver mutations (25, 26). Their model, created by combining evolutionary simulations of cancer development with analysis of cancer sequencing data, provides explanations for phenomena such as spontaneous regression, slow progression, and heterogeneous growth rates.

As only a few prognostic markers have been reported for the MSI patient group, our finding has important clinical implications. The RCC2 mutation test is a simple PCR test and can be performed alongside assessment of MSI status which is already implemented in clinical guidelines. A simple assay determining the 5’ UTR RCC2 mutation status in tumors with MSI could further guide therapeutic decision-making for this subgroup of patients, particularly for those with stage II cancer, who are not routinely offered adjuvant treatment. Although MSI patients have generally better outcome than MSS patients, they have little benefit from conventional 5-fluorouracil treatment (27). Therefore, high-risk MSI patients identified by the RCC2 mutation test could be candidates for alternative adjuvant treatment.

The RCC2 gene maps to chromosome band 1p36 from which allelic loss is an early and common event in colorectal adenomas and carcinomas (28, 29). Several genes have been suggested to be targets of this loss, and the current data add RCC2 as a candidate. The chromosome band 1p36 is most often lost in colorectal cancers with MSS, which are typically in the triploid range and exhibit close to complete overlap with the CIN phenotype. The cause(s) underlying the CIN phenotype is presently unknown, but defects in components involved in the regulation of mitosis and cell-cycle checkpoints are suggested as initiators (30). Mutations found across the RCC2 coding sequence in about 3% of all colorectal cancers, and deletions of 1p sequences, may in part explain the variation of RCC2 expression in about 3% of all colorectal cancers, and deletions of 1p sequences, may in part explain the variation of RCC2 staining among colorectal cancers. It should be noted that none of the five missense mutations nor the single frameshift mutation recorded by TCGA were recurrent. The latter leading to a premature stop codon disrupting the seven-bladed beta-propeller structure of RCC2 which would most likely produce a nonfunctional protein that is rapidly degraded, but as a consequence the total amount of wt RCC2 may be reduced. Because of the rareness of this mutation its clinical value is marginal.

IHC analysis of RCC2 in the consecutive colorectal cancer series revealed that patients with weak cytosolic RCC2 staining had a poor prognosis relative to patients with strong cytosolic RCC2 staining (Fig. 4). Statistical tests for interaction demonstrated particular relevance for female patients post hoc, and subgroup analyses showed striking differences in the MSS group, for both cytosolic and nuclear staining. These findings seemingly contradict the previous results, which indicated that the 5’ UTR RCC2 mutation leads to reduced protein expression and a good prognosis. However, the mutation data relate to MSI tumors and a moderate reduction in protein amount. In MSS tumors, the ploidy stem line is, in contrast to MSI tumors, typically in the triploid range, and several pathway disturbances may lead to a decreased protein half-life.

An intriguing explanation for why reduced RCC2 protein expression in MSS tumors is associated with poor prognosis can be drawn from a study that identified RCC2 as a key player in the integrin α6β1-fibronectin signaling network (10). These experiments in MEF and B16-F10 cells suggested that RCC2 serves as a negative regulator of Rac1 and Arf6, and showed that RCC2 knockdown resulted in accelerated cell spreading, cell adhesion, and reduced directional cell migration. The authors suggested that RCC2 regulates and limits the required signaling by Rac1 and Arf6 to enable proper membrane protrusion and delivery. A recent study shows that RCC2 achieves this by binding to coronin-1C, which is crucial for Rac1 activation (31). This is concordant with our observations of increased cell spreading and change in cytoskeletal organization following knockdown of RCC2 in HCT15 cells. We speculate that strong cytosolic staining for the RCC2 protein in colorectal cancer indicates active Rac1 and Arf6 inhibition, and that weak RCC2 staining relates to lack of inhibition leading to an increased migratory and metastatic potential. The latter is in keeping with a significant correlation between cytosolic RCC2 expression and tumor stage, as stage I/II tumors more often have strong cytosolic staining, and stage III/IV tumors more often have weak cytosolic staining. In line with this scenario, another recent study demonstrated that RCC2 forms a complex with the well-established F-actin regulator cortactin (32), providing further support for a connection between RCC2 and cell motility and invasion. Cortactin, together with among others Rac1, is tightly involved in the generation of cell membrane protrusions, such as lamellipodias and invadopodias, and several studies have suggested that cortactin plays a role in cancer development (33). As MSS tumors already suffer from a deranged mitotic apparatus, they might not experience severe negative effects following a reduced level of RCC2 protein. Instead, reduced levels of RCC2 protein might primarily lead to deregulation of Rac1, Arf6, coronin-1C and/or cortactin, followed by enhanced migratory and invasive capacities. A tentative model of this network has been proposed (34), placing RCC2 between cortactin and Rac1, enabling a dynamic interplay among these partners with probable relevance to cancer development.

The present data add cancer as an important dimension to the current RCC2 framework. We demonstrate functional consequences of ‘partial’ as well as ‘total’ protein absence in colorectal cancer models. RCC2 is the first prognostic marker validated for the MSI subgroup. By simple PCR or protein expression assay, RCC2 may serve as a prognostic biomarker for patients with either MSI or MSS tumors.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: J. Bruun, T.C. Ahlquist, A. Lindblom, R.A. Lothe Development of methodology: J. Bruun, M. Kolberg, T.C. Ahlquist, R.A. Lothe Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Bruun, M. Kolberg, T.C. Ahlquist, E.C. Rayrøvik, M.A. Merok, A. Lindblom, X.-F. Sun, A. Nesbakken, R.A. Lothe Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Bruun, M. Kolberg, T.C. Ahlquist, T. Nome, E. Leithe, G.E. Lind, G. Bjerkøy, T. Johannsen, A. Svindland, K. Liestøl, A. Nesbakken, R.I. Skotheim, R.A. Lothe Writing, review, and/or revision of the manuscript: J. Bruun, M. Kolberg, T.C. Ahlquist, E.C. Rayrøvik, T. Nome, E. Leithe, G.E. Lind, M.A. Merok, A. Lindblom, X.-F. Sun, K. Liestøl, A. Nesbakken, R.I. Skotheim, R.A. Lothe Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T.C. Ahlquist, M.A. Merok, T.O. Rognum, A. Nesbakken, R.A. Lothe Study supervision: R.A. Lothe Other (responsible for the surgical pathology including diagnostics and selection of the colorectal material that were used in the study): A. Svindland
Acknowledgments
The authors are grateful for all advice from Kirsti Solberg Landsverk at the Flow Cytometry Core Facility at The Cancer Research Institute, Oslo University Hospital HE–The Norwegian Radium Hospital.

Grant Support
This study was supported by The Norwegian Cancer Society (R.A. Lothe: grant no. PR-2006-0442, supporting J. Bruun as PhD student and T.C. Ahlquist as postdoctoral research associate; R.I. Skodheim: grant no. PR-2007-0166) and by the South-Eastern Norway Regional Health Authority (R.A. Lothe: grant HS014 gruppe 2010: "Genome medicine of colorectal cancer").

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Received December 19, 2014; revised March 12, 2015; accepted March 29, 2015; published OnlineFirst April 24, 2015.

References
Regulator of Chromosome Condensation 2 Identifies High-Risk Patients within Both Major Phenotypes of Colorectal Cancer

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Clin Cancer Res  Published OnlineFirst April 24, 2015.

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Access the most recent version of this article at:
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