Regulator of chromosome condensation 2 identifies high-risk patients within both major phenotypes of colorectal cancer

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TRANSLATIONAL RELEVANCE

Colorectal cancer has a high incidence and mortality rate. Prognosis and postoperative treatments are still based on clinicopathological parameters alone. Patients with the MSI phenotype tumors generally have a good prognosis. Here we show that a mutation in the regulator of chromosome condensation 2, \textit{RCC2}, gene can identify high-risk patients within the stage II MSI group. Furthermore, reduced RCC2 protein expression identifies female patients with poor outcome within the MSS group. Subgroup analyses suggest that RCC2 is able to stratify clinically important patient groups: patients above 75 years of age, patients with stage II and those with rectal cancer. Hence, RCC2 risk stratification can potentially guide clinical decision making for a large number of CRC patients. Importantly, both the mutation assay and the protein expression assessment can be done rapidly by cost-effective routine technologies.
ABSTRACT

Purpose: Colorectal cancer (CRC) has high incidence and mortality worldwide. Patients with microsatellite instable (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 CRC 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 CRC 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, independently of clinical high-risk parameters, and stratifies clinically important patient subgroups with MSS tumors, including old patients (>75 years), stage II patients and those with rectal cancer.

Conclusions: Impaired RCC2 affects functional and clinical endpoints of CRC. High-risk patients with either MSI or MSS tumors can be identified with cost-effective routine RCC2 assays.
INTRODUCTION

Each year, approximately 1.4 million patients are diagnosed with colorectal cancer (CRC), and only half of them survive five years\(^4\). 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 non-hereditary MSI tumors have better prognosis than those with microsatellite stable (MSS) tumors\(^3\text{-}^5\), 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 this subgroup. For the other 85% of CRC, 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 CRC\(^7\), 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 CRC 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)\(^9\). 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 CRC in two independent 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 CRC (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 CRC patients admitted to Swedish (n=44) and Norwegian (n=37) 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, Table S1).

Validation and further experiments were based on a population-based consecutive series consisting of 1290 CRC patients admitted to Oslo University Hospital (OUS) – Aker, Norway in 1993-2003 (see Fig. S1). According 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 neighbouring the capital of Norway, city of Oslo, and close to 100% of the CRC patients who were treated in this hospital were included in the study. Of the 1290 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, Table S2). More than 95% of patients were of Caucasian ethnicity (based on name-origin). All patient samples were analyzed retrospectively.

The study was approved by the Regional Committee for Medical 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 informed consent is obtained from patients being enrolled to the study.

Cell lines
HCT15, HT29 and HeLa CCl-2 cell lines were purchased from the American Type Culture Collection (ATCC, VA, USA) and maintained according to the ATCC recommendations. Identities were verified by fingerprinting according to the AmpFlSTR Identifiler PCR Amplification Kit (Life Technologies by Thermo Fisher Scientific, MA, USA), and matched to the profiles reported by ATCC. Cell lines were regularly tested for mycoplasma contamination according to the MycoAlert Mycoplasma Detection Assay (Lonza Cologne AG, Germany).
Determination of MSI

MSI status was determined using the consensus markers provided by the National Cancer Institute as previously described\textsuperscript{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 data Table S4). Electropherograms were visually examined for indels by two researchers independently (TA and ECR), against corresponding fragments from DNA from four different disease-free individuals (Fig. S6). Assays were duplicated in tandem runs using different PCR machines. Each gene was informative for at least 159 of the 209 samples.

Mutation verification by sequencing

In order 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 CRC tissue samples and 8 CRC 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)\textsuperscript{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., CA, USA), and sequencing was subsequently performed on an Illumina Genome Analyzer IIx for nine samples (tumor and normal tissue). Whole-genome paired-end sequencing was performed by BGI (Hong Kong, China) for the four CRC cell lines: HCT15, HCT116, HT29 and SW480. The sequence reads were aligned by BWA version 0.6.1 (Li & Durbin, 2009). Mutation data from all sequenced tumors by The Cancer Genome Atlas (TCGA) was accessed via the cBioPortal (http://www.cbioportal.org/public-portal/)\textsuperscript{14} on December 16, 2014.

Reporter gene assays

A dual luciferase reporter system (Promega, CA, USA) was used to determine reporter activity from vector constructs with mutated and wild-type \textit{RCC2} (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, Cambridge, UK), 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, MO, USA), recognizing an N-terminal epitope of the β-isoform. Antibodies were employed at dilutions of 1:500 and 1:1000, respectively. For immunocytochemistry and immunohistochemistry, rabbit polyclonal anti-RCC2 antibodies were obtained from Novus Biologicals (Cat. No. NB110-40619, Cambridge, UK), 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 RCC2HSS125252 (I) and RCC2HSS125253 (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 CU G 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, Paisley, UK) and the transfection procedure was carried out following the manufacturer's instructions, at a final concentration of 80 nM.

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 elsewhere15.

**Flow cytometry**

Flow cytometry analysis of cell cycle distributions and levels of apoptosis was carried out using a 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 elsewhere15.

**Immunohistochemistry**

The immunohistochemical (IHC) analysis was performed on FFPE tissue as described16 (details in Supplementary data).

**Statistics**

All statistics were performed using the SPSS 21.0 software (SPSS, IL, USA). Five-year overall survival (OS) and five-year time-to-recurrence (TTR) plots were generated using the Kaplan-Meier method. The logrank test was used to compare survival curves. TTR and OS were defined according to the guidelines given by Punt et al.17 where time-to-
recurrence 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, non-cancer death and post-operative death (<3 months). Overall survival 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 was included as background variables. Tumor location was also included in the protein expression analyses for informative purposes, and it did not affect the 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 post-operative 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 since there are very few cases in the distant colon and the rectum category as compared to the proximal category. Also, for the covariate Histopathological grade, the G1 and the G2 category were combined to make larger and more robust groups for comparison. We believe this is reasonable since 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) vs 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 clinicopathological data, Wilcoxon rank-sum test (exact) was used to compare two groups with regard to an ordinal variable and Fisher’s exact test was used to compare categorical variables. Student’s 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, an analysis of variance 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 CRCs with MSI

The mutation frequencies of 42 known target genes in MMR deficient CRC 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. 2A). 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) in order to identify the best candidate markers, which were then analyzed in the larger Validation series (n=122). From the six candidates identified by univariate five-year TTR analysis (ACVR2A, AXIN2, EP300, MRE11A, OGT and RCC2, Table S5), only the RCC2 mutation was found to be significantly associated with outcome in both clinical series (Fig. 1B-C). Stratification by tumor stage in the Validation series showed that for stage II patients after five 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 (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 three 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’s exact tests showed that mutations in $RCC2$ were not significantly associated ($P<0.05$) with other clinicopathological data, except surgical resection status (R-status, $P=0.039$) (Table S1-3).

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

To search for mutations elsewhere in the $RCC2$ gene, we used in-lab data sets from massive parallel sequencing of selected CRC 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 Hong Kong, China), paired-end RNA sequencing data sets 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 CRCs (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 publically available data: The Cancer Genome Atlas (TCGA) accessed through the cBioPortal$^{14}$. The provisional TCGA data set comprises somatic mutation data from exome sequencing of 223 CRCs. Here, $RCC2$ was mutated in 6 cases (2.7%), of which 5 were missense mutations (4 in MSI and one 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 (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$^{18}$. 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 \textit{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 \textit{RCC2} 5'UTR \((A)_9\) mutation as compared to the wild-type \((A)_{10}\). A wild-type or mutant 5'UTR \textit{RCC2} fragment was inserted immediately upstream of the luciferase reporter gene in an SV40 promoter-based plasmid (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 to the wild-type \((P=0.0057, n=17, \text{two-tailed paired Student’s } t\text{-test}, \text{Fig. } 2)\). Statistical significance was also evident from an analysis of variance of the relative 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 \((P=0.62, n=15, \text{two-tailed paired Student’s } t\text{-test})\).

\textbf{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 \textit{RCC2} in regulation of cell growth and proliferation. Two different siRNA constructs were employed to knock down \textit{RCC2}, both of which reduced the \textit{RCC2} protein level by more than 90\% compared to cells transfected with negative control siRNA sequences (mock) (Fig. 3). Depletion of \textit{RCC2} was associated with a significant 40-45\% reduction in cell numbers (Fig. 3). Concurrently, \textit{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 \textit{RCC2}-depleted cells exhibited an altered, more elongated actin fiber pattern with predominant stress- and filopodia-like fibers (Fig. 3 and Fig. S4).

\textbf{RCC2} knockdown causes G2-M arrest and increased apoptosis

Next, the effect of knockdown of \textit{RCC2} in HCT15 cells on cell cycle progression and apoptosis was determined by flow cytometry. Depletion of \textit{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 to mock transfected cells (Fig. 3). In addition, the level of aneuploid (>4N) cells was elevated for \textit{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% versus 1.7%, \( P=0.0077, n=3 \)), as detected by a TUNEL-assay (Fig. 3).

**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 IHC in a consecutive single-hospital series on a tissue microarray of primary colorectal cancers \((n=903; 781\text{ and } 797\text{ evaluable tissue cores for cytosolic and nuclear staining respectively Fig. S1})\), including the two main subgroups of CRC, MSI \((n=128, \text{ validation series})\) and MSS \((n=714; 619\text{ and } 630\text{ evaluable tissue cores for cytosolic and nuclear staining, respectively})\) tumors.

For cytosolic staining, both OS and TTR analyses showed significant associations with patient outcome \((P=5.2\times10^{-7} \text{ and } P=8.3\times10^{-5}, \text{ respectively, Figure 4})\). 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 \((P=0.0059\text{ for the interaction test and } P=0.0015\text{ for RCC2})\) and for the TTR analyses \((P=0.0037\text{ for the interaction test and } P=1.4\times10^{-5}\text{ for RCC2})\). Subsequent subgroup analysis (TTR) for gender revealed significant associations confined to female patients \((P=6.2\times10^{-6}, \text{ Fig. 4})\). The strong prognostic value of RCC2 cytosolic staining in females was confirmed in separate multivariate models for gender \((P=1.5\times10^{-5}, \text{ HR } 0.37 , \text{ CI } 0.24-0.58 \text{ for females and } P=0.65, \text{ HR } 0.90 , \text{ CI } 0.56-1.43 \text{ for men})\). Interestingly, further stratification on MSS/MSI status showed that this relationship was confined to patients with MSS (Fig. 4). MSI tumors had stronger cytoplasmic expression compared to MSS tumors \((P=0.012, \text{ Fisher’s exact test, } n=712)\), however, the number of patients with MSI and events were 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 \((P=2.1\times10^{-4}, n=777)\). 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/MSS-status, and tumor location, differentiation and stage) nuclear expression of RCC2 showed significant interaction with gender \((P=0.016, \text{ HR } 3.0, \text{ CI } 1.2-7.3 \text{ for the interaction test and } P=0.001, \text{ HR } 2.9, \text{ CI } 1.6-5.3 \text{ for RCC2})\) (Fig. 4). Nuclear staining stratified by gender and MSS/MSI-status showed significant associations for female patients \((P=0.045)\) and to MSS patients \((P=0.013, \text{ Fig. 4})\).
Further exploratory subgroup analyses show interesting associations between reduced RCC2 expression and old age, rectal cancer and early stage (I/II) disease (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 CRC. 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 CRCs, but particularly for female patients with MSS tumors, and that mutations in the RCC2 coding sequence are rare.

MNRs in UTRs are evolutionary conserved, indicating a functional role19. Repeat sequences are prone for alterations in cancers with defect mismatch repair (MSI) and some of these targets are involved in the regulation of gene and protein expression. UTRs are known to affect mRNA nuclear export, cytoplasmic localization, translational efficiency and stability20. The majority of translational control occurs at the level of initiation, thus implicating the 5’UTR region as an important site for translational regulation21. 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 RCC2 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 sequence22.

From global quantification studies of mammalian gene expression it is found that protein levels are predominantly controlled at the translational level23. RCC2 mRNA and protein levels display average half-lives of 9h and 48h, respectively. However, the translation rate constant in NIH 3T3 cells is about 230 proteins per mRNA per hour, much higher than the average of 14023. 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 cells9. 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. 3) that showed increased levels of apoptosis, as well as the cell cycle analysis that demonstrated increased levels of >4N cells. 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 since 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 HCT1.5 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 and accumulate alongside tumor progression in a balance with “classical” driver mutations\textsuperscript{24, 25}. Their model, created by combining evolutionary simulations of cancer development with analysis of cancer sequencing data, provide explanations for phenomena such as spontaneous regression, slow progression and heterogeneous growth rates.

Since only 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 already is 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-FU treatment\textsuperscript{26}. 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\textsuperscript{27, 28}. 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 CRCs 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\textsuperscript{29}. Mutations found across the RCC2 coding sequence in about 3% of all CRCs, and deletions of 1p sequences, may in part explain the variation of RCC2 staining among CRCs. 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 non-functional protein that is rapidly degraded, but as a consequence the total amount of wt RCC2 may be reduced. Due to the rareness of this mutation its clinical value is marginal.

IHC analysis of RCC2 in the consecutive CRC series revealed that patients with weak cytosolic RCC2 staining had a poor prognosis relative to patients with strong cytosolic RCC2 staining (Figure 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 α5β1-fibronectin signaling network10. 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 activation30. 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 CRC 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 cortactin31, 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 development32. 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\textsuperscript{33}, placing RCC2 between cortactin and RAC1, enabling a dynamic interplay among these partners with probable relevance to cancer development.

The present data adds cancer as an important dimension to the current RCC2 framework. We demonstrate functional consequences of “partly” as well as “total” protein absence in CRC 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.
AUTHOR CONTRIBUTIONS

J.B. participated in the study design, performed all functional studies and IHC experiments and scoring, participated in the genetic analyses, interpreted all results, performed all statistics and drafted the manuscript. M.K. performed IHC experiments and independent scoring, and participated in the statistical analyses. T.A., E.C.R. and G.E.L performed and scored the mutation analyses of all repetitive tracts. J.B., T.A., T.N. and R.I.S. performed massive parallel sequencing of own samples and evaluated the TCGA dataset. E.L., G.B. and T.J. participated in design and interpretation of the functional assays. M.A.M., T.O.R., A.N., A.L and X-F.S collected the patient samples, provided the MSI data, collected and quality controlled the clinical data. A.N. participated in the survival analyses. A.S. performed morphological identification of qualified tumor areas for TMA construction. K.L. participated in the statistical analyses. R.A.L. conceived and coordinated the study, was responsible for the study design and research biobank, and participated in evaluation and discussion of results and in the drafting of the manuscript. All authors participated in manuscript writing and in scientific discussions.

ACKNOWLEDGEMENTS

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DECLARATION OF INTERESTS

We declare that we have no competing interests.
REFERENCES


FIGURE LEGENDS

**Fig. 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 (logrank test) for CRC patients with MSI tumors stratified by the *RCC2* 5’UTR mutation in the test series (B), 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 CRC (E). Nucleotide-level somatic mutation data in CRC from the complete length of *RCC2* (F). Red pins represent mutations identified from own samples and yellow pins represent mutations in the dataset from The Cancer Genome Atlas. Orange: UTRs; Blue: coding sequence; Green: protein domains. Abbreviations: RLD - RCC1-like domain; NLS - Nuclear localization signal (putative). Abbreviations: wt – wild type; mut – mutant.

**Fig. 2.** The *RCC2* 5’UTR mutation causes a reduced protein expression. Constructs where wild type or mutated 5’UTR from *RCC2* were placed upstream of the coding region of the firefly luciferase gene, were transiently transfected into HCT15, HT29 and HeLa cells, followed by determination of luciferase activity in cell extracts. *P*-value from Student’s *t*-test for paired samples (two-tailed). The y-axis represents the relative firefly luciferase expression (normalized to the *Renilla* luciferase expression) from the mutated to the wild-type construct.

**Fig. 3.** siRNA knockdown of *RCC2* in HCT15 cells has functional consequences on cell growth and morphology. Depletion of *RCC2* causes reduced proliferation and increased cell spreading of HCT15 cells (A-B). HCT15 cells were either transfected with negative control siRNA (mock) or with two different siRNA sequences against *RCC2* (siRNA1 and siRNA2). The cells were assayed after four days of transfection. Representative Western immunoblots showing knockdown of *RCC2* by the two different siRNAs with quantification bar charts (A). Verification of knockdown was obtained by three independent experiments for each of the two siRNAs. The protein expression was normalized relative to cells transfected with mock. (B) Quantification of cell proliferation. Cells were counted after four days of growth on a 35 mm Petri dish. Each bar represents the average cell number from three independent experiments. Error bars indicate the standard error of the mean. *P*-value from Student’s *t*-test for paired samples (two-tailed). Representative images of the morphology of mock-transfected cells (left) and *RCC2*-depleted cells (right) (B). The morphological changes following *RCC2* depletion are accompanied by a rearranged actin filament network (C-E). HCT15 cells were either transfected with negative control siRNA (mock) (C), or with siRNA sequences against *RCC2* (E-F). After four days of transfection, the cells were fixed, stained
with anti-RCC2 antibodies and phalloidin to label actin, and visualized using immunofluorescence confocal microscopy. An apparent increase in the number of large multinuclear cells was observed in cells with reduced RCC2 protein (asterisk). Scale bar represents 20 µm and applies to all images. Depletion of RCC2 causes cell cycle arrest in the G2-M phase and increased levels of apoptosis (F-H). HCT15 cells were either left untreated or transfected with negative control siRNA (mock) or with siRNA sequences against RCC2 (siRNA1) (F) Visualizations of representative cell cycle phase distributions for each treatment, as seen by flow cytometry. (G) Quantification bar charts for cell cycle phase distributions. (H) Quantification bar charts for levels of apoptosis, as seen by TUNEL-assay. Error bars indicate the standard error of the mean from at least three independent experiments with at least three biological replicates for each experimental variable. P-values indicate significant differences in cell cycle phase distributions between mock- and siRNA-transfected cells (two-tailed Student’s t-test for paired samples).

Fig. 4. Weak staining for the RCC2 protein is associated with poor patient outcome, particularly for female CRC patients with MSS tumors. (A-H) Differential immunohistochemical staining of RCC2 in colorectal carcinomas. Staining of tumor sections using an RCC2 selective antibody demonstrated a specific staining in both the cytosol and in 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 400x magnification. (A-D) Strong-, moderate-, weak- and negative nuclear staining, respectively. (E) Strong nuclear and cytosolic staining. (F) Predominant cytosolic 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 CRC series (n=903). Green curves represent strong staining and blue curves represent weak staining. (I) Five-year overall survival. (J-L) Five-year time-to-recurrence 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 (logrank test) was used to draw the plots and determine statistical differences between patients with strong and weak RCC2 staining.
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<th>Multivariate analysis(^b)</th>
<th>5-year TTR (%)</th>
<th>P</th>
<th>HR</th>
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Table 1. Univariate and multivariate analyses of RCC2 5’UTR mutation status in the Validation series. \(^a\)Kaplan-Meier estimate (logrank test). \(^b\)Cox regression model (Wald test) - all included parameters are displayed in the table. \(^c\)Hazard ratios are given per year of age. *Excluded from the statistical analyses. Abbreviations: G1 – High differentiation; G2 – Moderate differentiation; G3 – Poor differentiation.
<table>
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<tr>
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<tr>
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<td>Weak</td>
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<td>237 (26)</td>
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<td>G1</td>
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<sup>a</sup> Univariate analysis includes 5-year OS/TTR (%), P-value, HR, and 95% CI.

<sup>b</sup> Multivariate analysis includes 95% CI and P-value.
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<td>365(51)</td>
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<td>Male</td>
<td>349(49)</td>
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| Agec | - | 1.03 | 1.01-1.04 | 0.0066 |

| RCC2 |  |
| cytosolic staining |  |
| Weak | (238) | 60.2 | 1 |
| Strong | (381) | 75.2 | 8.3x10^{-5} | 0.38 | 0.24-0.58 | 1.4x10^{-5} |

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<td>Proximal colon</td>
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**Interaction parameter**

| Gender x RCC2 | 2.58 | 1.36-4.90 | 0.0037 |

**Table 2.** Univariate and multivariate analyses of cytosolic RCC2 in situ protein expression in a consecutive CRC series. \(^a\)Kaplan-Meier estimate (logrank test). \(^b\)Cox regression model (Wald test) - all included parameters are displayed in the table. \(^c\)Hazard ratios are given per year of age.

*Excluded from the statistical analyses. 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 radiological evidence of residual cancer, locally or distant.
Figure 1

A

Mutation frequency (%)

ACVR2A  PCNLX2  MAR7OS  MRE11A  PTHLH  RAF1  E2F4  RCC2  CASP5  RAD50  MSH3  SEMG1  ACY2  SLC3A2  OGT  EPHB2  GB2R4  BAX  TECER2  TSC2  EGFR2  SPN  RBBP8  PRKDC  MSH6  PTK6  CRK  SRC  ATP7B  APC  RAS  BAX  MSH6  PRKDC  CDC6  MRE11A  MSH3  SMC1  SMC3  BARD1  BAX  MSH6  PTM2  ATM  PALB2  BARD1  BAX  MSH6  SMC1  SMC3  BARD1  BAX  MSH6  PTM2  ATM  PALB2

B

Test series

Proportion without event

RCC2wt  n = 20, 7 events

P = 0.045
n = 37

0 10 20 30 40 50 60
0.0 0.2 0.4 0.6 0.8 1.0

Time to recurrence (months)

Patients at risk
RCC2mut  RCC2wt
17  15  14  13  12  12
20  17  14  13  13  12

D

Validation series (R0)

Proportion without event

RCC2mut  n = 45, 5 events

RCC2wt  n = 15, 5 events

P = 0.038
n = 60

0 10 20 30 40 50 60
0.0 0.2 0.4 0.6 0.8 1.0

Time to recurrence (months)

Patients at risk
RCC2mut  RCC2wt
45  44  43  43  41  37
15  14  12  12  10  10

E

Validation series (stage II)

Proportion without event

RCC2mut  n = 54, 6 events

RCC2wt  n = 19, 7 events

P = 0.0065
n = 73

0 10 20 30 40 50 60
0.0 0.2 0.4 0.6 0.8 1.0

Time to recurrence (months)

Patients at risk
RCC2mut  RCC2wt
54  51  50  47  45  43
19  17  16  14  12  11

F

- Frame-shift deletion
- Missense
- Deletion
- Synonymous

NLS (~26-36 aa)
RLD domain (121-507 aa)
Figure 2

HCT15 HT29 HeLa

$P = 0.0057$

$n = 17$
Figure 3

A) RCC2 expression (%) - RCC2 and β-actin levels.

B) Histogram showing # Cells with p-values: Untransfected vs Mock (P = 0.011), Mock vs siRNA1 (P = 0.023).

C) Hoechst and RCC2 staining images.

D) RCC2 siRNA stained images.

E) RCC2 siRNA stained images.

F) Flow cytometry histograms for Untransfected, Mock, and siRNA.

G) β-actin expression histograms with p-values: Untransfected vs Mock (P = 0.012), Mock vs siRNA1 (P = 0.011), siRNA1 vs siRNA2 (P = 0.016).

H) Histogram showing Mock vs siRNA2 with P = 0.0077.
Cytosolic staining

Overall survival

Proportion without event

1.0

0.8

0.6

0.4

0.2

0

0 10 20 30 40 50 60

Months

P = 5.2x10^-7

n = 462, 192 events

n = 192, 193 events

P = 8.3x10^-7

n = 618, 300 events

n = 185, 94 events

P = 0.79

n = 781

n = 612, 300 events

n = 185, 94 events

P = 0.55

n = 630

R0 patients

All cases

Female R0 patients

Male R0 patients

Female R0 patients with MSS

Male R0 patients with MSS

Proportion without event

0 10 20 30 40 50 60

Months

Overall survival

Time-to-recurrence

Female R0 patients

Male R0 patients

Female R0 patients with MSS

Male R0 patients with MSS

Proportion without event

0 10 20 30 40 50 60

Months

Time to recurrence (months)

Figure 4
Regulator of chromosome condensation 2 identifies high-risk patients within both major phenotypes of colorectal cancer

Jarle Bruun, Matthias Kolberg, Terje C. Ahlquist, et al.

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