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Short Report

Alternative splicing expands the prognostic impact of *KRAS* in microsatellite stable primary colorectal cancer

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KRAS **mutation is a well-known marker for poor response to targeted treatment and patient prognosis in microsatellite stable (MSS) colorectal cancer (CRC). However, variation in clinical outcomes among patients wild-type for *KRAS* underlines that this is not a homogeneous population. Here, we evaluated the prognostic impact of *KRAS* alternative splicing in relation to mutation status in a single-hospital series of primary MSS CRCs ($N = 258$). Using splicing-sensitive microarrays and RNA sequencing, the relative expression of *KRAS-4A* versus *KRAS-4B* transcript variants was confirmed to be down-regulated in CRC compared to normal colonic mucosa ($N = 41$; $p \leq 0.001$). This was independent of mutation status, however, gene set enrichment analysis revealed that the effect of splicing on KRAS signaling was specific to the *KRAS* wild-type subgroup, in which low relative *KRAS-4A* expression was associated with a higher level of KRAS signaling ($p = 0.005$). In concordance, the prognostic value of *KRAS* splicing was also dependent on mutation status, and for patients with Stage I–III *KRAS* wild-type MSS CRC, low relative *KRAS-4A* expression was associated with inferior overall survival (HR: 2.36, 95% CI: 1.07–5.18, $p = 0.033$), a result not found in mutant cases ($p_{interaction} = 0.026$). The prognostic association in the wild-type subgroup was independent of clinicopathological factors, including cancer stage in multivariable analysis (HR: 2.68, 95% CI: 1.18–6.09, $p = 0.018$). This suggests that *KRAS* has prognostic value beyond mutation status in MSS CRC, and highlights the importance of molecular heterogeneity in the clinically relevant *KRAS* wild-type subgroup.**

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Colorectal cancer (CRC) is a heterogeneous disease, both at the molecular level and with respect to disease outcome and treatment response. There is a need for biomarkers to improve the stratification of patients into therapeutically relevant subgroups. Currently, microsatellite instability (MSI) and *KRAS* mutation status are among the few biomarkers recommended for clinical use.[1] *KRAS* is primarily a predictive factor, however, studies indicate that mutated *KRAS* also confers poor prognosis in CRC, but this is limited to the microsatellite stable (MSS) subtype,[2–4] and possibly also to subgroups within MSS. Recently, the negative prognostic value of *KRAS* mutations was suggested to be specific to the "epithelial-like" MSS cancers of the gene expression-based consensus molecular subtypes (CMS) 2/3,[4] highlighting the clinical importance of evaluating biomarkers in the context of molecular subtypes. Overall, patients with *RAS* (*KRAS*/*NRAS*) wild-type metastatic CRC benefit from anti-epidermal growth factor receptor (EGFR) treatment,[5] although resistance occurs in more than half.[6] This indicates that *KRAS* wild-type CRC is not a homogeneous entity, and although a few additional resistance

What's new?

Patients with microsatellite stable (MSS) colorectal cancer (CRC) that lacks *KRAS* mutation benefit from targeted therapy. Nonetheless, variations in clinical outcome suggest that *KRAS* wild-type CRC is a heterogeneous disease. Here, two *KRAS* transcript variants, KRAS-4A and *KRAS*-4B, generated through alternative splicing, were investigated in relation to *KRAS* mutation status and MSS CRC prognosis. Aberrant splicing resulting in low expression of the *KRAS*-4A transcript variant, relative to the *KRAS*-4B transcript, was associated with increased *KRAS* signaling and poor patient prognosis specifically in *KRAS* wild-type MSS CRC. The findings suggest that *KRAS* splicing is of prognostic relevance in *KRAS* wild-type CRC.

factors have been identified,[7] there is a need for detailed biological understanding of this clinically important subgroup.

KRAS is expressed as two transcript variants caused by alternative splicing,[8] *KRAS-4A* and *KRAS-4B*, both of which will encode oncogenic proteins when *KRAS* is mutated. Although the evidence for differential functions is limited, mutated *KRAS-4A* and *KRAS-4B* differ in their ability to induce anchorage-independent growth and cell migration.[9] Furthermore, studies in mice indicate that wild-type *KRAS-4A* has tumor suppressive and pro-apoptotic activity, while wild-type *KRAS-4B* is anti-apoptotic.[10,11] *KRAS* splicing has been shown to be altered in CRC, by reduced relative expression of the *KRAS-4A* transcript,[12] indicating that the balance of *KRAS* splice variants may have a role in CRC tumorigenesis. In contrast to *KRAS* mutations, few studies have evaluated the clinical relevance of *KRAS* splice variants. Although the expression level of the KRAS-4A protein has been shown to be prognostic in CRC,[13] another study comparing the expression of *KRAS-4A* relative to *KRAS-4B* at the transcript level failed to show a prognostic association in Stage I–II CRC.[14]

Here, we analyzed the mutation status-dependent prognostic impact of *KRAS* splicing in the MSS subtype in a single-hospital series of primary CRCs.

Material and Methods

Patient samples

Altogether 258 fresh frozen primary MSS CRCs from a population-representative series of patients who underwent surgical resection at Oslo University Hospital, Oslo, Norway, in the time period 2005–2014 were included. All samples were analyzed for gene-expression using splicing-sensitive platforms, either by microarrays (N = 168), RNA sequencing (N = 128) or both (N = 38 overlapping samples). The microarray dataset represented a consecutive series of Stage I–IV MSS CRCs and has previously been published[15] (Gene Expression Omnibus accession number GSE96528), while the RNA sequencing dataset was selected to include mostly Stage II or III MSS cancers (Supporting Information Table 1). Additionally, normal mucosa samples taken from disease-free areas of the colon of 41 CRC patients were analyzed either by microarrays (N = 29) or RNA sequencing (N = 12), 30 of which also had their corresponding primary tumor analyzed. The study is part of a project approved by the Regional Committee for Medical and Health Research Ethics, South Eastern Norway (REC number 1.2005.1629). Written informed consent was obtained from all patients.

Total RNA was isolated using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen GmBH, Hilden, Germany). Genomic DNA was isolated using either a standard phenol/chloroform extraction method or metallic beads (Maxwell 16 DNA Purification Kit; Promega, Madison, WI). All procedures were performed according to the manufacturer's recommendations.

MSI status, determined by analysis of the Bethesda marker panel, and mutation hotspots in *KRAS* (exon 2: codons 12 and 13, exon 3: codons 59 and 61), assessed using Sanger sequencing, have previously been published.[15] Additionally, mutations in *KRAS* codons 117 and 146 were analyzed by amplification of exon 4 using the primers: sense 5′-TGACAAAAGTTGTGGACAGGT-3′ and antisense 5′-AAGAAGCAATGCCCTCTCA-3′. When referring to *KRAS* at the transcript level, the 5′-untranslated exon number 1 at the DNA level is denoted Exon 0, and exon number 5 at the DNA level is Exon 4A, according to previous reports on *KRAS* splice variants (Fig. 1*a*).[8]

Splicing-sensitive gene expression microarrays

Microarray analyses were done using GeneChip Human Transcriptome Arrays 2.0 (Thermo Fisher Scientific, Waltham, MA), which have probes interrogating both individual exons and known splicing junctions. Fragmented and labeled single-stranded cDNA was prepared from 100 ng total RNA and hybridized to arrays as recommended by the manufacturer. For alternative splicing analysis, CEL files were background corrected, quantile normalized and summarized according to a custom CDF file using the aroma.affymetrix R package and the robust multi-array average (RMA) algorithm. The CDF file was generated using the CDFfromGTF function in the R package EventPointer,[16] which grouped the probes of the array into probesets according to splice graphs, representing all annotated splicing events of each gene, built by mapping the probes to the Ensembl transcriptome (GRCh37 version 90). After pre-processing, a percent spliced in (PSI) index for each identified splicing event in each sample was estimated using the getPSI command from the R package EventPointer. The relative abundance of the two splice variants of *KRAS* was estimated as the PSI for *KRAS-4A*, defined as the expression signal of exonic and junction probes supporting exon 4A inclusion, relative to the total expression signal at the splicing event (*i.e.*, for probes supporting inclusion and exclusion of

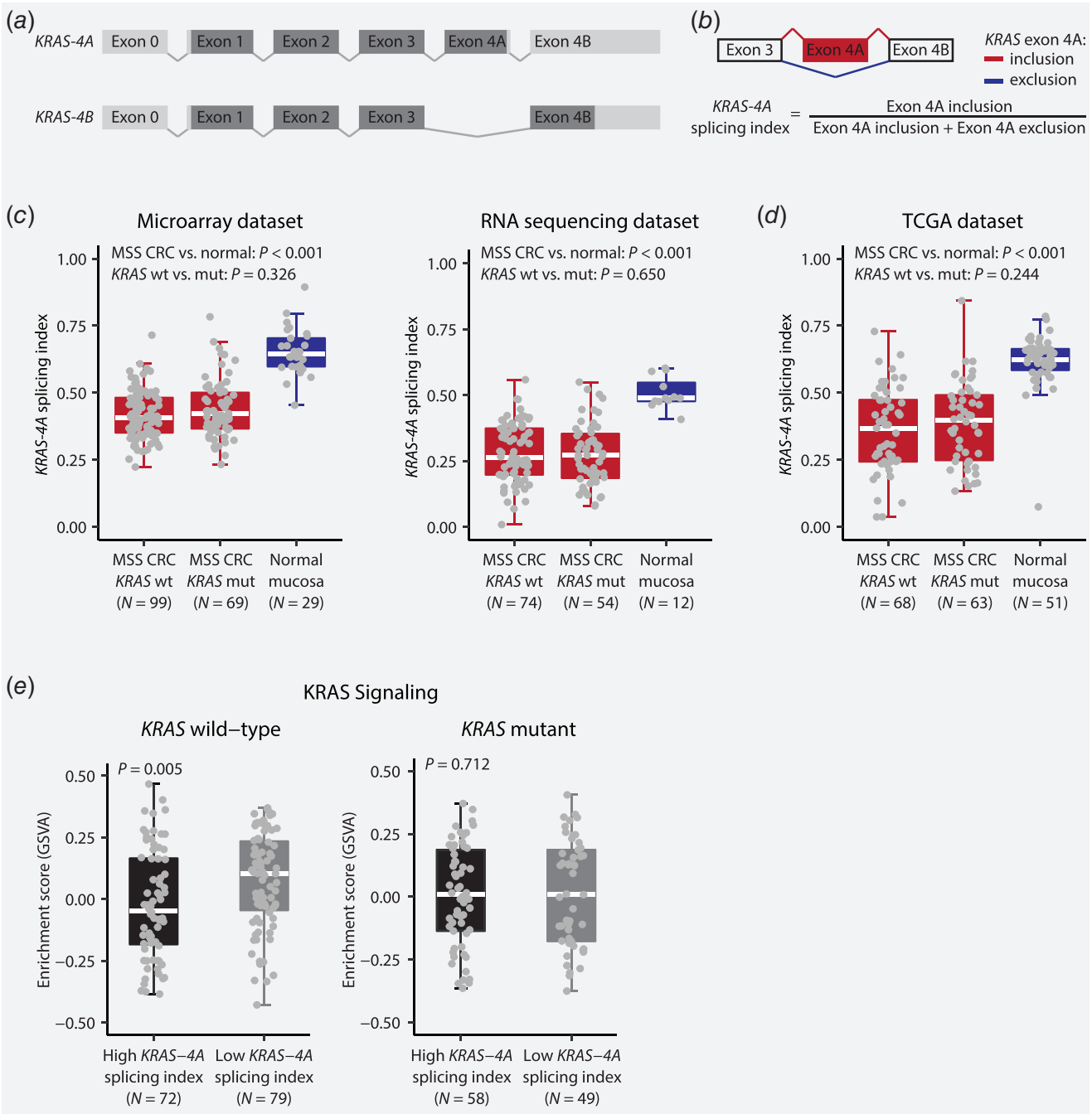

Figure 1. Expression of *KRAS*-4*A* and *KRAS*-4*B* splice variants in MSS CRC. (*a*) Schematic of the two splice variants of *KRAS* with protein coding regions in dark gray and non-protein coding regions in light gray. To follow convention when referring to *KRAS* at the transcript level, the 5′-untranslated exon number 1 at the DNA level is denoted Exon 0, and exon number 5 at the DNA level is Exon 4A. (*b*) *KRAS* splicing (denoted as the *KRAS*-4*A* splicing index) was estimated using the signal of exonic and junction probes or RNA sequencing reads mapping to the exon 4A inclusion (red) and exclusion (blue) splice variants of *KRAS*. The *KRAS*-4*A* splicing index was significantly decreased in MSS CRCs compared to normal colonic mucosa (*c*) in the microarray and RNA sequencing datasets, as well as (*d*) in an independent cohort of TCGA MSS CRC patients. No association was observed between the *KRAS*-4*A* splicing index and *KRAS* mutation status. (*e*) *KRAS* wild-type CRCs with low *KRAS*-4*A* splicing indices had a higher level of KRAS signaling compared to tumors with high *KRAS*-4*A* splicing indices. *KRAS* mutant cases showed no association between *KRAS* splicing and KRAS signaling.

exon 4A), and hereafter referred to as the *KRAS-4A* splicing index. For gene level analysis, CEL files were preprocessed using the modified Signal Space Transformation algorithm of RMA implemented in the Affymetrix Expression Console Software (version 1.4.1.46). For genes annotated by more than one transcript cluster, the entry from RefSeq was used.

RNA sequencing

RNA sequencing libraries were generated from 500 ng total RNA using the TruSeq Stranded Total RNA Library Prep Gold kit (Illumina, San Diego, CA), and submitted to 2×101 base-pair paired-end sequencing using the Illumina HiSeq 2500 system at the Oslo University Hospital Genomics Core Facility (The Norwegian Radium Hospital, Oslo, Norway). The demultiplex software package CASAVA (version 1.8.2) was applied to generate raw FASTQ files. For alternative splicing analysis, RNA sequencing reads were processed using the SpliceSeq software[17] (version 2.1, default parameters), with a reference splice graph database built using the GRCh38 reference genome downloaded from the NCBI FTP server and feature annotation from Ensembl (GRCh38 version 85) with all transcript support levels. The sample-wise PSI indices of *KRAS-4A* estimated by SpliceSeq corresponded with the microarray analysis and are similarly referred to as the *KRAS-4A* splicing index. For gene level analysis, sequencing reads were aligned to the GRCh38 reference genome (downloaded from https://ccb.jhu.edu/software/hisat2/index.shtml) using HISAT2[18] (version 2.0.4), and the resulting BAM files were preprocessed with the standard GATK/Picard[19] pipeline. Read counts were calculated using the htseq-count tool from the python package HTSeq[20] (version 0.9.1) with feature annotation from GENCODE[21] (GRCh38; gencode.v27.annotation.gtf). Normalized gene expression levels were calculated as fragments per kilobase of transcripts per million mapped reads (FPKM). The FPKM values were log2 transformed after a constant of 0.005 was added to FPKM values of 0 to avoid infinite values.

Gene set enrichment analysis

The microarray and RNA sequencing gene level expression datasets were matched by HGNC symbols (N = 18,069 common protein-coding genes) and merged by batch correction using the ComBat function in the R package SVA, and for overlapping samples, the expression data from the microarray dataset was used. Single-sample gene set enrichment analysis for a set of 200 genes up-regulated by KRAS activation (HALLMARK_KRAS_SIGNALING_UP) obtained from the Molecular Signatures Database[22] was performed with the GSVA R package as described in Sveen *et al.*[15]

TCGA COADREAD samples

PSI indices of *KRAS-4A* for 131 MSS CRCs and 51 normal mucosa samples were downloaded from the TCGA SpliceSeq database.[23] Information on MSI and *KRAS* mutation status was obtained from TCGA.[24]

Statistical analyses

All statistical tests were performed in the R environment (version 3.4.2), including Mann–Whitney U and Wilcoxon signed rank tests using the wilcox.exact function in the R package exactRankTests, and Spearman's rank correlation test using the function cor.test. The Cox proportional hazards regression model with Wald test was applied for univariable and multivariable survival analysis using the coxph function in the R package survival. Kaplan–Meier survival curves were generated using the R package survminer and the ggsurvplot function. Survival analysis was performed on the microarray and RNA sequencing datasets in combination, and for overlapping samples, the dichotomized splicing index estimated by microarray analysis was used. Five-year overall survival was used as endpoint, defined as the time from surgery to death from any cause, censored at last follow-up or 5 years after surgery. All tests were 2-sided, and p-values lower than 0.05 are reported as significant.

Results

The *KRAS-4A* splice variant is down-regulated in CRC

The relative abundance of the two splice variants of *KRAS*, *KRAS-4A* and *KRAS-4B* (Figs. 1*a* and 1*b*), was analyzed by splicing-sensitive microarrays and/or RNA sequencing in 258 Stage I–IV MSS CRCs (Supporting Information Fig. 1). Quality control showed good correlation of the *KRAS-4A* splicing index among the 38 samples analyzed on both platforms (Spearman's $\rho = 0.65$, $p < 0.001$). In comparison with normal colonic mucosa, the *KRAS-4A* splicing index was significantly down-regulated in CRC in both datasets ($p < 0.001$; Fig. 1*c*), also in paired analysis of matching tumor–normal samples ($p \leq 0.001$; Supporting Information Fig. 2*a*). Stratification according to mutation status showed that *KRAS* mutations did not influence *KRAS* splicing (Fig. 1*c*). These results were validated in an independent patient cohort of 131 MSS CRCs, obtained from the TCGA SpliceSeq database, where the *KRAS-4A* splicing index was significantly lower in MSS CRCs compared to normal mucosa, independently of *KRAS* mutation status (Fig. 1*d* and Supporting Information Fig. 2*b*).

To evaluate the impact of *KRAS* splicing on downstream signaling, sample-wise enrichment scores were calculated for a gene expression signature of KRAS activity. In general, KRAS signaling was higher in CRCs than in normal colonic mucosa ($p < 0.001$; Supporting Information Fig. 3). Furthermore, dichotomization of the CRCs according to the median *KRAS-4A* splicing index within the two datasets revealed significantly higher KRAS signaling in samples with the lowest splicing indices ($p = 0.016$), indicating splicing-associated up-regulation of KRAS signaling. Interestingly, this was found to be significant only for *KRAS* wild-type tumors ($p = 0.005$) and not in the *KRAS* mutant subgroup ($p = 0.712$; Fig. 1*e*).

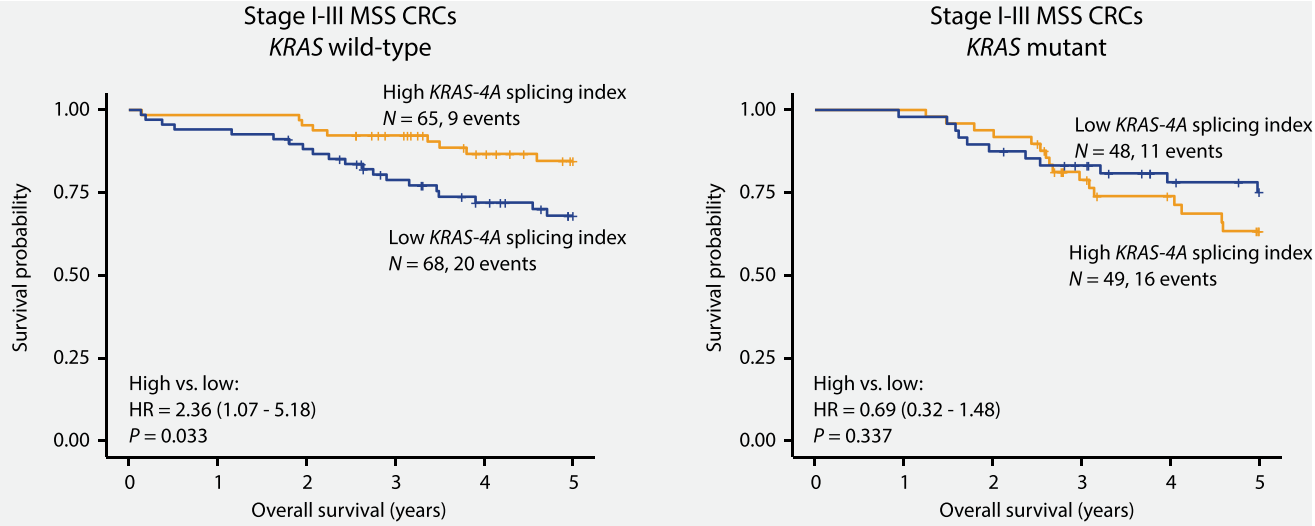

Figure 2. Mutation status-dependent poor-prognostic impact of *KRAS* splicing. Among patients with Stage I–III MSS CRC, a low *KRAS-4A* splicing index (below median) was associated with poor survival specifically in the *KRAS* wild-type subgroup (5-year overall survival: 85% *vs.* 68%), but not in patients harboring tumors with *KRAS* mutations.

The *KRAS-4A* splicing index has prognostic impact dependent on mutation status

Considering the mutation status-dependent impact of *KRAS* splicing on downstream signaling, we also analyzed prognostic associations according to mutation status in Stage I–III MSS CRCs. Patients from the microarray and sequencing datasets were separately dichotomized by their median *KRAS-4A* splicing index within the two datasets and combined (N = 230). Among all patients, there was no association between the *KRAS-4A* splicing index and overall survival (HR: 1.29, 95% CI: 0.76–2.18, p = 0.346). However, stratification according to mutation status revealed that low *KRAS-4A* splicing indices were associated with a significantly lower 5-year overall survival rate than high splicing indices specifically in the *KRAS* wild-type subgroup (HR: 2.36, 95% CI: 1.07–5.18, p = 0.033), and not in the subset of patients with mutant *KRAS* (HR: 0.69, 95% CI: 0.32–1.48, p = 0.337, $p_{interaction}$ = 0.026; Fig. 2), consistent with the corresponding impact of splicing on KRAS signaling. The negative prognostic value of low *KRAS-4A* splicing indices in *KRAS* wild-type cases was retained in multivariable

Table 1. Univariable and multivariable analysis of the prognostic impact of the *KRAS-4A* splicing index in *KRAS* wild-type patients with Stage I–III MSS CRCs

	Five-year overall survival			
	Univariable analysis		Multivariable analysis[1]	
Variable	HR (95% CI)	*p*	HR (95% CI)	*p*
KRAS-4A splicing index				
High (above median)	1		1	
Low (below median)	2.36 (1.07–5.18)	0.033	2.68 (1.18–6.09)	0.018
Gender				
Female	1		1	
Male	1.45 (0.69–3.03)	0.327	1.14 (0.54–2.41)	0.737
Age[2]	1.04 (1.00–1.07)	0.025	1.05 (1.01–1.09)	0.012
Location				
Right	1		1	
Left or rectum	1.27 (0.52–3.13)	0.597	1.85 (0.70–4.86)	0.213
Stage				
Stage I and II	1		1	
Stage III	1.20 (0.57–2.51)	0.633	1.26 (0.60–2.66)	0.536

[1]Included all variables in table. N = 133.
[2]Continous variable.

analysis including patient age and gender, as well as tumor stage and location (HR: 2.68, 95% CI: 1.18–6.09, p = 0.018, Table 1). Similar results were also seen for *KRAS* wild-type patients that had not received post-operative chemotherapy, although statistically significant only in the multivariable analysis (multivariable HR: 2.82, 95% CI: 1.11–7.16, p = 0.029, Supporting Information Table 2 and Supporting Information Fig. 4). Furthermore, a similar prognostic association was found when analyzing the *KRAS-4A* splicing index as a continuous variable among *KRAS* wild-type Stage I–III MSS CRCs, although statistically significant only in the sequencing dataset (multivariable HR: 0.001, 95% CI: 0.00–0.30, p = 0.017).

Discussion

Our study shows that the clinical relevance of *KRAS* aberrations in MSS CRC is not limited to genetic changes, but also extends to the RNA splicing process. In a single-hospital series of MSS CRCs investigated for *KRAS* splicing, relative down-regulation of the *KRAS-4A* versus *KRAS-4B* splice variant was associated with high KRAS signaling and a poor patient outcome. However, both effects were specific to the *KRAS* wild-type subgroup, consistent with the presumption that the effect of mutations outweighs the effect of aberrant splicing in this gene. Nonetheless, although targeting the MAP kinase pathway may be effective in *KRAS* wild-type CRC, the proportion of patients that are sensitive to anti-EGFR therapy is modest, highlighting the need for improved biological knowledge in this subgroup. Along with mutations in *PIK3CA* and *MET* amplification,[7] *KRAS* amplification has been shown to cause resistance in a small proportion of patients.[25] Based on our finding of an association with increased KRAS signaling and poor patient outcome, albeit in the primary setting, we hypothesize that consideration of *KRAS* splicing may also contribute to optimize the treatment of *KRAS* wild-type CRC.

We confirm previous results showing that *KRAS* splicing is altered in CRC compared to normal colonic mucosa by relative down-regulation of *KRAS-4A* versus *KRAS-4B* splice variants, and that this is not dependent on the presence of *KRAS* mutations.[12] Furthermore, it has been shown also on the protein level that the expression level specifically of the KRAS-4A variant may be prognostic in *KRAS* wild-type CRC.[13] However, in the latter study, the prognostic impact of KRAS-4A and KRAS-4B protein expression was investigated separately. Studies in preclinical models have shown that *KRAS-4A* and *KRAS-4B* differ in their ability to regulate apoptosis, proliferation and metastasis-related processes,[9–11] suggesting that the impact of *KRAS* splicing on patient outcome might ultimately be dependent on the overall balance of *KRAS-4A* and *KRAS-4B* expression. Furthermore, a study of carcinogen-induced colonic adenomas in *KRAS* wild-type mice unable to express *KRAS-4A* revealed higher levels of *KRAS-4B* expression and concomitant evidence of an increased expression of the KRAS downstream effectors ERK1/2 and AKT,[11] consistent with higher KRAS signaling observed in tumors with low relative *KRAS-4A* expression in our dataset. In addition, an increase of the proliferation marker Ki-67 and a decrease of the apoptosis marker cleaved caspase-3 in mice depleted of *KRAS-4A* expression indicated that *KRAS-4A* has tumor suppressor activity in the *KRAS* wild-type setting,[11] providing a biological rationale for the poor outcome observed for patients with low relative *KRAS-4A* expression. It should be noted that the size of the patient series is limited and a search in the public domain for splicing-sensitive expression data with corresponding clinical information identified no suitable external validation series. However, our hypothesis that *KRAS* splicing is a surrogate marker for activated *KRAS* in the wild-type setting is intriguing and awaiting validation studies.

In conclusion, our study indicates that *KRAS* has prognostic value beyond mutation status in MSS CRC, and suggests relevance of investigating *KRAS* splicing for optimization of targeted treatment of *KRAS* wild-type CRC.

Authors Contribution

Conception and design: A.S., R.A.L.; Acquisition of data: I.A.E., J.M.S., R.I.S., A.N.; Analysis and interpretation of data: I.A.E., A.S., R.A.L.; Drafting of the manuscript: I.A.E., A.S., R.A.L.; Study supervision: A.S., R.A.L. All authors were involved in revision of the manuscript and have approved the final version.

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