Germline variation in RASAL2 may predict survival in patients with RAS-activated colorectal cancer

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Abstract
Background: Therapeutic agents that specifically target patients with RAS mutant colorectal cancer (CRC) are needed. We sought potential drug targets by relating genome-wide association study and survival data in patients with advanced CRC profiled for mitogen-activated protein kinase (MAPK) pathway mutations.

Methods: In total, 694 patients from the clinical trials COIN and COIN-B had MAPK-activated CRCs (assigned as KRAS, NRAS, or BRAF mutant). Genome-wide single nucleotide polymorphism (SNP), gene, and gene-set analyses were performed to identify determinants of survival. For rs12028023 in RAS protein activator-like 2 (RASAL2), we studied its effect by MAPK pathway activation status (by comparing to 760 patients without MAPK-activated CRCs), MAPK gene mutation status, surface area of the primary tumor (as a marker of proliferation), and expression on RASAL2.

Results: In MAGMA genome-wide analyses, RASAL2 was the most significant gene associated with survival (p = 2.0 x 10^-5). Patients carrying the minor (A) allele in the lead SNP, rs12028023 in intron 1 of RASAL2, had a median increase in survival of 167 days as compared with patients carrying the major allele. rs12028023 improved survival in patients with RAS mutant (hazard ratio \[HR\] = 0.62, 95% confidence intervals \[CI\] = 0.5–0.8, \[p\] = 3.4 x 10^-5) but not BRAF mutant (\[p\] = 0.87) CRCs. The rs12028023 A-allele was associated with reduced surface area of the primary tumor (Beta = -0.037, standard error \[SE\] = 0.017, \[p\] = 3.2 x 10^-2) and reduced RASAL2 expression in cultured fibroblasts (\[p\] = 1.6 x 10^-11).

Conclusion: Our data demonstrate a prognostic role for RASAL2 in patients with MAPK-activated CRCs, with potential as a therapeutic target.

KEYWORDS
colorectal cancer, MAPK-activation, RAS, RASAL2, survival

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Monoclonal antibodies against the epidermal growth factor receptor, such as cetuximab, have shown benefit in KRAS and, KRAS and NRAS (RAS), wild-type advanced colorectal cancer (CRC) when either used as a monotherapy, or in combination with chemotherapy. In contrast, targeted treatments for patients with RAS mutant disease are only just emerging. Given that around half of all CRCs are RAS mutant, this represents a clear unmet clinical need. AMG 510 (Sotorasib), an inhibitor of KRAS G12C, traps mutant KRAS in its inactive GDP-bound state and has shown effectiveness in a Phase 2 trial of nonsmall-cell lung cancer. MRTX849 (Adagrasib) also binds KRAS G12C and inhibits intercellular signaling, and has shown promising efficacy in patients with colorectal, nonsmall-cell lung, endometrial, pancreatic, and ovarian cancers. However, both treatments are only effective in cancers harboring G12C, which occurs in just 1–3% of CRCs. Identifying drug targets for improved survival in patients with RAS mutant CRCs therefore remains challenging.

RAS protein activator-like 2 (RASAL2) encodes a RAS GTPase-activating protein (GAP), which negatively regulates the RAS signaling pathway by converting RAS-GTP to RAS-GDP. RASAL2 was identified as a tumor suppressor in prostate cancer and its inactivation promotes progression and metastasis in colorectal, lung, ovarian and luminal B breast cancers. However, RASAL2 has also shown pro-oncogenic roles in triple-negative breast and hepatocellular cancers. Furthermore, RASAL2 is upregulated in metastatic CRCs with higher expression associated with lymph node involvement and distant metastasis. Knockdown of RASAL2 in multiple CRC cell lines decreases cell proliferation, anchorage-dependent and -independent growth, cell invasion, and migration and may represent a potential candidate for targeted therapy.

Relating germline variation to outcome in patients with RAS mutant cancers offers the prospect of identifying novel therapeutic targets. To explore this possibility, we analyzed genome-wide association study (GWAS) and survival data on 1589 patients with advanced CRC from the clinical trials COIN and COIN-B. Patients’ tumors were profiled for mutations in the mitogen-activated protein kinase (MAPK) and Akt pathways, to help stratify our survival analyses by MAPK pathway activation status.

2 | MATERIALS AND METHODS

2.1 | Patients and samples

In total, 2671 unrelated patients with metastatic or locally advanced CRC were recruited into the MRC clinical trials COIN (NCT00182715) and COIN-B (NCT00640081) and treated with oxaliplatin and...
**TABLE 1** Clinicopathological features of patients with and without MAPK-activated tumors.

<table>
<thead>
<tr>
<th>Clinicopathological factor</th>
<th>Patients with MAPK-activated CRCs (n = 694)</th>
<th>Patients without MAPK-activated CRCs (n = 760)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>436</td>
<td>535</td>
<td>2.2 × 10⁻³</td>
</tr>
<tr>
<td>Female</td>
<td>258</td>
<td>225</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>Median (years) 64</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>Response at 12-weeks</td>
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<td></td>
<td></td>
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<tr>
<td>Responders</td>
<td>295</td>
<td>452</td>
<td>1.9 × 10⁻¹¹</td>
</tr>
<tr>
<td>Nonresponders</td>
<td>293</td>
<td>203</td>
<td></td>
</tr>
<tr>
<td>No data</td>
<td>106</td>
<td>105</td>
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<td>Overall survival</td>
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</tr>
<tr>
<td>Median (95% CI; days)</td>
<td>433 (397–465)</td>
<td>611 (569–659)</td>
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<tr>
<td>WHO performance status</td>
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<td></td>
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<tr>
<td>0</td>
<td>330</td>
<td>356</td>
<td>4.7 × 10⁻²</td>
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<tr>
<td>1</td>
<td>301</td>
<td>359</td>
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</tr>
<tr>
<td>2</td>
<td>63</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>Site of primary tumor</td>
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<td></td>
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</tr>
<tr>
<td>Left colon</td>
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<td>235</td>
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<tr>
<td>Right colon</td>
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<tr>
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<td>2</td>
<td>0.3</td>
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<td>Status of primary tumor</td>
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<tr>
<td>3</td>
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</tr>
<tr>
<td>4</td>
<td>694</td>
<td>760</td>
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<tr>
<td>Timing of metastases</td>
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<tr>
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<tr>
<td>Liver only</td>
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<tr>
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<td>386</td>
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<tr>
<td>Nonliver</td>
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<td>175</td>
<td>23</td>
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<tr>
<td>Number of metastatic sites</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>220</td>
<td>290</td>
<td>38.2</td>
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<tr>
<td>2</td>
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<td>22.2</td>
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<td>MAPK activated</td>
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<tr>
<td>Mutation status</td>
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<tr>
<td>KRAS mutation</td>
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(Continues)
fluoropyrimidine chemotherapy, with or without cetuximab. Patients were combined for survival analyses since there was no evidence of heterogeneity in overall survival (OS; time from trial randomization to death or end of trial) between patients when analyzed by trial, trial arm, type of chemotherapy received, or cetuximab use.\textsuperscript{22} Assessment of response was performed at 12 weeks; response was defined as complete or partial response using RECIST 1.0 guidelines and no response was defined as stable or progressive disease.

### 2.2 | Germline genotyping

DNA was extracted from blood samples from 2244 patients by conventional methods and genotyped using Affymetrix Axiom Arrays.\textsuperscript{23} After quality control (QC), genotype data were available on 1950 patients. Prediction of untyped single nucleotide polymorphisms (SNPs) was carried out using IMPUTE2 v2.3.0\textsuperscript{24} based on data from the 1000 Genomes Project as reference.\textsuperscript{25,26} Discordant sex, individual and SNP missingness, heterozygosity, relatedness, principal component analysis (PCA), minor allele frequency (MAF), and Hardy–Weinberg Equilibrium (HWE) QC steps were performed as previously described.\textsuperscript{22} In brief, we excluded SNPs with MAFs <5%, poor imputation scores (INFO score <0.8), missingness >0.02, or HWE exact test \( p < 1.0 \times 10^{-6} \). Survival data were missing on two patients, leaving 1948 for analysis.

### 2.3 | Somatic genotyping

Tumor samples were not available, or were of insufficient quantity, in 301 of the 1948 patients. DNA was extracted from formalin-fixed paraffin embedded CRC for the remaining 1647 patients and screened for KRAS (codons 12, 13, and 61), NRAS (codons 12 and 61), BRAF (codons 594 and 600) and PIK3CA (codons 542, 545, 546, and 1047) mutations using Pyrosequencing and Sequenom technologies.\textsuperscript{27} Microsatellite instability (MSI) status in tumors was determined using the markers BAT-25 and BAT-26. Overall, KRAS mutations (G12A, G12D, G12V, G12C, G12R, G12S, G13C, G13D, G13S, G13R, G61H, G61L, Q61R, and four remained uncharacterized) were identified in 637/1589 (40.1%), NRAS mutations (G12C, Q61K, Q61L, Q61H, Q61R, and one remained uncharacterized) in 54/1546 (3.5%), BRAF mutations (D594G and V600E) in 143/1554 (9.2%) and PIK3CA mutations (E542K, E545K, Q546K, H1047L, and H1047R) in 212/1448 (14.6%) CRCs. MSI was detected in 45/1237 (3.6%) CRCs. Of those also tested for BRAF mutations, 13/45 (28.9%) CRCs with MSI carried BRAF V600E as compared with 93/1185 (7.8%) without MSI (\( p = 3.1 \times 10^{-6} \)), consistent with their sporadic nature.\textsuperscript{28} MAPK-activated CRCs were assigned as those carrying KRAS, BRAF, or NRAS mutations. In total, 829 patients with MAPK-activated CRCs had corresponding GWAS data. We excluded patients with potentially Akt-activated tumors (those with PIK3CA mutations, \( n = 108 \)), MSI (\( n = 20 \)), and those in whom covariate data were lacking (\( n = 7 \) for platelet count, primary tumor surface area, time to metastases or synchronous/metachronous metastases). Of the remaining 760 patients, 521 (75.1%) carried KRAS mutations, 44 (6.3%) NRAS mutations, 120 (17.3%) BRAF mutations, and 9 (1.3%) had combinations of these mutations (Figure 1 and Table 1). For comparison, we analyzed 760 patients without MAPK-activated tumors (i.e., those with KRAS, NRAS, and BRAF wild-type CRC) and a further subset whose CRCs carried PIK3CA mutations as a marker of Akt-activation (\( n = 87 \) patients with covariate data).

### 2.4 | Statistical analyses

We previously identified clinicopathological factors associated with survival in patients from COIN and COIN-B.\textsuperscript{22} Due to the number of covariates added to the regression models, dimensionality reduction was performed using PCA to reduce the risk of overfitting. A threshold of 70% total variance explained was used to select the number of principal components to include,\textsuperscript{29} the first five were selected (but only four were necessary when analyzing patients with NRAS mutations). We carried out the GWAS for OS under an additive model. All analyses performed by MAPK gene mutation status were multivariate.

Gene and gene-set analysis were performed on the summary statistics from the association analysis to identify genes containing significant numbers of highly associated SNPs and significantly enriched gene sets. The threshold for significance at gene level was \( p < 2.5 \times 10^{-6} \).
**FIGURE 2** Legend on next page.
a Bonferroni correction for 20,000 independent tests. Correction for multiple testing for gene-set analysis was made by adjusting p-values for the false discovery rate to produce q-values, held to a significance threshold of q < 0.05.

2.5 Bioinformatic analyses

Regional association plots were created using LocusZoom (http://locuszoon.org). PCA, survival analyses, and manhattan/quantile-quantile plots were performed using the psych (https://cran.r-project.org/web/packages/psych/index.html), gwasurvivr, and qqman R (https://www.r-project.org) packages, respectively.

Gene and gene-set analyses were performed using MAGMA v1.09b (https://ctg.canc.nl/software/magma). SNPs were annotated to genes (including those 35 kb before the genes transcription zone and 10 kb after) using the --annotate command and the gene location file for hg19: “NCBI37.3.loc.” SNP p-values were assessed with the linkage disequilibrium between them using the multi = snp-wise and --gene-model commands. This model takes advantage of the sum of the $-\log_{10}(p)$ for all SNPs, as well as the top SNP associations within each gene, to assess the association of their constituent genes. Genes were annotated to sets by gene-ontology terms. A competitive model (--set-result command) was used to assess each gene-set’s association with OS.

Expression quantitative trait loci (eQTL) analysis was performed by searching the Genotype-Tissue Expression (GTEx) project database (https://gtexportal.org/home/) for associations between SNPs and gene expression.

3 RESULTS

Patients with MAPK-activated CRCs were defined as those carrying KRAS, NRAS, or BRAF mutations and that did not have Akt-activating mutations (n = 108) or MSI (n = 20). After QC, 694 patients had MAPK-activated CRCs (Figure 1). Patients with MAPK-activated CRCs had more right-sided primary tumors, worse response at 12 weeks and poorer survival (median OS 433 days) as compared with patients without MAPK-activated CRCs (KRAS, NRAS, and BRAF wild-type, n = 760, median OS 611 days; hazard ratio [HR] = 1.57, 95% confidence interval [CI] = 1.39–1.77, p = 2.6 × 10^-13; Table 1). Genomewide SNP, gene and gene-set analyses were performed to identify determinants of survival using the first five principal components as covariates, which explained 71.9% of the total variance for previously established prognostic factors. No detectable genomic inflation was observed (lambda = 1.08). No SNPs passed the threshold for genome-wide significance ($p < 5.0 \times 10^{-8}$).

In MAGMA gene analysis, RASAL2 at 1q25.2, was the most significant gene associated with survival in patients with MAPK-activated CRCs ($p = 2.0 \times 10^{-7}$) (Figure 2), although it did not achieve formal genome-wide significance. Patients carrying the minor (A) allele in the lead SNP, rs12028023 in intron 1 of RASAL2, had a median increase in survival of 167 days as compared with patients carrying the major (G) allele (HR = 0.63, 95% CI = 0.5–0.8, $p = 1.3 \times 10^{-5}$, Figure 3). In contrast, rs12028023 genotype was not associated with survival in patients without MAPK-activated tumors (HR = 1.00, 95% CI = 0.81–1.23, $p = 0.98$) nor a subset whose CRCs carried PIK3CA mutations as a marker of Akt-activation (HR = 1.72, 95% CI = 0.87–3.37, $p = 0.12$); the difference in the relationship between patient groups was significant ($p_{\text{geno}} = 2.1 \times 10^{-3}$ and $5.3 \times 10^{-3}$, respectively). Cetuximab administration did not influence the prognostic effect of rs12028023, regardless of the MAPK-activation status (MAPK-activated $p_{\text{test}} = 0.29$, nonactivated $p_{\text{test}} = 0.49$).

The rs12028023 A-allele was also associated with improved response at 12-weeks in patients with MAPK-activated cancers (77/128, 60.2% of patients carrying the A allele responded compared with 212/447, 47.4% with the G allele, OR = 1.62, 95% CI = 1.11–2.36, $p = 1.2 \times 10^{-2}$). This relationship was not seen in patients without MAPK-activated cancers (93/134, 69.4% vs. 352/513, 68.6%, OR = 0.98, 95% CI = 0.70–1.51, $p = 0.91$).

We dissected the prognostic role of RASAL2 by MAPK gene mutation status. The rs12028023 A-allele was associated with improved survival in patients with KRAS (median increase of 191 days, HR = 0.63, 95% CI = 0.5–0.8, $p = 1.0 \times 10^{-4}$) and NRAS (median increase of 407 days, HR = 0.22, 95% CI = 0.05–0.9, $p = 3.8 \times 10^{-2}$) mutant CRCs (combined RAS mutant–median increase of 186 days, HR = 0.62, 95% CI = 0.5–0.8, $p = 3.4 \times 10^{-5}$), but not in patients with BRAF mutant CRCs (HR = 1.05, 95% CI = 0.6–1.8, $p = 0.87$; Figure 4). Although there was a trend for a predictive effect on RAS compared with RAF mutant backgrounds, this did not reach statistical significance (for KRAS versus BRAF mutant, $p_{\text{test}} = 0.097$, NRAS versus BRAF mutant, $p_{\text{test}} = 4.6 \times 10^{-2}$, combined RAS versus BRAF mutant, $p_{\text{test}} = 8.5 \times 10^{-2}$).

The rs12028023 A-allele was associated with reduced surface area of the primary tumor (Beta = −0.037, standard error [SE] = 0.017, 95% CI = −0.058–0.018, $p = 0.037$).

FIGURE 2 Relationship between gene, genotype and survival in 694 patients with mitogen-activated protein kinase-activated colorectal cancers. (A) Manhattan plot of gene associations with overall survival (OS). Genes are ordered by chromosome position and plotted against the $-\log_{10}(p)$ for their association with OS. The red line represents the threshold for genome-wide significance ($p = 2.5 \times 10^{-8}$). (B) Regional locus zoom plot shows the analysis for single nucleotide polymorphisms (SNPs) and recombination rates. $-\log_{10}(p)$ (y-axis) of the SNPs are shown according to their chromosomal positions (x-axis) for an area 200 kb upstream and downstream of RASAL2. The sentinel SNP (purple) is labeled by its rsID. The color intensity of each symbol reflects the extent of linkage disequilibrium with the sentinel SNP, deep blue ($r^2 = 0$) through to dark red ($r^2 = 1.0$). Genetic recombination rates, estimated using 1000 Genomes Project samples, are shown with a blue line. Physical positions are based on NCBI build 37 of the human genome. Also shown are the relative positions of genes and transcripts mapping to the region of association. Genes have been redrawn to show their relative positions; therefore, maps are not to physical scale.
\( p = 3.2 \times 10^{-2} \) in patients with MAPK-activated CRCs. rs12028023 was an eQTL for RASAL2 in cultured fibroblasts \( (p = 1.6 \times 10^{-11}) \) with the A-allele associated with decreased RASAL2 expression.

Five gene sets (Golgi cisterna membrane, cisterna and stack, monoamine transport, and Cul4A-RING E3 ubiquitin ligase complex), were significantly associated with survival in patients with MAPK-activated CRCs after adjusting for multiple testing \( (q < 0.05) \).

**FIGURE 3** Kaplan–Meier plot of the relationship between rs12028023 genotype and overall survival in patients with mitogen-activated protein kinase-activated colorectal cancers. Time in days plotted against survival probability for patients homozygous for the major allele (GG) and heterozygous (GA) or homozygous for the minor allele (AA). Shaded areas represent 95% confidence intervals. The number of patients still at risk at each time point is shown beneath. 95% CI, 95% confidence intervals; HR, hazard ratio.

4 | DISCUSSION

To help identify novel therapeutic targets in patients with MAPK-activated CRCs, we studied the relationship between germline variation and survival in patients with somatically profiled advanced CRC. RASAL2 was the most significant gene associated with survival in patients with MAPK-activated CRCs. Although RASAL2 did not pass formal genome-wide significance in our screen, its direct interaction with RAS (as 1 of only 14 known RAS GAPs\(^38\)) suggests it is highly unlikely to have been identified by chance. Given that we only had 694 patients with MAPK-activated CRCs, it is more likely that we had too few cases to achieve the stringent threshold for genome-wide significance. It is noteworthy that the rs12028023 A-allele specifically improved survival in patients with KRAS and NRAS mutant cancers, but not in those with BRAF mutant cancers, supporting a direct effect on the upstream RAS signaling pathway. The lack of association in patients with BRAF mutant cancers was unlikely to be due to the small numbers of samples \( (n = 120) \) since we observed this effect in a much smaller group with NRAS mutant cancers \( (n = 44) \). Furthermore, rs12028023 did not influence survival in patients without MAPK-activated CRCs, nor the subset with Akt-activation, highlighting its specificity to this pathway.

Carriers of the rs12028023 A-allele were predicted to have reduced RASAL2 expression and a median increase in survival of 167 days in patients with MAPK-activated CRCs and 186 days in the subset with RAS-mutant CRCs. Importantly, others have shown that reduced RASAL2 expression is also associated with improved survival.
FIGURE 4  Relationship between inherited genetic variation in RASAL2 and survival by mitogen-activated protein kinase gene mutation status. Regional locus zoom plots for single nucleotide polymorphism (SNP) associations with overall survival in patients with colorectal cancers carrying (A) KRAS mutations \((n = 521)\), (B) NRAS mutations \((n = 44)\) and (C) BRAF mutations \((n = 120)\). Plots show results of the analysis for SNPs and recombination rates. \(-\log_{10}(p)\) (y-axis) of the SNPs are shown according to their chromosomal positions (x-axis) for an area 200 kb upstream and downstream of RASAL2. The sentinel SNP (purple) is labeled by its rsID. The color intensity of each symbol reflects the extent of linkage disequilibrium with the sentinel SNP, deep blue \((r^2 = 0)\) through to dark red \((r^2 = 1.0)\). Genetic recombination rates, estimated using 1000 Genomes Project samples, are shown with a blue line. Physical positions are based on NCBI build 37 of the human genome. Also shown are the relative positions of genes and transcripts mapping to the region of association. Genes have been redrawn to show their relative positions; therefore, maps are not to physical scale. Hazard ratio (HR), 95% confidence intervals (CI), and p-values are given for rs12028023.
in two independent cohorts of patients with CRC, although these were not molecularly stratified by MAPK-activation status. However, these data suggest that RASAL2 may represent a potential therapeutic target via modulation of its expression and warrant further investigation. Interestingly, we noted that the rs12028023 A-allele was associated with reduced surface area of the primary tumor in patients with MAPK-activated CRCs, potentially supporting a link between reduced RASAL2 expression and decreased proliferation. These data are consistent with in vitro models of RASAL2 knockdown. Furthermore, given RASAL2’s role in tumourigenesis in other cell types, we speculate that it may represent a target for intervention in a broader range of cancers.

AUTHOR CONTRIBUTIONS
Jeremy P. Cheadle obtained funding for and directed this study. The study was designed by Christopher Wills and Jeremy P. Cheadle. Timothy S. Maughan was Chief Investigator of COIN and provided clinical advice and supported the translational research. David Fisher facilitated access to the clinical data, Nada A. Al-Tassan oversaw the genotyping and Richard S. Houlston oversaw the imputation and quality control. Christopher Wills undertook all of the statistical analyses with supervision from Valentina Escott-Price and Jeremy P. Cheadle. Christopher Wills and Jeremy P. Cheadle interpreted the data with input from Katie Watts and Valentina Escott-Price. Christopher Wills wrote the first draft of the article with subsequent input from Jeremy P. Cheadle, and all authors provided comments.

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CONFLICT OF INTEREST STATEMENT
The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT
The GWAS summary statistics are available through the NHGRI-EBI GWAS Catalog at https://www.ebi.ac.uk/gwas/, under study accession number GCST90244553. Further details and other data that support the findings of this study are available from the corresponding author upon request.

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