

KRAS inhibitors may prevent colorectal cancer metachronous metastasis by suppressing TGF- β mediated epithelial-mesenchymal transition

YAOYU GUO^{1*}, CHULING HU^{1*}, KUNTAI CAI^{1*}, GUOJIE LONG², DU CAI¹, ZHAOLIANG YU¹,
XINXIN HUANG¹, ZERONG CAI¹, PEISHAN HU¹, YUFENG CHEN¹, FENG GAO¹ and XIAOJIAN WU¹

¹Guangdong Institute of Gastroenterology, Department of General Surgery, Guangdong Provincial Key Laboratory of Colorectal and Pelvic Floor Diseases, The Sixth Affiliated Hospital, Sun Yat-sen University, Guangzhou, Guangdong 510655, P.R. China; ²Department of General Surgery (Department of Pancreatic Hepatobiliary Surgery), The Sixth Affiliated Hospital, Sun Yat-sen University, Guangzhou, Guangdong 510655, P.R. China

Received August 9, 2024; Accepted October 22, 2024

DOI: 10.3892/mmr.2024.13389

Abstract. In colorectal cancer (CRC), KRAS mutations enhance metachronous metastasis, a condition without prognostic biomarkers or preventive measures. The present study demonstrated that KRAS mutation may be a risk factor for CRC metachronous metastasis through meta-analysis of public databases. A risk scoring model was constructed using machine learning for predicting metachronous metastasis in KRAS-mutant CRC. Wound healing and Transwell assay indicated that KRAS inhibitors strongly suppress migration and invasion capabilities of high-risk CRC cells and these findings were validated through *ex vivo* organoid and a mouse model of splenic-liver metastasis. Mechanistically, RNA sequencing, reverse transcription-quantitative PCR and western blot analyses revealed that KRAS inhibitors suppressed epithelial-mesenchymal transition (EMT) and transforming growth factor β (TGF- β) signaling. Notably, addition of TGF- β 1 protein partially reversed the inhibitory effects of KRAS inhibitors on CRC. These results suggested that KRAS inhibitors may prevent CRC metachronous metastasis by downregulating TGF- β -mediated EMT, suggesting they can be used prophylactically in high-risk KRAS-mutant CRC.

Introduction

Colorectal cancer (CRC) is one of the most prevalent malignancies worldwide, with nearly 2 million new cases reported annually (1). Although treatment strategies incorporating surgery and chemotherapy are used for CRC, up to 23% of patients still experience distant recurrence, known as metachronous metastasis (2). Such patients often have a poor prognosis, with 5-year survival rate <30% (3,4). Moreover, the effectiveness of current first-line chemotherapy in preventing metachronous metastasis of CRC is limited (5,6). This calls for in-depth characterization of the molecular profile of distant recurrence of CRC following surgery to guide the development of more effective interventions for high-risk patients.

KRAS is an oncogene involved in the development and progression of CRC, with ~40% of CRC cases harboring KRAS mutation (7). Evidence from previous investigations has indicated that KRAS mutation drives epithelial-mesenchymal transition (EMT), promoting CRC migration and invasion (8,9). KRAS mutation might serve as a risk factor for metachronous metastasis in CRC (10-12). Therefore, a novel risk assessment model and targeted therapy tailored for patients with KRAS mutation are needed to offer new perspectives for prevention of metachronous metastasis in CRC.

KRAS has long been considered an undruggable target. However, studies have led to the development of KRAS inhibitors (13,14). Adagrasib, a small molecule which traps KRAS^{G12C} in the inactive state (GDP-bound), has been demonstrated to be efficacious in a CRC clinical trial (15). A recent study developed a pan-KRAS inhibitor, BI2865, which binds to a residue in the switch II binding pocket (His95) present only in KRAS, sparing other RAS family proteins, which showed satisfactory results in a pre-clinical trial (16). However, whether such KRAS inhibitors prevent CRC metastasis remains to be clarified. Existing literature suggests that KRAS mutations drive EMT via TGF- β signaling in CRC (8,17). Thus, it was hypothesized that KRAS inhibitors suppress CRC metastasis by restraining EMT.

Correspondence to: Professor Feng Gao or Professor Xiaojian Wu, Guangdong Institute of Gastroenterology, Department of General Surgery, Guangdong Provincial Key Laboratory of Colorectal and Pelvic Floor Diseases, The Sixth Affiliated Hospital, Sun Yat-sen University, 26 Erheng Road, Yuancun, Tianhe, Guangzhou, Guangdong 510655, P.R. China
E-mail: gaof57@mail.sysu.edu.cn
E-mail: wuxjian@mail.sysu.edu.cn

*Contributed equally

Key words: KRAS inhibitor, metastasis, colorectal cancer, machine learning, meta-analysis

The present study aimed to verify the association between KRAS mutation and metachronous metastasis of CRC and develop a tool for identifying patients with high-risk CRC who are likely to experience metachronous metastasis, as well as assess the potential of KRAS inhibitors on preventing CRC metachronous metastasis and explore the underlying mechanism.

Materials and methods

Meta-analysis. A systemic literature review was performed on the PubMed database (pubmed.ncbi.nlm.nih.gov), Embase (embase.com) and Cochrane library (cochrane.org). The search was conducted using the following terms: [(KRAS) or (K-RAS)] and [(colorectal cancer) or (colon cancer) or (rectal cancer)] and [(metachronous metastasis) or (distant recurrence) or (distant relapse)]. The study was performed in line with the guidelines of PRISMA (18). Inclusion criteria were as follows: i) Study included patients who were initially diagnosed with stage I-III CRC and underwent curative surgery; ii) KRAS mutational status was provided and iii) follow-up information including distant recurrence after surgery was reported. Duplicates, non-English literature, reviews, editorials, case reports, abstracts and studies unrelated to CRC were excluded (Fig. S1A). A systematic full-text review was conducted on eligible literature, to collect information, including the first author name, publication date, sample size, KRAS mutation rate and metachronous survival rate. The odds ratio (OR) and 95% confidence interval (CI) were utilized as the outcome measures. The OR >1 indicated that the KRAS mutation was a risk factor for metachronous metastasis. Heterogeneity was examined using I^2 and Cochran's Q statistics. An I^2 value >50% indicated substantial heterogeneity. Statistical significance was determined based on $P < 0.05$ obtained by the Wald test. The results were presented using a forest plot. Publication bias in the studies was determined using a funnel plot. Figures were plotted with R (Version:4.0.3; R-project.org).

Data acquisition. Clinical information, KRAS mutation status and transcriptome sequencing data of 1,001 patients with CRC were downloaded from the Omics Study of CRC (COCC) in China (icgc-argo.org/page/114/cgcc) cohort of International Cancer Genome Consortium Accelerating Research in Genomic Oncology (ICGC-ARGO) project. Moreover, clinical data and KRAS mutational status were derived from the MSKCC (Memorial Sloan-Kettering Cancer Center) cohort reported by Chatila *et al* (19) and Sidra-LUMC (Leiden University Medical Center) AC-ICAM (Atlas and Compass of Immune-Cancer-Microbiome interactions) cohort data reported by Roelands *et al* (20) were retrieved from cbiportal (cbiportal.org). Transcriptome sequencing data were downloaded from the Gene Expression Omnibus (GEO; accession no. GSE209746; ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE209746) and Genotypes and Phenotypes (accession no. phs002978.v1.p1; ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs002978.v1.p1). Transcriptome data of 42 KRAS-MUT CRC cell lines were acquired from the Cancer Cell Line Encyclopedia (CCLE) database (sites.broadinstitute.org/ccle/datasets).

Model construction. To construct the metachronous metastatic risk prediction model for patients with KRAS-mutant CRC, 286 cases with KRAS mutation from the ICGC-ARGO cohort were selected as the training cohort. In addition, 77 KRAS-mutant cases from the Sidra-LUMC AC-ICAM cohort and 32 KRAS-mutant cases from the MSKCC cohort with eligible gene expression data were enrolled as independent validation cohorts. Inclusion criteria were as follows: i) Patients with stage I-III CRC who underwent curative surgery and ii) CRC with KRAS-mutation. Cases with no sequencing data and incomplete clinical information were excluded. In the training cohort, Lasso-Cox regression was performed to identify metastasis-associated genes and establish KRAS metachronous-metastasis (KMM) score ($\text{KMM score} = \sum_i \text{coefficient}(\text{gene}_i) \times \text{expression}(\text{gene}_i)$). The patients were stratified into high- and low-risk groups based on the unsupervised KMM score cutoff (ARGO, -0.2184; Sidra-LUMC AC-ICAM, -0.0362; MSKCC, -31.1379) determined by the Youden index. Kaplan-Meier, differential gene expression and gene set enrichment analysis (GSEA) were performed using R (Version:4.0.3; r-project.org) to compare differences in metastasis, gene expression and pathway activation pattern between high- and low-risk groups.

Cell culture. The cell lines were purchased from Meisen Chinese Tissue Culture Collections (Zhejiang Meisen Cell Technology, Ltd.) and authenticated using short tandem repeat analysis. The cell lines were confirmed to be KRAS-mutant CRC (SW480: KRAS-G12V; HCT116: KRAS-G13D; SW837: KRAS-G12C; DLD1: KRAS-G13D; HCT15: KRAS-G13D) making them suitable models for testing KRAS inhibitors (21). The cells were cultured in DMEM (SW480, HCT116 and SW837) or RPMI-1640 (both Gibco; Thermo Fisher Scientific, Inc.) (DLD1 and HCT15) supplemented with 10% fetal bovine serum (HyClone, Cytiva) and 1% penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.) in 5% CO₂ environment at 37°C. Monthly mycoplasma testing was performed on the cells. BI-2865 (1 μM), adagrasib (60 nM), oxaliplatin (1 μM) and 5-fluorouracil (5 μM) were purchased from TargetMol. After drug addition, all the cells were incubated at 37°C for 48 h. The human recombinant TGF- β 1 protein was purchased from MedChemExpress.

Colony formation assay. SW837 cells were dissociated by digestion with trypsin, resuspended in complete culture DMEM as single cells and seeded into six-well plates (20,000 cells/well) at 37°C. The medium was changed every 3 days and the cells were cultured for 12 days at 37°C. The cell colonies were fixed with 20% methanol for 15 min at room temperature, stained with crystal violet (0.5%) for 15 min at room temperature and then imaged. The colony is defined to consist of at least 50 cells. Colonies were quantified using ImageJ (Version:1.53q; imagej.net/ij).

Wound healing assay. The cells (HCT116, SW480, DLD1, HCT15 and SW837) were seeded in a six-well plate at a density of 5×10^5 cells/well and incubated overnight at 37°C to form a 100% confluent monolayer. Complete medium was replaced with serum-free medium (RPMI-1640 for DLD1 and HCT15; DMEM for HCT116, SW480 and SW837) as previously

described (22). A scratch was created on the cell surface using a 200- μ l pipette tip. After incubating at 37°C for 48 h, wounds were photographed under Olympus IX73 phase-contrast inverted light microscopy on 40x magnification and images were analyzed using ImageJ (Version:1.53q; imagej.net/ij).

Transwell assay. A total of 1×10^5 cells (HCT116, SW480, DLD1, HCT15 and SW837) was resuspended in 200 μ l serum-free medium (RPMI-1640 for DLD1 and HCT15; DMEM for HCT116, SW480 and SW837) and seeded into the upper chamber of a Transwell plate (8 μ m pore size; Corning, Inc.). The upper chamber was pre-coated with Matrigel (1:20, cat. no. 356234; Corning, Inc.) at 37°C for 30 min. Next, 500 μ l medium (RPMI-1640 for DLD1 and HCT15; DMEM for HCT116, SW480 and SW837) containing 10% fetal bovine serum (HyClone, Cytiva) was added to the lower chamber. After incubation at 37°C for 48 h, cells were fixed with 4% paraformaldehyde for 15 min at room temperature and then stained with 1% crystal violet solution for 15 min at room temperature. A total of five randomly selected fields of view/plate was visualized under Olympus IX73 inverted light microscope on 200x magnification. Finally, the images were processed using ImageJ (Version:1.53q; imagej.net/ij).

Organoid culture. The source and culture of organoids was performed using a previously published method (23).

Cell viability assay. The cells (HCT116, SW480, DLD1, HCT15 and SW837) were cultured in 96-well plates (2,000 cells per well, with three replicates for each group. After incubating at 37°C for 24 h, cells were treated with varying concentrations of drugs and incubated at 37°C for 96 h. To test the IC₅₀ of HCT116, SW480, DLD1 and HCT15, the gradient concentrations of oxaliplatin and 5-fluorouracil are 1 nM, 10 nM, 100 nM, 500 nM, 1 μ M, 5 μ M, 10 μ M, 50 μ M, 100 μ M and 1 mM. To test the IC₅₀ of SW837, concentrations of adagrasib are 100 pM, 1 nM, 5 nM, 10 nM, 50 nM, 100 nM, 500 nM, 1 μ M, 5 μ M and 10 μ M. The concentration of adagrasib for growth curve assay (SW837) is 500 nM. The number of viable cells was determined using the CellTiter-Glo Luminescent Cell Viability Assay (cat. no. #G7573, Promega Corporation), following the manufacturer's protocol.

Establishment of cell lines with acquired resistance to adagrasib. 1×10^6 SW837 cells were cultured with 60 nM adagrasib at 37°C for 72 h. Next, 50% confluent cells were treated with adagrasib at IC₅₀ (60 nM) for 72 h, followed by a 96 h incubation at 37°C. This cycle was repeated once and the remaining cells were cultured with adagrasib (60 nM) for 3 passages. The resistant cells were continuously cultured in the presence of adagrasib (60 nM) at 37°C for 72 h and the passaged parental cells were utilized for cellular assays.

Western blotting. Total protein of cells was extracted using RIPA cell lysis buffer, containing 150 mM NaCl, 50 mM Tris-HCl, 0.5% sodium deoxycholate, 200 mM NaF, 200 mM PMSF, 1.0% NP-40 and 1 mM EDTA, supplemented with a protease inhibitor cocktail (Roche Diagnostics). Protein lysate was determined using BCA method. Equal amounts of protein (15 μ g/lane), boiled with a pre-stained protein marker

(cat. no. M221, Beijing Kangrun Chengye Biotechnology Co., Ltd.) was subjected to 10% SDS-PAGE and then transferred to PVDF membranes for immunoblotting analysis. After blocking with TBST (0.1% Tween-20) containing 5% non-fat dry milk powder for 2 h at room temperature, the membranes were incubated overnight at 4°C with primary antibodies. The membranes were incubated with secondary HRP-conjugated antibodies at room temperature for 2 h and then protein bands were visualized with ECL western-blotting kit (Tanon, Cat. No.: 180-5001) using ChemiDoc (Bio-Rad Laboratories, Inc.) system. The density of the bands was determined using ImageJ (Version:1.53q; imagej.net/ij). The antibodies are presented in Table SI.

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from the cells (SW480, HCT116 and SW837) using the RNeasy Mini kit (Qiagen GmbH) according to the manufacturer's instructions. A total of 1 μ g extracted RNA was reverse-transcribed to cDNA using the RT kit (cat. no. #AT341-02, Transgen Biotech Co., Ltd.) before qPCR using the PCR kit (cat. no. #N30920, Transgen Biotech Co., Ltd.) on a Biorad CFX Real-time PCR system. Each qPCR experiment was performed in triplicate and the average values were calculated for each gene. 18S rRNA served as the internal control for normalization. The relative expression level of mRNA was calculated using $2^{-\Delta\Delta C_q}$. The primer sequences are provided in Table SII.

RNA sequencing (seq). Total RNA of SW837 cells was isolated using the RNAeasy Mini kit (Qiagen GmbH, cat. no. 74104) following the manufacturer's instructions. Integrity of RNA was verified using 4200 Bioanalyzer (Agilent). Subsequently, mRNA libraries were prepared using the TruSeq Stranded Total RNA Sample Preparation kit on the Ribo Zero Gold (Illumina, Inc.). Concentration of mRNA libraries were measured using Qubit 3.0 fluorometer dsDNA HS Assay (Thermo Fisher Scientific). 0.5 nM prepared libraries were loaded on the NovaSeq 6000 platform (Illumina, Inc.) and sequenced under 150 bp paired end mode using NovaSeq 6000 S4 Reagent Kit v1.5 (300 cycles, cat. No. 20028312). For RNA-seq analysis, raw sequencing reads were first processed with fastp (version 0.12.5; github.com/OpenGene/fastp) to remove low-quality sequences and adapters. The cleaned reads were aligned to the human reference genome (GRCh38, hg38) using STAR (version 2.7.0f; github.com/alexdobin/STAR) under default parameters (24,25). The gene expression levels were quantified using RNA-seq by Expectation-Maximization (26). Differentially expressed genes were identified by normalizing raw counts and applying the DESeq2 package (version 1.44.0; bioconductor.org/packages/release/bioc/html/DESeq2.html), based on the threshold of $\log_2$ fold-change > 1 and an adjusted P-value < 0.05 . Pathway enrichment analysis was conducted using GSEA desktop software (version 4.3.3; gsea-msigdb.org/gsea/downloads.jsp), focusing on Kyoto Encyclopedia of Genes and Genomes and Hallmark gene sets (27-29).

Animal experiments. A total of 12 male BALB/c nude mice (age, 6 weeks; weight, 18-22 g were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. and housed in the Biological Resource Centre of the sixth

affiliated hospital, Sun Yat-sen University, Guangzhou, China. The mice were given ad libitum access to food and water under a 12/12-h dark/light cycle with 23–25°C temperature, 55–60% controlled humidity. 5×10^6 SW480 cells were treated with either DMSO or BI2865 ($1 \mu\text{M}$) at 37°C for 96 h. Cells were washed with PBS and cultured in a complete DMEM (Gibco; Thermo Fisher Scientific, Inc.) at 37°C for 48 h and 2×10^6 cells in $50 \mu\text{l}$ sterile saline were injected into the spleen of nude mice (6 mice/group). Mice were palpated every 2 days to monitor tumor progression. Animal health and behavior (signs of distress including hunched posture, ruffled coat, lack of appetite and dehydration) were monitored daily. Mice demonstrating $\geq 15\%$ weight loss within 12 weeks were euthanized. No animal reached the humane endpoint during the experiment. Body weight of the animals was measured every 2 days. After 12 weeks, mice were euthanized by CO_2 inhalation using a flow rate of 3 l/min for 3 min. After respiratory arrest, mice were continually exposed to CO_2 for ≥ 15 min to ensure death. Following animal death confirmation (loss of heartbeat), livers were collected, photographed and preserved in 4% paraformaldehyde at room temperature for 24 h for further hematoxylin-eosin staining. Fixed liver samples were embedded in paraffin and then sliced into $5 \mu\text{m}$ sections. The sections were stained using hematoxylin for 8 min and eosin for 5 min at room temperature. After staining, the sections were scanned using PANNORAMIC DESK II DW (3DHISTECH, Hungary) system. Scanned images were processed using 3DHISTECH CaseViewer (version: 2.4.0, 3dhitech.com). The animal experiment was conducted in compliance with animal protocols approved by the Animal Research Committee of the Sixth Affiliated Hospital of Sun Yat-sen University, Guangzhou, China (approval no. SYSU-IACUC-2022-080601).

Statistical analysis. In survival analysis, clinical data obtained from the ICGC-ARGO, MSKCC and Sidra-LUMC AC-ICAM databases were screened. The exclusion criteria were as follows: i) Cases without KRAS mutation status; ii) patients initially diagnosed with stage IV CRC; iii) distant recurrence information missing and iv) local recurrence. The remaining patients were included in subsequent analysis of metachronous-metastasis free survival (MFS; Fig. S2A–C). Metachronous metastasis was defined as distant metastasis following initial diagnosis or primary surgery (30). To investigate the impact of chemotherapy on metachronous metastasis in CRC, propensity score matching was performed to eliminate bias toward clinical decision-making of chemotherapy caused by postoperative pathological staging. Matched cases were included in the subsequent survival analysis. The Kaplan-Meier method was employed to estimate cumulative MFS rates; P-values, hazard ratio (HR) and 95% CI were assessed using log-rank test. R (Version:4.0.3; r-project.org) was utilized to construct Kaplan-Meier curves. Propensity score matching was performed using SPSS 26.0 (IBM Corp.).

All experiments were replicated three times and data are presented as the mean $\pm$ SD. Statistical differences between two groups were determined using two-tailed unpaired Student's t test. Multiple groups were compared with two-way ANOVA followed by Dunnett's multiple comparisons or one-way ANOVA followed by Bonferroni's post hoc test. All statistical analyses were performed using GraphPad Prism

software (version: 9.5.0; Dotmatics). $P < 0.05$ was considered to indicate a statistically significant difference.

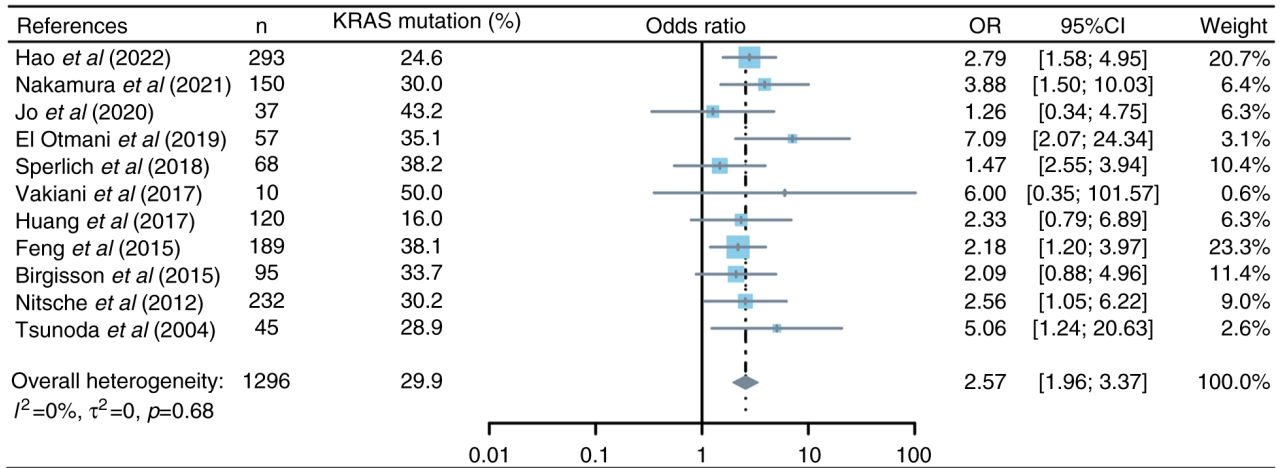
Results

KRAS mutation is a risk factor for CRC metachronous metastasis. KRAS mutation is a predictive factor for metachronous metastasis of CRC (31–33). The present study included meta-analysis of the available literature. The initial search identified 210 records (Fig. S1A), which were screened, leading to the exclusion of 117 articles. A review of reference lists of the remaining articles resulted in 11 relevant articles which were pooled for the meta-analysis (Fig. 1A). These articles comprised 1,296 patients, of whom 387 had mutant KRAS (10,12,32,34–41). The results from these studies were generally consistent, with KRAS positively associated with CRC metachronous metastasis (Fig. 1A). Analysis of the funnel plot did not find significant publication bias (Fig. S1B). Further investigations using ICGC-ARGO database indicated that the KRAS mutation was negatively associated with postoperative MFS of patients with CRC (Figs. 1B and S2A; Table SIII). Similar trends were observed in two other online databases, however differences were not significant (Figs. 1C and D and S2B and C; Table SIII) (19,20). Comparison of MFS between patients with or without chemotherapy following propensity score matching (Table SIV) showed that adjuvant chemotherapy significantly prevented distant recurrence in KRAS-WT CRC, while the efficacy was markedly limited in patients with KRAS-MUT (Fig. 1E–J). These results demonstrate that the KRAS mutation may be a risk factor for CRC metachronous metastasis and first-line chemotherapy does not significantly improve metastasis-free survival in patients with KRAS-MUT CRC after curative surgery.

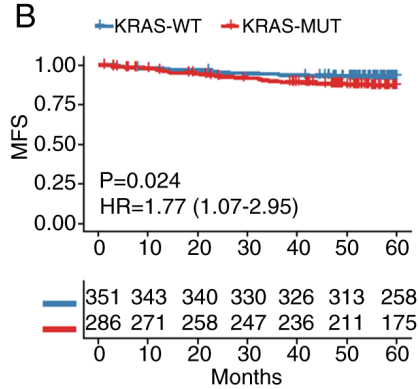
Machine-learning method stratifies KRAS-MUT CRC based on the risk of metachronous metastasis. A metastatic risk prediction model consisting of 36 genes (Fig. S3A–C) was constructed and validated using complete RNA sequencing data and follow-up information from 395 KRAS-MUT CRC cases across three cohorts. Using KMM score, patients in the training cohort (COCC, $n=286$) and two validation cohorts (Sidra-LUMC AC-ICAM, $n=77$; MSKCC, $n=32$) were categorized as high- or low-risk. Kaplan-Meier curve analysis showed that patients in the high-risk group had significantly worse MFS (Fig. 2A–C). Furthermore, differential gene expression analysis demonstrated significantly higher expression of genes associated with the epithelial-mesenchymal transition (EMT) and TGF- β pathway in the high-compared with the low-risk group (Fig. 2D) in the ICGC-ARGO cohort. GSEA revealed that EMT and TGF- β pathways were also highly activated in the high-risk group (Fig. 2E). However, significant differences were not observed in the heatmaps of MSKCC and AC-ICAM due to the relatively small sample sizes in these cohorts (Fig. S3D). These findings suggested that the highly activated EMT and TGF- β pathways may contribute to tumor metastasis in patients with KRAS-MUT CRC, which can be identified by KMM signature.

KRAS inhibitors suppress migration and invasion ability of KRAS-MUT CRC cells. Using the aforementioned KMM scoring model, the KRAS-MUT CRC cell lines were classified

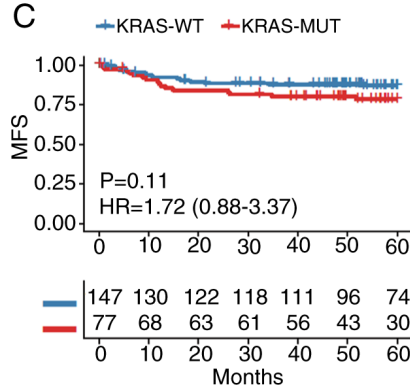
A



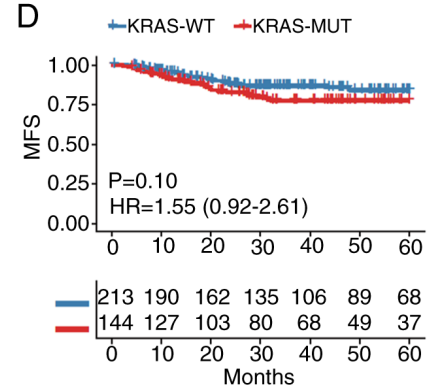
B



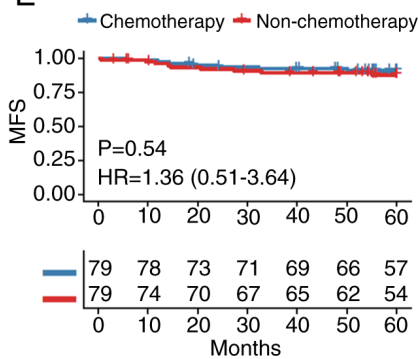
C



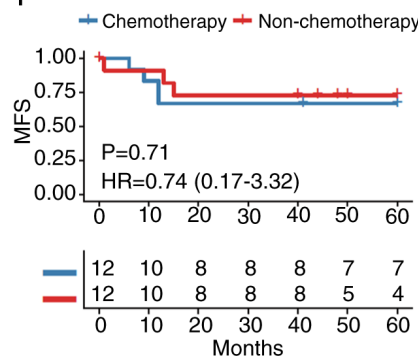
D



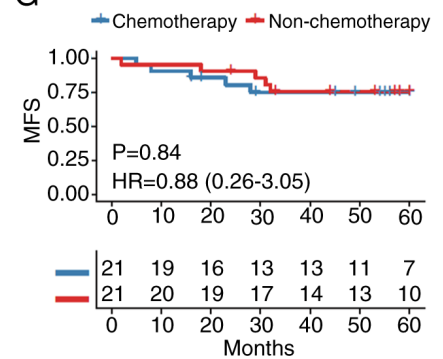
E



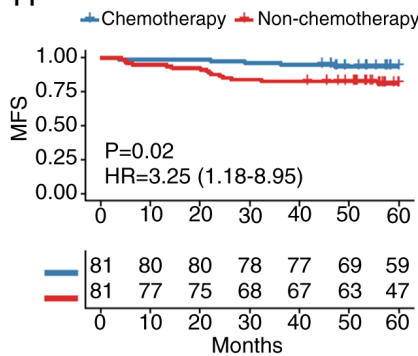
F



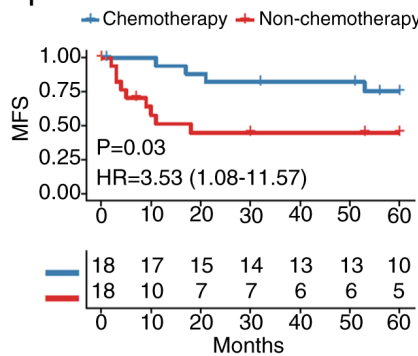
G



H



I



J

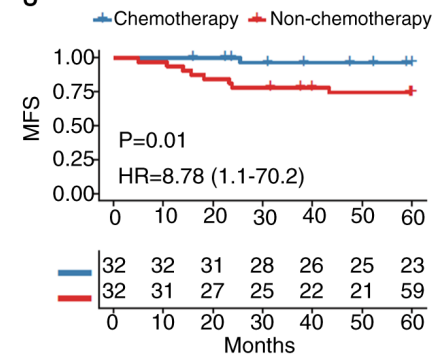


Figure 1. KRAS mutation is associated with CRC metachronous metastasis. (A) Association between KRAS mutation status and CRC metachronous metastasis in 11 studies. A random-effects model with inverse-variance method was used in the meta-analysis. MFS between patients with KRAS-MUT and KRAS-WT CRC in (B) ARGO, (C) Sidra-LUMC AC-ICAM and (D) MSKCC cohort. Comparison of MFS between patients with KRAS-MUT with or without chemotherapy in (E) ARGO, (F) Sidra-LUMC AC-ICAM and (G) MSKCC cohort. Comparison of MFS between patients with KRAS-WT with or without chemotherapy in (H) ARGO, (I) Sidra-LUMC AC-ICAM and (J) MSKCC cohort. CRC, colorectal cancer; MFS, metachronous-metastasis free survival; MUT, mutant; WT, wild-type; ARGO, Accelerating Research in Genomic Oncology; LUMC AC-ICAM, Leiden University Medical Center Atlas and Compass of Immune-Cancer-Microbiome interactions; MSKCC, Memorial Sloan-Kettering Cancer Center; HR, hazard ratio.

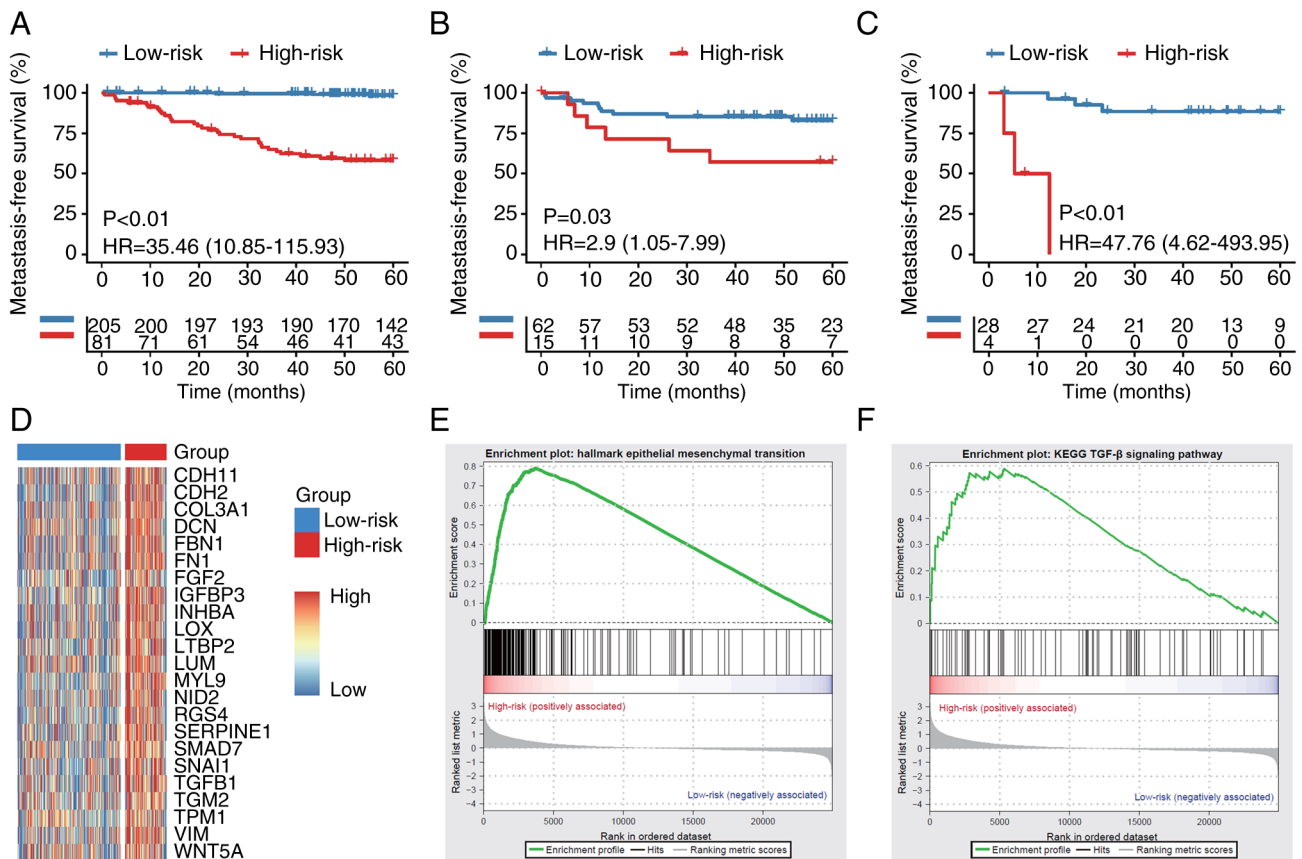


Figure 2. Machine-learning method stratifies KRAS-MUT CRC according to risk of metachronous metastasis. MFS analysis for KMM high and low risk patients in (A) training (ICGC-ARGO) and validation cohorts (B) Sidra-LUMC AC-ICAM cohort and (C) MSKCC cohort. (D) Differentially expressed genes of EMT and TGF- β pathways between high and low risk groups in ICGC-ARGO patients with KRAS-MUT CRC. (E) Hallmark pathway analysis showing genes in the ICGC-ARGO high risk group were enriched in the EMT pathway. (F) KEGG pathway analysis indicated that genes upregulated in the ICGC-ARGO high risk group were enriched in the TGF- β pathway. KRAS-MUT, KRAS mutant; CRC, colorectal cancer; MFS, metachronous-metastasis free survival; KMM, KRAS metachronous-metastasis; EMT, epithelial-mesenchymal transition; KEGG, Kyoto Encyclopedia of Genes and Genomes; ICGC-ARGO, International Cancer Genome Consortium Accelerating Research in Genomic Oncology; LUMC AC-ICAM, Leiden University Medical Center Atlas and Compass of Immune-Cancer-Microbiome interactions; MSKCC, Memorial Sloan-Kettering Cancer Center.

based on the gene expression profile in the CCLE database (Fig. 3A). Based on the scoring results, two KRAS-MUT CRC cell lines with high (HCT116 and SW480) and low KMM score (DLD1 and HCT15) were selected to evaluate their sensitivity to chemotherapy and KRAS inhibitors. KMM score stratification was not associated with chemo-sensitivity (Fig. S4). CRC cell lines were treated with 5-fluorouracil and oxaliplatin to assess the effect of first-line chemotherapy on migration and invasion ability. Wound healing and Transwell experiments showed that chemotherapy significantly decreased the migration and invasion ability of KMM low-risk CRC cell lines (Fig. S5A-D) but had no significant effect on KMM high-risk CRC cell lines (Fig. S5E-H). SW620 was a KMM low-risk cell line, although it shares the same origin as SW480 (42). GSEA using GSE228010 dataset indicated that the EMT pathway was more enriched in SW480 compared with SW620 (Fig. S6A). Furthermore, unlike the EMT morphology of SW480, SW620 exhibited a typical epithelial morphology during cell culture (Fig. S6C).

BI-2865 is a recently designed pan-KRAS inhibitor that inhibits mutant-KRAS (KRAS-G12V in SW480 and KRAS-G13D in HCT116) (16). Wound healing and Transwell assay demonstrated that BI-2865 effectively

inhibited migration and invasion capacity of SW480 cells (Fig. 3B and C) and HCT116 cells (Fig. 3D and E). BI2865 treatment of SW480 and HCT116 cells transformed the mesenchymal morphology of the cells to an epithelial-like morphology, suggesting KRAS inhibitor may promote mesenchymal-epithelial transition (Fig. S6). Since BI-2865 has not been tested in clinical settings, results were compared with US Food and Drug Administration-approved KRAS inhibitor adagrasib (43). Long-term *in vitro* treatment of SW837, a KMM high-risk CRC cell line harboring KRAS-G12C mutation (Fig. 3A), was performed to simulate the extended application of adagrasib in clinical scenarios (Fig. S7A). Acquired resistance to KRAS inhibitors is a challenge during treatment of CRC (44). Prolonged treatment induced resistance, characterized by increased IC50 and maintenance of p-ERK levels under the relatively high dosage of adagrasib compared with SW837-parental (P; Fig. S7B-E) cells. Although it exhibited acquired resistance to adagrasib, SW837-resistant (R) cell line showed significantly lower migration and invasion ability compared with SW837-P cells (Fig. 3F-G), indicating that adagrasib stabilized tumor cells and prevented metastasis. BI-2865 was used to treat previously established organoid, PDO-CC0117,

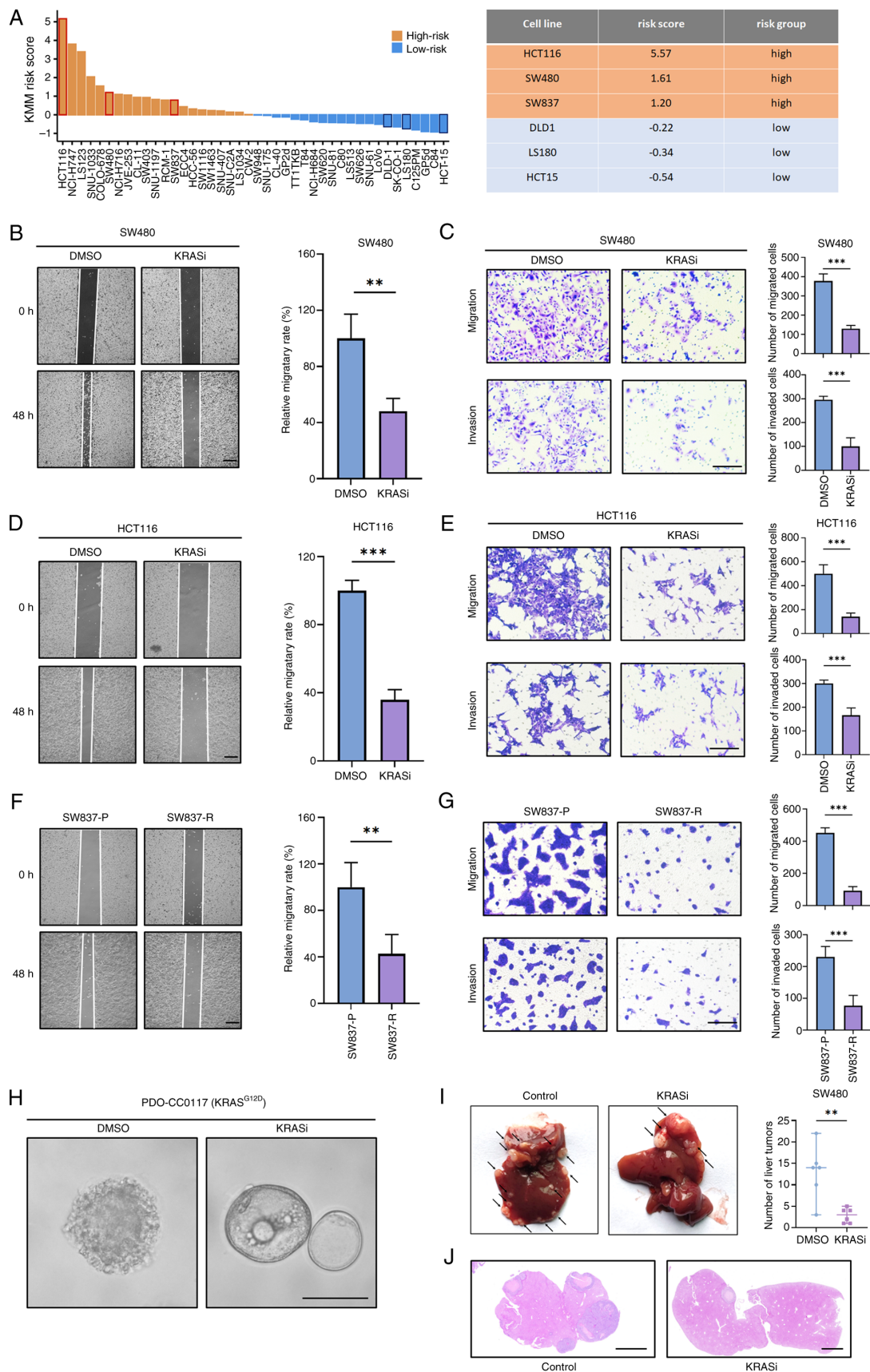


Figure 3. KRAS inhibitors inhibit migration and invasion of CRC. (A) KMM score of Cancer Cell Line Encyclopedia CRC cell lines. Effect of BI-2865 on the migration and invasion phenotype of SW480 cells was examined using (B) wound healing and (C) Transwell assays. Scale bar, 200 μ m. Effect of BI-2865 on the migration and invasion phenotype of HCT116 cells was examined using (D) wound healing and (E) Transwell assays. Scale bar, 200 μ m. (F) Migration and (G) invasion of SW837-P and SW837-R. Scale bar, 200 μ m. (H) Effects of BI-2865 treatment of KRAS^{G12P} CRC organoid PDO-CC0117. The cystic structure was maintained and spike-like features were decreased. Scale bar, 200 μ m. (I) Representative liver metastasis of SW480 cells treated with or without BI-2865 before trans-splenic injection into nude mice. Arrows indicate metastatic nodules. (J) Hematoxylin-eosin staining of liver tissue samples. Scale bar, 2 mm. **P<0.01, ***P<0.001. KRASi, KRAS inhibitor; CRC, colorectal cancer; KMM, KRAS metachronous metastasis; P, parental; R, resistant.

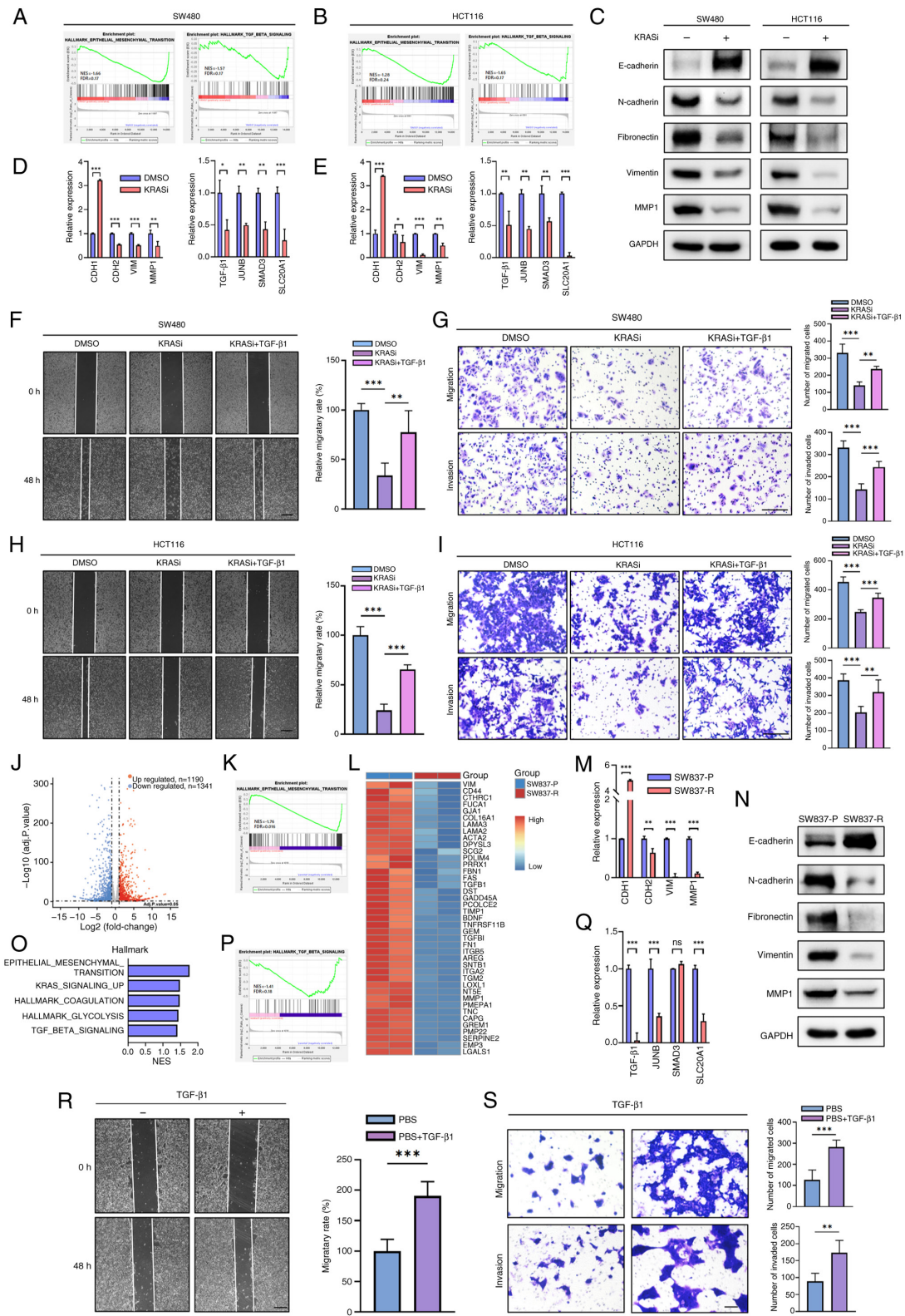


Figure 4. KRAS inhibitor abrogates TGF- β and EMT pathways in colorectal cancer cell lines. Gene Set Enrichment Analysis of Hallmark in GSE228010 showing enrichment of the EMT and TGF- β signaling pathways following BI-2865 treatment in (A) SW480 and (B) HCT116 cells. (C) Protein expression of EMT markers in SW480 and HCT116 following treatment with DMSO or BI-2865. Validation of EMT- and TGF- β -associated genes by reverse transcription-quantitative PCR in (D) SW480 and (E) HCT116 cell lines. Migration and invasion of SW480 cells was investigated using (F) wound healing assay and (G) Transwell assay. Scale bar, 200 μ m. Migration and invasion of HCT116 cells was investigated using (H) scratch wound healing assay and (I) Transwell assay. Scale bar, 200 μ m. (J) Differentially expressed genes in SW837-P and SW837-R cells. (K) Hallmark pathway analysis indicated that downregulated genes in SW837-R were enriched in EMT. (L) Relative mRNA levels of EMT genes in SW837-P/R cells. (M) Western blot analysis of EMT markers in SW837-P/R cells. (N) Top five down-regulated enrichment pathways in SW837-R and SW837-P cells. (O) Hallmark pathway analysis indicated that downregulated genes in SW837-R were enriched in TGF- β pathways. (P) Relative mRNA levels of TGF- β -related genes in SW837-P/R cells. Migration and invasion capacity of SW837-R cells assessed by (R) wound healing and (S) Transwell assay. * P <0.05, ** P <0.01, *** P <0.001. EMT, epithelial-mesenchymal transition; KRASi, KRAS inhibitor; P, parental; R, resistant; NES, normalized enrichment score; FDR, false discovery rate; VIM, vimentin; ns, not significant; CDH, cadherin; SLC20A1, solute carrier family 20 member 1.

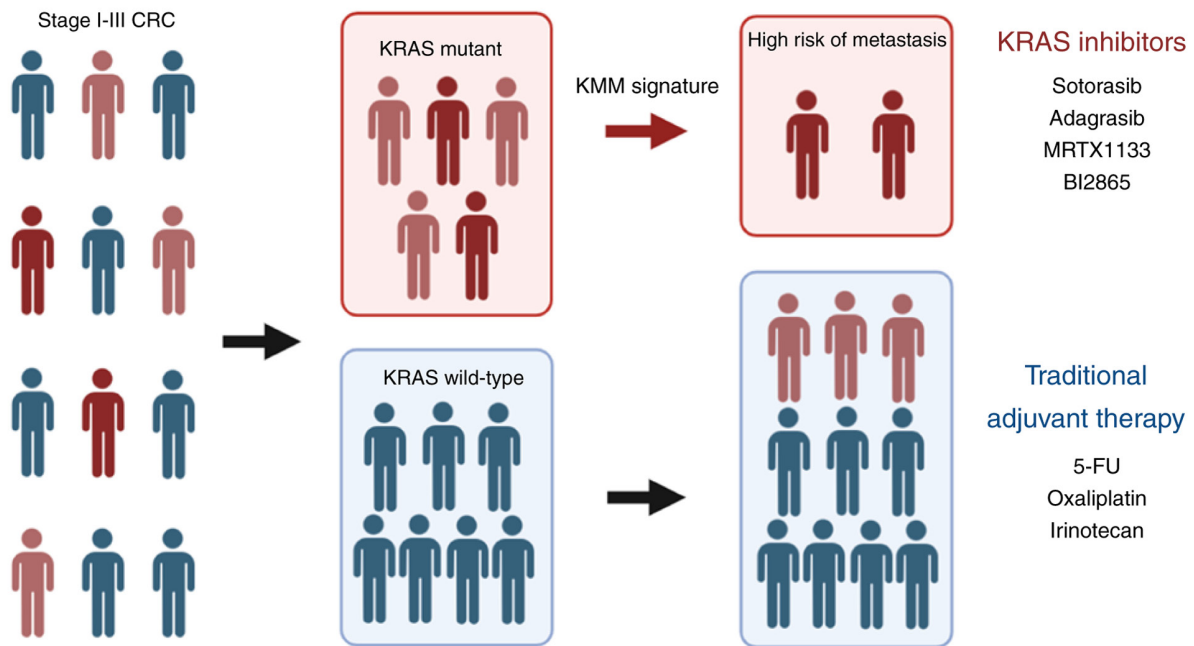


Figure 5. Potential use of KRAS inhibitors in preventing metachronous metastasis CRC. CRC harboring KRAS mutation is resistant to traditional chemotherapy and is likely to develop metachronous metastasis even following curative surgery. KMM signature may identify patients at risk of distant recurrence who may require prophylactic use of KRAS inhibitors. CRC, colorectal cancer; FU, fluorouracil; KMM, KRAS metachronous metastasis.

harboring KRAS^{G12D} mutation (21). Although treatment-naïve PDO-CC0117 organoids exhibited a solid structure with spike-formation, which indicated migration activity, those treated with BI-2865 had relatively normal cystic morphology, suggesting that the KRAS inhibitor exerted stabilizing effects (Fig. 3H). Effect of the KRAS inhibitor on the metastatic ability of CRC was assessed *in vivo*. Body weight of the animals was measured every 2 days after intrasplenic tumor injection (Fig. S8). No metastasis outside the liver was noted in any mice. BI2865-treated group had significantly reduced liver metastases compared with the control group (Fig. 3I and J). Collectively, these results indicate that KRAS inhibitors suppress the metastatic ability of high-risk CRC by inhibiting metachronous metastasis of KRAS-MUT CRC.

KRAS inhibitors suppress TGF- β -mediated pathway in CRC cell lines. KRAS-MUT enhances migration and invasion capabilities of CRC via EMT and TGF- β signaling pathway (8,9,45,46). GSEA using the GSE228010 dataset demonstrated that the BI-2865 treatment decreases the EMT and TGF- β pathways in SW480 and HCT116 cells (16), as verified by RT-qPCR (Fig. 4A and B, D and E). Enrichment analysis using GSE116823 dataset showed that KRAS knockdown inhibited EMT and TGF- β in HCT116 (Fig. S9A and B). Consistent with RT-qPCR, western blot revealed that BI-2865 can decrease levels of EMT markers such as N-cadherin, vimentin, fibronectin and MMP1 while upregulating the levels of E-cadherin (Fig. 4C). Given that KRAS regulates EMT via the TGF- β pathway, cells were treated with TGF- β 1, which partially rescued the inhibitory effect of BI-2865 on migration and invasion capacity of SW480 and HCT116 cells (Fig. 4F-I).

Transcriptomes of SW837-P and SW837-R were compared using RNA-seq, which identified 2,531 differentially expressed

genes (Fig. 4J). Pathway enrichment analysis indicated significant downregulation of the EMT and TGF- β pathways in SW837-R compared with SW837-P cells (Fig. 4K and L, O and P). These findings were validated by RT-qPCR (Fig. 4M and Q). Western blot analysis confirmed downregulation of N-cadherin, fibronectin, vimentin and MMP1, along with the upregulation of E-cadherin in SW837-R (Fig. 4N). The results indicated that, although acquired resistance occurred, prolonged treatment with adagrasib downregulated EMT and TGF- β pathways in SW837 and decreased migration and invasion abilities, and these effects were rescued by exogenous TGF- β 1 administration (Fig. 4R and S). Collectively, these results indicated that KRAS inhibitors may inhibit CRC metastatic potential by suppressing TGF- β -mediated EMT.

Discussion

CRC is one of the most prevalent malignancies accounting for 10% of new cancer cases worldwide (1). Although several treatments, including surgery, neoadjuvant chemotherapy and radiotherapy have been developed for the treatment of non-stage IV CRC, up to 23% of patients develop metachronous metastasis (2). The present study found that the KRAS mutation is a risk factor for CRC metachronous metastasis and constructed a model for assessing metachronous metastasis risk in patients with KRAS-MUT CRC. *In vitro* and *in vivo* experiments indicated that KRAS inhibitors effectively suppressed the metastatic potential of high-risk CRC, providing a novel approach for CRC adjuvant therapy (Fig. 5).

KRAS is an oncogene with the highest mutation frequency in CRC, with a cumulative mutation rate of 49% reported in a recent study (47). Ample evidence suggests an association between KRAS mutation and CRC metastasis, with KRAS-MUT associated with higher incidence of stage IV

CRC compared with wild-type (48,49). However, whether KRAS-MUT increases the risk of CRC metachronous metastasis is unclear. Studies have indicated that KRAS mutations are associated with metachronous lung metastasis but not liver metastasis in CRC (50,51). The present meta-analysis revealed that KRAS-MUT was a risk factor for CRC metachronous metastasis, highlighting the role of KRAS in metastasis. As a single factor, KRAS mutation is not a robust predictive factor, and thus, the association between KRAS and metastasis is highly variable (52). Therefore, the present analyzed public omics databases to develop a tool for predicting metachronous metastasis risk in KRAS-MUT CRC, which showed good results. However, to improve the performance of the KMM tool, which was primarily based on gene expression omics, future studies need to incorporate clinical data, radiomics, genomics and proteomics information to establish a multiomics model.

The treatment of KRAS-MUT CRC is a clinical challenge due to the lack of a specific therapeutic approach. KRAS mutations may decrease the efficacy of chemotherapy (53) and cause EGFR-targeted therapy resistance (54). Moreover, the KRAS-MUT can reduce tumor-infiltrated T lymphocytes, resulting in the establishment of a suppressive anti-tumor immune microenvironment in CRC, suggesting that immunotherapy may not be a preferred option for KRAS-MUT CRC (55). Thus, alternative adjuvant treatment options should be explored for management of KMM high-risk CRC. In recent years, several KRAS inhibitors, including adagrasib and BI-2865, have been developed, some of which effectively treat KRAS-MUT CRC (56). However, current KRAS inhibitors are only approved for the treatment of metastatic CRC, which is not responsive to first-line treatment (43). To the best of our knowledge, no study has reported their ability to prevent tumor metastasis (57). The present study demonstrated that adagrasib, an FDA-approved KRASG12C inhibitor, and pan-KRAS inhibitor BI-2865, which targets multiple mutational subtypes, inhibited migration and invasion capabilities of KRAS-MUT CRC. To the best of our knowledge, the present study is the first to provide preclinical evidence for the benefits of KRAS inhibitors and offers a prophylactic strategy for management of metachronous metastasis in KRAS-MUT CRC. Since the potency of adagrasib and BI-2865 against KRAS-MUT but not KRAS-wild-type cancer has been well documented (16,58), the present study did not further validate the efficacy of KRAS inhibitors in KRAS wild-type CRC. Microsatellite status is a commonly used clinical signature in CRC (59,60). HCT116 is an microsatellite instability-high CRC, while SW480 and SW837 are microsatellite stable CRC (61). All three cell lines are KMM high-risk. KRAS inhibitors suppressed migration and invasion capability in both MSI (HCT116) and MSS (SW480 and SW837) CRC cell lines, indicating microsatellite instability status did not affect phenotype.

EMT affects metastatic behavior of colorectal cancer (62). EMT level in primary tumors may be a risk factor for distant relapse (63), which is consistent with the present finding that EMT was enriched in the KMM high-risk group. The oncogenic KRAS drives EMT in CRC (64). However, a recent study suggested that long-term KRAS inhibition induces EMT reactivation in lung cancer, implying that the relationship between KRAS and EMT may exhibit different patterns depending on

cancer heterogeneity (65). KRAS signaling exerts crosstalk with TGF- β in CRC (66). Meanwhile, as a canonical upstream regulator of EMT, inhibition of TGF- β pathway blocks EMT in CRC (8). Therefore, it was hypothesized that the KRAS inhibitor can suppress EMT via KRAS-mediated TGF- β signaling; the results confirmed this hypothesis, indicating that KRAS inhibitors prevent the metastatic potential of CRC. SW480 has a SMAD4 mutation while HCT116 has a TGFBR2 mutation, both of which are key factors in the TGF- β signaling pathway (67,68). Nevertheless, studies have shown EMT phenotype of SW480 and HCT116 can be activated by either exogenous SMAD4 or TGF- β protein, indicating that the TGF- β signaling pathway is functional in these cell models (67,69). Thus, it is reasonable to use KRAS inhibitors to suppress this pathway to inhibit EMT and invasion/metastasis.

In conclusion, KRAS mutation is a risk factor for metachronous metastasis. The present study constructed a scoring model for predicting the risk of distant relapse in KRAS-MUT CRC. KRAS inhibitors suppressed the EMT process that drives the migration and invasion properties of CRC by blocking the TGF- β signaling pathway, offering a potential prophylactic treatment for metachronous metastasis.

Acknowledgements

Not applicable.

Funding

The present study was supported by National Natural Science Foundation of China (grant no. 82203072) and Tianshan Talents Leading Medical Talents in Guangdong Province Cooperative Expert Studio (grant no. KSYJ2022001).

Availability of data and materials

The data generated in the present study may be found in the Gene Expression Omnibus database at the National Center for Biotechnology Information under accession number PRJNA1085403 or at the following URL: ncbi.nlm.nih.gov/bioproject/1085403.

Authors' contributions

YG, XW and FG conceived and designed the study. YG and CH wrote the manuscript and analyzed data. KC, GL and XH performed experiments. DC, ZY, ZC, PH and YC analyzed data and reviewed the manuscript. PH and ZY confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Sixth Affiliated Hospital, Sun Yat-sen University (approval no. 2024ZSLYEC-192). The animal experiment was conducted in compliance with animal protocols approved by the Institutional Animal Care and Use Committee at Sun Yat-sen University (approval no. SYSU-IACUC-2022-080601).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Siegel RL, Miller KD, Wagle NS and Jemal A: Cancer statistics, 2023. *CA Cancer J Clin* 73: 17-48, 2023.
2. Cohen R and Platell CF: Metachronous colorectal cancer metastasis: Who, what, when and what to do about it. *J Surg Oncol* 129: 71-77, 2024.
3. Hansdotter P, Scherman P, Nikberg M, Petersen SH, Holmberg E, Rizell M, Naredi P and Syk I: COLOFOL study group: Treatment and survival of patients with metachronous colorectal lung metastases. *J Surg Oncol* 127: 806-814, 2023.
4. Reboux N, Jooste V, Goungounga J, Robaszekiewicz M, Noursbaum JB and Bouvier AM: Incidence and survival in synchronous and metachronous liver metastases from colorectal cancer. *JAMA Netw Open* 5: e2236666, 2022.
5. Martin J, Petrillo A, Smyth EC, Shaïda N, Khwaja S, Cheow HK, Duckworth A, Heister P, Praseedom R, Jah A, *et al*: Colorectal liver metastases: Current management and future perspectives. *World J Clin Oncol* 11: 761-808, 2020.
6. Pfannschmidt J, Dienemann H and Hoffmann H: Surgical resection of pulmonary metastases from colorectal cancer: A systematic review of published series. *Ann Thorac Surg* 84: 324-338, 2007.
7. Biller LH and Schrag D: Diagnosis and treatment of metastatic colorectal cancer: A review. *JAMA* 325: 669-685, 2021.
8. Boutin AT, Liao WT, Wang M, Hwang SS, Karpinets TV, Cheung H, Chu GC, Jiang S, Hu J, Chang K, *et al*: Oncogenic Kras drives invasion and maintains metastases in colorectal cancer. *Genes Dev* 31: 370-382, 2017.
9. Lemieux E, Cagnol S, Beaudry K, Carrier J and Rivard N: Oncogenic KRAS signalling promotes the Wnt/ β -catenin pathway through LRP6 in colorectal cancer. *Oncogene* 34: 4914-4927, 2015.
10. Feng Q, Liang L, Ren L, Chen J, Wei Y, Chang W, Zhu D, Lin Q, Zheng P and Xu J: A specific KRAS codon 13 mutation is an independent predictor for colorectal cancer metachronous distant metastases. *Am J Cancer Res* 5: 674-688, 2015.
11. Ilm K, Kemmner W, Osterland M, Burock S, Koch G, Herrmann P, Schlag PM and Stein U: High MACC1 expression in combination with mutated KRAS G13 indicates poor survival of colorectal cancer patients. *Mol Cancer* 14: 38, 2015.
12. Hao M, Li H, Wang K, Liu Y, Liang X and Ding L: Predicting metachronous liver metastasis in patients with colorectal cancer: Development and assessment of a new nomogram. *World J Surg Oncol* 20: 80, 2022.
13. Herzberg BO and Manji GA: KRAS: druggable at last. *Oncologist* 28: 283-286, 2023.
14. Abubaker J, Bavi P, Al-Haqawi W, Sultana M, Al-Harbi S, Al-Sanea N, Abduljabbar A, Ashari LH, Alhomoud S, Al-Dayel F, *et al*: Prognostic significance of alterations in KRAS isoforms KRAS-4A/4B and KRAS mutations in colorectal carcinoma. *J Pathol* 219: 435-445, 2009.
15. Nusrat M and Yeager R: KRAS inhibition in metastatic colorectal cancer: An update. *Curr Opin Pharmacol* 68: 102343, 2023.
16. Kim D, Herdeis L, Rudolph D, Zhao Y, Böttcher J, Vides A, Ayala-Santos CI, Pourfarjam Y, Cuevas-Navarro A, Xue JY, *et al*: Pan-KRAS inhibitor disables oncogenic signalling and tumour growth. *Nature* 619: 160-166, 2023.
17. Ji Q, Liu X, Han Z, Zhou L, Sui H, Yan L, Jiang H, Ren J, Cai J and Li Q: Resveratrol suppresses epithelial-to-mesenchymal transition in colorectal cancer through TGF- β 1/Smads signaling pathway mediated Snail/E-cadherin expression. *BMC Cancer* 15: 97, 2015.
18. Moher D, Liberati A, Tetzlaff J and Altman DG; Group P: Preferred reporting items for systematic reviews and meta-analyses: The PRISMA statement. *PLoS Med* 6: e1000097, 2009.
19. Chatila WK, Kim JK, Walch H, Marco MR, Chen CT, Wu F, Omer DM, Khalil DN, Ganesh K, Qu X, *et al*: Genomic and transcriptomic determinants of response to neoadjuvant therapy in rectal cancer. *Nat Med* 28: 1646-1655, 2022.
20. Roelands J, Kuppen PJK, Ahmed EI, Mall R, Masoodi T, Singh P, Monaco G, Raynaud C, de Miranda NFCC, Ferraro L, *et al*: An integrated tumor, immune and microbiome atlas of colon cancer. *Nat Med* 29: 1273-1286, 2023.
21. Medico E, Russo M, Picco G, Cancelliere C, Valtorta E, Corti G, Buscarino M, Isella C, Lamba S, Martinoglio B, *et al*: The molecular landscape of colorectal cancer cell lines unveils clinically actionable kinase targets. *Nat Commun* 6: 7002, 2015.
22. Kim WK, Kwon Y, Jang M, Park M, Kim J, Cho S, Jang DG, Lee WB, Jung SH, Choi HJ, *et al*: β -catenin activation down-regulates cell-cell junction-related genes and induces epithelial-to-mesenchymal transition in colorectal cancers. *Sci Rep* 9: 18440, 2019.
23. Yu Z, Deng P, Chen Y, Liu S, Chen J, Yang Z, Chen J, Fan X, Wang P, Cai Z, *et al*: Inhibition of the PLK1-coupled cell cycle machinery overcomes resistance to oxaliplatin in colorectal cancer. *Adv Sci (Weinh)* 8: e2100759, 2021.
24. Chen S, Zhou Y, Chen Y and Gu J: fastp: An ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* 34: i884-i890, 2018.
25. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M and Gingeras TR: STAR: Ultrafast universal RNA-seq aligner. *Bioinformatics* 29: 15-21, 2013.
26. Li B and Dewey CN: RSEM: Accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* 12: 323, 2011.
27. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES and Mesirov JP: Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA* 102: 15545-15550, 2005.
28. Kanehisa M, Furumichi M, Sato Y, Matsuura Y and Ishiguro-Watanabe M: KEGG: Biological systems database as a model of the real world. *Nucleic Acids Res*: gkae909, 2024 (Epub ahead of print).
29. Liberzon A, Birger C, Thorvaldsdóttir H, Ghandi M, Mesirov JP and Tamayo P: The molecular signatures database (MSigDB) hallmark gene set collection. *Cell Syst* 1: 417-425, 2015.
30. Engstrand J, Stromberg C, Nilsson H, Freedman J and Jonas E: Synchronous and metachronous liver metastases in patients with colorectal cancer-towards a clinically relevant definition. *World J Surg Oncol* 17: 228, 2019.
31. Kuan TC, Chang SC, Lin JK, Lin TC, Yang SH, Jiang JK, Chen WS, Wang HS, Lan YT, Lin CC, *et al*: Prognosticators of long-term outcomes of TNM stage II colorectal cancer: Molecular patterns or clinicopathological features. *World J Surg* 43: 3207-3215, 2019.
32. Jo P, Bernhardt M, Nietert M, König A, Azizian A, Schirmer MA, Grade M, Kitz J, Reuter-Jessen K, Ghadimi M, *et al*: KRAS mutation status concordance between the primary tumor and the corresponding metastasis in patients with rectal cancer. *PLoS One* 15: e0239806, 2020.
33. Lin CC, Lin JK, Lin TC, Chen WS, Yang SH, Wang HS, Lan YT, Jiang JK, Yang MH and Chang SC: The prognostic role of microsatellite instability, codon-specific KRAS, and BRAF mutations in colon cancer. *J Surg Oncol* 110: 451-457, 2014.
34. Tsunoda A, Iijima T, Tsunoda Y, Nakao K, Miyaki M and Kusano M: Association of K-ras mutations with liver metastases from colorectal carcinoma. *Anticancer Res* 24: 2471-2476, 2004.
35. Vakiani E, Shah RH, Berger MF, Makohon-Moore AP, Reiter JG, Ostrovskaya I, Attiye H, Cercek A, Shia J, Iacobuzio-Donahue CA, *et al*: Local recurrences at the anastomotic area are clonally related to the primary tumor in sporadic colorectal carcinoma. *Oncotarget* 8: 42487-42494, 2017.
36. Sperlich A, Balmert A, Doll D, Bauer S, Franke F, Keller G, Wilhelm D, Mur A, Respondek M, Friess H, *et al*: Genetic and immunological biomarkers predict metastatic disease recurrence in stage III colon cancer. *BMC Cancer* 18: 998, 2018.
37. Nitsche U, Rosenberg R, Balmert A, Schuster T, Slotta-Huspenina J, Herrmann P, Bader FG, Friess H, Schlag PM, Stein U and Janssen KP: Integrative marker analysis allows risk assessment for metastasis in stage II colon cancer. *Ann Surg* 256: 763-771, 2012.
38. Nakamura Y, Yokoyama S, Matsuda K, Tamura K, Mitani Y, Iwamoto H, Mizumoto Y, Murakami D, Kitahata Y and Yamaue H: Preoperative detection of KRAS mutated circulating tumor DNA is an independent risk factor for recurrence in colorectal cancer. *Sci Rep* 11: 441, 2021.
39. Huang SC, Huang SF, Chen YT, Chang Y, Chiu YT, Chang IC, Wu HI and Chen JS: Overexpression of MutL homolog 1 and MutS homolog 2 proteins have reversed prognostic implications for stage I-II colon cancer patients. *Biomed J* 40: 39-48, 2017.

40. El Otmani I, El Agy F, El Baradai S, Bouguenouch L, Lahmidani N, El Abkari M, Benajah DA, Toughrai I, El Bouhaddouti H, Mouaqit O, *et al*: Analysis of molecular pretreated tumor profiles as predictive biomarkers of therapeutic response and survival outcomes after neoadjuvant therapy for rectal cancer in moroccan population. *Dis Markers* 2020: 8459303, 2020.
41. Birgisson H, Edlund K, Wallin U, Pählman L, Kulthma HG, Mayrhofer M, Micke P, Isaksson A, Botling J, Glimelius B and Sundström M: Microsatellite instability and mutations in BRAF and KRAS are significant predictors of disseminated disease in colon cancer. *BMC Cancer* 15: 125, 2015.
42. Verhagen MP, Xu T, Stabile R, Joosten R, Tucci FA, van Royen M, Trerotola M, Alberti S, Sacchetti A and Fodde R: The SW480 cell line as a model of resident and migrating colon cancer stem cells. *iScience* 27: 110658, 2024.
43. Dhillon S: Adagrasib: First approval. *Drugs* 83: 275-285, 2023.
44. Awad MM, Liu S, Rybkin II, Arbour KC, Dilly J, Zhu VW, Johnson ML, Heist RS, Patil T, Riely GJ, *et al*: Acquired resistance to KRAS^{G12C} inhibition in cancer. *N Engl J Med* 384: 2382-2393, 2021.
45. Flum M, Dicks S, Teng YH, Schrempp M, Nyström A, Boerries M and Hecht A: Canonical TGFβ signaling induces collective invasion in colorectal carcinogenesis through a Snail1- and Zeb1-independent partial EMT. *Oncogen* 41: 1492-1506, 2022.
46. Sánchez-Tilló E, Pedrosa L, Vila I, Chen Y, Györfy B, Sánchez-Moral L, Siles L, Lozano JJ, Esteve-Codina A, Darling DS, *et al*: The EMT factor ZEB1 paradoxically inhibits EMT in BRAF-mutant carcinomas. *JCI Insight* 8: e164629, 2023.
47. Lee JK, Sivakumar S, Schrock AB, Madison R, Fabrizio D, Gjoerup O, Ross JS, Frampton GM, Napalkov P, Montesin M, *et al*: Comprehensive pan-cancer genomic landscape of KRAS altered cancers and real-world outcomes in solid tumors. *NPJ Precis Oncol* 6: 91, 2022.
48. Huang D, Sun W, Zhou Y, Li P, Chen F, Chen H, Xia D, Xu E, Lai M, Wu Y and Zhang H: Mutations of key driver genes in colorectal cancer progression and metastasis. *Cancer Metastasis Rev* 37: 173-187, 2018.
49. Meng M, Zhong K, Jiang T, Liu Z, Kwan HY and Su T: The current understanding on the impact of KRAS on colorectal cancer. *Biomed Pharmacother* 140: 111717, 2021.
50. Pereira AAL, Rego JFM, Morris V, Overman MJ, Eng C, Garrett CR, Boutin AT, Ferrarotto R, Lee M, Jiang ZQ, *et al*: Association between KRAS mutation and lung metastasis in advanced colorectal cancer. *Br J Cancer* 112: 424-428, 2015.
51. Tie J, Lipton L, Desai J, Gibbs P, Jorissen RN, Christie M, Drummond KJ, Thomson BN, Usatoff V, Evans PM, *et al*: KRAS mutation is associated with lung metastasis in patients with curatively resected colorectal cancer. *Clin Cancer Res* 17: 1122-1130, 2011.
52. Guo L, Wang Y, Yang W, Wang C, Guo T, Yang J, Shao Z, Cai G, Cai S, Zhang L, *et al*: Molecular profiling provides clinical insights into targeted and immunotherapies as well as colorectal cancer prognosis. *Gastroenterology* 165: 414-428.e7, 2023.
53. Iseas S, Sendoya JM, Robbio J, Coraglio M, Kujaruk M, Mikolaitis V, Rizzolo M, Cabanne A, Ruiz G, Salanova R, *et al*: Prognostic impact of an integrative landscape of clinical, immune, and molecular features in non-metastatic rectal cancer. *Front Oncol* 11: 801880, 2022.
54. Misale S, Di Nicolantonio F, Sartore-Bianchi A, Siena S and Bardelli A: Resistance to anti-EGFR therapy in colorectal cancer: From heterogeneity to convergent evolution. *Cancer Discov* 4: 1269-1280, 2014.
55. Lal N, White BS, Goussous G, Pickles O, Mason MJ, Beggs AD, Tanriere P, Willcox BE, Guinney J and Middleton GW: KRAS mutation and consensus molecular subtypes 2 and 3 are independently associated with reduced immune infiltration and reactivity in colorectal cancer. *Clin Cancer Res* 24: 224-233, 2018.
56. Caughey BA and Strickler JH: Targeting KRAS-mutated gastrointestinal malignancies with small-molecule inhibitors: A new generation of breakthrough therapies. *Drugs* 84: 27-44, 2024.
57. Erlanson DA and Webster KR: Targeting mutant KRAS. *Curr Opin Chem Biol* 62: 101-108, 2021.
58. Hallin J, Engstrom LD, Hargis L, Calinisan A, Aranda R, Briere DM, Sudhakar N, Bowcut V, Baer BR, Ballard JA, *et al*: The KRAS^{G12C} inhibitor MRTX849 provides insight toward therapeutic susceptibility of KRAS-mutant cancers in mouse models and patients. *Cancer Discov* 10: 54-71, 2020.
59. Popat S, Hubner R and Houlston RS: Systematic review of microsatellite instability and colorectal cancer prognosis. *J Clin Oncol* 23: 609-618, 2005.
60. Storojeva I, Boulay JL, Heinimann K, Ballabeni P, Terracciano L, Laffer U, Mild G, Herrmann R and Rochlitz C: Prognostic and predictive relevance of microsatellite instability in colorectal cancer. *Oncol Rep* 14: 241-249, 2005.
61. Berg KCG, Eide PW, Eilertsen IA, Johannessen B, Bruun J, Danielsen SA, Bjørnslett M, Meza-Zepeda LA, Eknæs M, Lind GE, *et al*: Multi-omics of 34 colorectal cancer cell lines-a resource for biomedical studies. *Mol Cancer* 16: 116, 2017.
62. Vu T and Datta P: Regulation of EMT in colorectal cancer: A culprit in metastasis. *Cancers (Basel)* 9: 171, 2017.
63. Wang K, Song K, Ma Z, Yao Y, Liu C, Yang J, Xiao H, Zhang J, Zhang Y and Zhao W: Identification of EMT-related high-risk stage II colorectal cancer and characterisation of metastasis-related genes. *Br J Cancer* 123: 410-417, 2020.
64. Chu PC, Lin PC, Wu HY, Lin KT, Wu C, Bekaii-Saab T, Lin YJ, Lee CT, Lee JC and Chen CS: Mutant KRAS promotes liver metastasis of colorectal cancer, in part, by upregulating the MEK-Spl-DNMT1-miR-137-YB-1-IGF-IR signaling pathway. *Oncogene* 37: 3440-3455, 2018.
65. Adachi Y, Kimura R, Hirade K, Yanase S, Nishioka Y, Kasuga N, Yamaguchi R and Ebi H: Scribble mis-localization induces adaptive resistance to KRAS G12C inhibitors through feedback activation of MAPK signaling mediated by YAP-induced MRAS. *Nat Cancer* 4: 829-843, 2023.
66. Vivekanandhan S and Mukhopadhyay D: Genetic status of KRAS influences transforming growth factor-beta (TGF-β) signaling: An insight into neuropilin-1 (NRP1) mediated tumorigenesis. *Semin Cancer Biol* 54: 72-79, 2019.
67. Pohl M, Radacz Y, Pawlik N, Schoeneck A, Baldus SE, Munding J, Schmiegel W, Schwarte-Waldhoff I and Reinacher-Schick A: SMAD4 mediates mesenchymal-epithelial reversion in SW480 colon carcinoma cells. *Anticancer Res* 30: 2603-2613, 2010.
68. Lee J, Ballikaya S, Schöniß K, Ball CR, Glimm H, Kopitz J and Gebert J: Transforming growth factor beta receptor 2 (TGFBR2) changes sialylation in the microsatellite unstable (MSI) Colorectal cancer cell line HCT116. *PLoS One* 8: e57074, 2013.
69. Hawinkels LJAC, Paauwe M, Verspaget HW, Wiercinska E, van der Zon JM, van der Ploeg K, Koelink PJ, Lindeman JH, Mesker W, ten Dijke P and Sier CF: Interaction with colon cancer cells hyperactivates TGF-β signaling in cancer-associated fibroblasts. *Oncogene* 33: 97-107, 2014.



Copyright © 2024 Guo *et al*. This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.