COLORECTAL CANCER: TARGETS, TREATMENT AND PREVENTION

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COLORECTAL CANCER: TARGETS, TREATMENT AND PREVENTION

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ORIGINAL RESEARCH
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Pro-Oncogenic c-Met/EGFR, Biomarker Signatures of the Tumor Microenvironment are Clinical and Therapy Response Prognosticators in Colorectal Cancer, and Therapeutic Targets of 3-Phenyl-2H-benzo[e][1,3]-Oxazine-2,4(3H)-Dione Derivatives

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Genetic and environmental factors play important roles in cancer progression, metastasis, and drug resistance. Herein, we used a multiomics data analysis to evaluate the predictive and prognostic roles of genetic and epigenetic modulation of c-MET (hepatocyte growth factor receptor)/epidermal growth factor receptor (EGFR) in colorectal cancer (CRC). First, we found that overexpressions of c-MET/EGFR were associated with the infiltration of tumor immune cells and cancer-associated fibroblasts, and were of prognostic relevance in CRC cohorts. We also observed that genetic alterations of c-MET/EGFR in CRC co-occurred with other gene alterations and were associated with overexpression of messenger (m)RNA of some cancer hallmark proteins. More specifically, DNA-methylation and somatic copy number alterations of c-MET/EGFR were associated with immune infiltration, dysfunctional T-cell phenotypes, and poor prognoses of the cohorts. Moreover, we describe two novel gefitinib-inspired small molecules derivatives of 3-phenyl-2H-benzo[e] [1,3]-oxazine-2,4(3H)-dione, NSC777205 and NSC777207, which exhibited wide-spectrum antiproliferative activities and selective cytotoxic preference for drug-sensitive and multidrug-resistant melanoma, renal, central nervous system, colon, and non-small cell lung cancer cell lines. We further provided in silico mechanistic evidence implicating c-MET/EGFR/phosphatidylinositol 3-kinase (PI3K)-mammalian target of rapamycin (mTOR) inhibition in anticancer activities of those compounds. Our overall structure-activity relationship study revealed that the addition of an $-OCH_3$ group to

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salicylic core of NSC777207 was not favorable, as the added moiety led to overall less-favorable drug properties as well as weaker anticancer activities compared to the properties and activities demonstrated by NSC777205 that has no $–OCH_3$ substituent group. Further *in vitro* and *in vivo* analyses in tumor-bearing mice are ongoing in our lab to support this claim and to unravel the full therapeutic efficacies of NSC777205 and NSC777207 in CRC.

Keywords: colorectal cancer, genetic and epigenetic alterations, cancer-associated fibroblast, immune infiltration, small molecule, NSC777205, NSC777207

INTRODUCTION

According to 2020 global cancer statistics, with more than 1.9 million new cases and 935,000 deaths, colorectal cancer (CRC) ranked third in terms of incidence, but second in terms of mortality in 2020 (Sung et al., 2021). CRC is regarded as a marker of socioeconomic development, as incidence rates tend to rise uniformly with an increasing human development index (HDI) in countries undergoing major transitions (Bray, 2014; Fidler et al., 2016). In particular, incidence rates have been steadily increasing in Asia, Europe, and South America (Arnold et al., 2017; Arnold et al., 2020). These increases correspond to adoption of lifestyles that are associated with risk factors, such as being overweight, having decreased physical activity, and becoming more sedentary (Siegel et al., 2020). In addition, increased consumption of cigarettes, alcohol, and red or processed meats, and decreased intake of dairy products, grains, and fibers in those regions have greatly contributed to the observed trends (Clinton et al., 2020).

Both genetic and environmental factors play important roles in the etiology of CRC. To date, several molecular hallmarks have been associated with CRC (Colussi et al., 2013). These hallmarks of CRC are acquired through progressive accumulation of genetic and epigenetic alterations that inactivate tumor-suppressor genes and activate oncogenes (Grady and Carethers, 2008; Kuipers et al., 2015). At the molecular level, activation of growth factor receptors (GFRs) was shown to be involved in the rapid growth of cancer cells (Normanno et al., 2006). The etiology of most cancers can be linked to aberrant intra- and intercellular communication associated with GFR-mediated pathways. Activated GFRs aid blood vessel formation, cell migration, metastasis, and the inhibition of apoptosis. Among these GFRs, the epidermal growth factor receptor (EGFR) and hepatocyte growth factor receptor (HGFR) play central roles in the pathogenesis and progression of different carcinoma types (Normanno et al., 2001).

HGFR, also known as tyrosine-protein kinase (c-Met), is a tyrosine kinase receptor (RTK) that in humans is encoded by the *MET* gene, while the EGFR belongs to the ErbB family of RTKs, and is a trans-membrane protein. The binding of ligands to the extracellular domain of these receptors induces the formation of receptor homo- or heterodimers and subsequent activation of the intrinsic tyrosine kinase domain (Oprita et al., 2021), which facilitates recruitment of proteins that initiate a signaling cascade, integrating numerous signaling pathways that lead to specific cellular responses that favor angiogenesis, high nutrient supplies, cell migration, tumor growth, and metastasis of CRC (Sattler et al., 2011; An et al., 2018).

Several treatment modalities exist, including immunotherapy, radiotherapy, neoadjuvant and palliative chemotherapies, laparoscopic surgery for primary disease, and more-aggressive resection of metastatic disease, and these provide alternatives for patients with primary and metastatic CRC (Stintzing, 2014; Kuipers et al., 2015). However, these treatment strategies have limited success rates in terms of prognoses and long-term survival. For these reasons, searching for relevant predictive biomarkers that can inform treatment decisions and developing novel therapeutic strategies with high efficacy and minimal side effects are impetuses for the research world. To this end, we used a multiomics data analysis to evaluate the predictive and prognostic roles of genetic and epigenetic modulation of c-MET/EGFR in CRC. Moreover, we describe two novel gefitinib-inspired small molecules, NSC777205 and NSC777207, with wide-spectrum antiproliferative activities and selective cytotoxic preferences for melanoma, renal, central nervous system (CNS), colon, and non-small cell lung cancer (NSCLC) cell lines, and provide *in silico* mechanistic evidence implicating c-MET/EGFR/phosphatidylinositol 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) inhibition in anticancer activities of these compounds.

MATERIALS AND METHODS

In Silico Evaluation of the Drug Likeness, Pharmacokinetics, Acute Toxicity, and Cytotoxic Activities of NSC777205 and NSC777207 Against Cancer Cell Lines

We analyzed the drug-likeness, PKs, medicinal chemistry, and toxicity of NSC777205 and NSC777207 using SwissADME software (http://www.swissadme.ch) (Daina et al., 2017), and computer-aided Prediction of Biological Activity Spectra (PASS) web resources (http://way2drug.com/dr) (Poroikov et al., 2019). We used the blood-brain barrier (BBB) Prediction Server (https://www.cbligand.org/BBB/) which operates based on support vector machine (SVM) and LiCABEDS algorithms on four types of fingerprints of 1,593 reported compounds (Liu et al., 2014) to analyze the BBB-permeation ability of NSC777205 and NSC777207 based on the permeation threshold of 0.02. In addition, we also used the

Brain Or IntestinaL EstimateD permeation method (BOILED-Egg) model (Daina and Zoete, 2016) to further analyze the brain- and intestinal-permeation abilities of the compounds based on their lipophilicity and polarity. We used the CLC-Pred (Cell Line Cytotoxicity Predictor) modules of the PASS server (http://www.way2drug.com/PASSonline) (Poroikov et al., 2019) created based on a training set of data on cytotoxicity retrieved from ChEMBLdb (vers. 23) (https://www.ebi.ac.uk/chembldb/), to predict the cytotoxic activities of NSC777205 and NSC777207 against various cancer cell lines.

Differential Expression and Survival Analysis of Tyrosine-Protein Kinase/Epidermal Growth Factor Receptor in Colorectal Cancer

Expression levels of *c-MET/EGFR* in CRC tissues from The Cancer Genome Atlas (TCGA) database were compared with expression levels in normal tissues using the TNMplot module of the Kaplan-Meier Plotter (https://www.tnmplot.com/). Furthermore, we used the Human Protein Atlas (HPA) database (www.proteinatlas.org) to assess the immunohistochemical (IHC) profile of *c-MET/EGFR* in tumor samples from CRC patients. We used the MEXPRESS algorithm (https://mexpress.be/index.html) to depict the mechanisms of c-MET/EGFR dysregulation in colorectal cancer (Koch et al., 2019). To analyze the prognostic relevance of the gene signatures, we used the PREdiction of Clinical Outcomes from Genomic profiles (PRECOG) server (https://precog.stanford.edu/precog_metaZ.datatable.php) (Gentles et al., 2015) to split CRC patient cohorts into high and low *c-MET/EGFR* expression groups, by setting the median expression level as the expression threshold. Kaplan-Meier survival plots were used to present the survival ratio of cohorts with hazard ratios (HRs), 95% confidence intervals (CIs), and log-rank test p values.

Analysis of the Effects of Tyrosine-Protein Kinase/Epidermal Growth Factor Receptor Expression, and Genetic and Epigenetic Alterations on Immune Infiltration, Dysfunctional T-Cell Phenotypes, and Prognostic Relevance in Colorectal Cancer

We used the cBioPortal server (http://www.cbioportal.org/) to mine the Colorectal Adenocarcinoma (TCGA, PanCancer Atlas) dataset for c-MET/EGFR genetic alterations including mutations, copy number alterations, gene mutation co-occurrences, and microbiome signatures and evaluated the prognostic relevance of the alterations in 594 CRC patients using survival analyses of the cohorts (Cerami et al., 2012; Gao et al., 2013). Protein-protein interaction (PPI) networks and functional enrichment analyses including Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and biological processes enriched in genes that were associated with c-MET/EGFR alterations were analyzed using the Search Tool for Retrieval of Interacting Genes (STRING, vers. 10.5, (https://www.string-db.org) with the adjusted threshold confidence set to 0.400 (Szklarczyk et al., 2019). We used the IMmune Estimation Resource (TIMER2.0) algorithm (http://timer.cistrome.org/) to analyze correlations of c-MET/EGFR expression with somatic copy number alterations and infiltration of six tumor-infiltrating immune cell subsets (B cells, cluster of differentiation 4 (CD4) T cells, CD8 T cells, macrophages, neutrophils, and dendritic cells) in CRC. We also used the TIMER server to analyze correlations of c-MET/EGFR expressions and cancer-associated fibroblast (CAF) infiltration. In addition, we analyzed the prognostic relevance of these associations by employing a protocol described in a previous study (Lawal et al., 2021a). Briefly, TCGA CRC cohorts on the TIMER server were categorized into four groups of low CAF+low c-MET/EGFR, low CAF+high c-MET/EGFR, high CAF+low c-MET/EGFR, and high CAF+high c-MET/EGFR and used Kaplan-Meier survival plots to analyze the cumulative survival of the cohorts. We also used Tumor Immune Dysfunction and Exclusion (TIDE) (http://tide.dfci.harvard.edu) (Jiang et al., 2018) to analyze the effects of genetic (somatic copy number) and epigenetic (DNA methylation) alterations of c-MET/EGFR on dysfunctional T-cell phenotypes, risk factors, and survival of CRC cohorts.

In Vitro Anticancer Screening of NSC777205 and NSC777207

NSC777205 and NSC777207 were evaluated for *in vitro* anticancer activities against 60 panels of human tumor cancer cell lines representing leukemia (six cell lines), NSCLC (eight cell lines), colon cancer (seven cell lines), CNS cancers (six cell lines), melanomas (nine cell lines), ovarian cancer (seven cell lines), renal cancer (eight cell lines), prostate cancer (two cell lines), and breast cancer (five cell lines) through the National Cancer Institute (NCI). Ranges of 5,000–40,000 viable cancer cells were seeded into each well of 96-well plates and incubated at 37 °C with 5% CO_2, 95% air, and 100% relative humidity for 24 h. After incubation, cells were treated with either NSC777205 or NSC777207 at a single dose of 10 μM and further incubated for 48 h (Shoemaker, 2006; Holbeck et al., 2010). A sulforhodamine B (SRB) staining protocol (Vichai and Kirtikara, 2006) was used to determine cell viability. After determining satisfactory antiproliferative activities of a single dose, both NSC777205, and NSC777207 were further subjected to multiple-dose screening at five concentrations of 0, 0.1, 1.0, 10, and 100 μM using the same protocol described above. The activity of each of the drug on each cell line was calculated using four measurements parameters as described here.

Growth inhibition (GI; %): $[(Ti-Tz)/(C-Tz)] \times 100$ for concentrations for which Ti>/ = Tz or $[(Ti-Tz)/Tz] \times 100$ for concentrations for which Ti < Tz.

50% GI (GI_{50}) (μM) = $[(Ti-Tz)/(C-Tz)] \times 100 = 50$.

Total growth inhibition (TGI; μM) = Ti = Tz.

50% Lethal concentration (LC_{50}; μM) = $[(Ti-Tz)/Tz] \times 100 = -50$.

In these equations, Tz is the absorbance at time 0; C is the absorbance of the control after 48 h without treatment; Ti is the absorbance of drug-treated cells after 48 h; GI_{50} is the concentration of the drug causing a 50% reduction in cell

growth; TGI is the concentration causing complete inhibition of cell growth, and LC_{50} is the concentration causing 50% cell death. For a drug whose maximum dose tested (100 μM) did not meet the required effect on a particular cell line, the value for that parameter was expressed as greater than the maximum concentration tested (>100 μM).

DTP-COMPARE Analysis and in Silico Identification of Mechanistic Targets for NSC777205 and NSC777207

We used the private COMPARE module of the NCI-COMPARE program (https://dtp.cancer.gov/databases_tools/compare.htm) to correlate anticancer activity fingerprints of NSC777205 and NSC777207 with NCI synthetic compounds, standard agents, and molecular targets based on established relationships between cell responses to therapy and gene expression profiles of the cell lines (Paull et al., 1989). The NSC numerical IDs were used as the "seed," whereas GI_{50}, TGI, and LC_{50} values were set as endpoints (Lawal et al., 2021b). Analyses were conducted based on program default settings of a minimum correlation of 0.1, minimum common cell line counts of 40, minimum coefficient of variation of 0.01, and maximum return of 200, while results were generated in rank ordered lists of the most highly correlated NCI compounds and targets. In addition to the COMPARE algorithm, we also identified potential targets of NSC777205 and NSC777207 using three different *in silico* target identification algorithms, including the PharmMapper Server, a pharmacophore mapping algorithm with statistical modules (http://lilab-ecust.cn/pharmmapper/index.html) (Liu et al., 2010), the SwissTargetPrediction algorithm (http://www.swisstargetprediction.ch/), which operates on the basis of similarity of the queried molecule with known active drugs (Gfeller et al., 2013), and the computer-aided PASS web resources (http://way2drug.com/dr) (Poroikov et al., 2019).

Molecular Docking Study of NSC777205 and NSC777207 With Various Targets

Three-dimensional (3D) molecular ball-and-stick models in mol2 format of NSC777205 and NSC777207 were obtained using the Avogadro molecular builder and visualization tool vers. 1.XX (http://avogadro.cc/) (Hanwell et al., 2012), while 3D models of standard drugs, including dactolisib (CID: 11977753), gefitinib (CID: 123631), copanlisib (CID: 135565596), and crizotinib (CID: 11626560), were retrieved in sdf file format from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). All mol2 and sdf files were converted to pdb files using the PyMOL Molecular Graphics System, vers. 1.2r3pre (Schrödinger; https://pymol.org/edu/?q=educational/), while pdbqt files of ligands were generated from pdb files using AutoDock Vina (vers. 0.8, Scripps Research Institute, La Jolla, CA, United States) (Trott et al., 2010). Crystal structures of PI3K (PDB:3APC), (B) c-MET (PDB: 3RHK), (C) EGFR (PDB: 5EDP), and (D) mTOR (PDB: 5FLC) were obtained from the Protein Data Bank (https://www.rcsb.org/) in protein data bank (PDB) file format and subsequently converted into the Auto Dock Pdbqt format using AutoDock Vina tools. All receptors were charged, hydrogen atoms were added, and water (H_20) molecules were removed prior to docking (Lawal et al., 2021b). Docking experiments were performed with AutoDock Vina software using default settings, and at a docking exhaustiveness of 8, with all bonds in the ligand rotated freely while considering the receptor to be rigid. A grid box of 40 × 40 × 40 Å in the X, Y, and Z dimensions and a spacing of 1.0 Å were used (Lawal et al., 2021c). Docking outcomes were visualized in 2D conformations using the Discovery studio visualizer vers. 19.1.0.18287 (BIOVIA, San Diego, CA, United States) (Visualizer, 2020) and the protein-ligand interaction profiler (https://plip-tool.biotec.tu-dresden.de/plip-web/plip/index) (Salentin et al., 2015).

Statistical Analysis

All statistical analyses were conducted according to each server's instructions. We used the Wilcoxon test to compare statistically significant differences in c-MET/EGFR expressions between CRC cohorts and healthy tissues. Purity adjustment and partial Spearman's correlations were used to analyze c-MET/EGFR expression correlations with infiltration of CAFs and various immune cells. Gene alteration co-occurrences on the cbioportal were considered significant only at an adjusted p value of <0.05. The KEGG and gene ontologies (GOs) were visualized using GraphPad prism software. DTP-COMPARE analyses were conducted using Pearson's correlations. All survival analyses are presented using Kaplan-Meier plots. GI was calculated relative to cells without drug treatment and the time-zero control. Statistical significance was denoted as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

RESULTS

Tyrosine-Protein Kinase/Epidermal Growth Factor Receptor are Deregulatory Expressed and are Associated With Oxaliplatin Resistance and Poor Prognoses of Colorectal Cancer Patients

We conducted a differential expression analysis between the CRC tumorous and adjacent normal tissues in TCGA database. We found that c-MET expression is higher (p = 1.24e-07) while EGFR expression is lower (p = 4.67e-04) in colorectal cancer cohorts compared to adjacent normal cohorts**.** (**Figure 1A**). Correlation analyses also indicated that c-MET expression was strongly correlated with EGFR expressions in COAD (r = 0.497, p = 5.95e-30) and READ (r = 0.545, p = 3.01e-14) (**Figure 1B**). Furthermore, we explored the HPA database for IHC data of c-MET/EGFR protein expressions based on tissue microarrays (TMAs) in CRC cohorts (**Figure 1C**). We found that all CRC patient samples presented for EGFR (% 66.66 COAD and 33.33% READ) and c-MET (58.33% COAD and 41.66% READ) expression profiling were positive for c-MET (Antibody: CAB005282) and EGFR (Antibody: HPA018530) signals

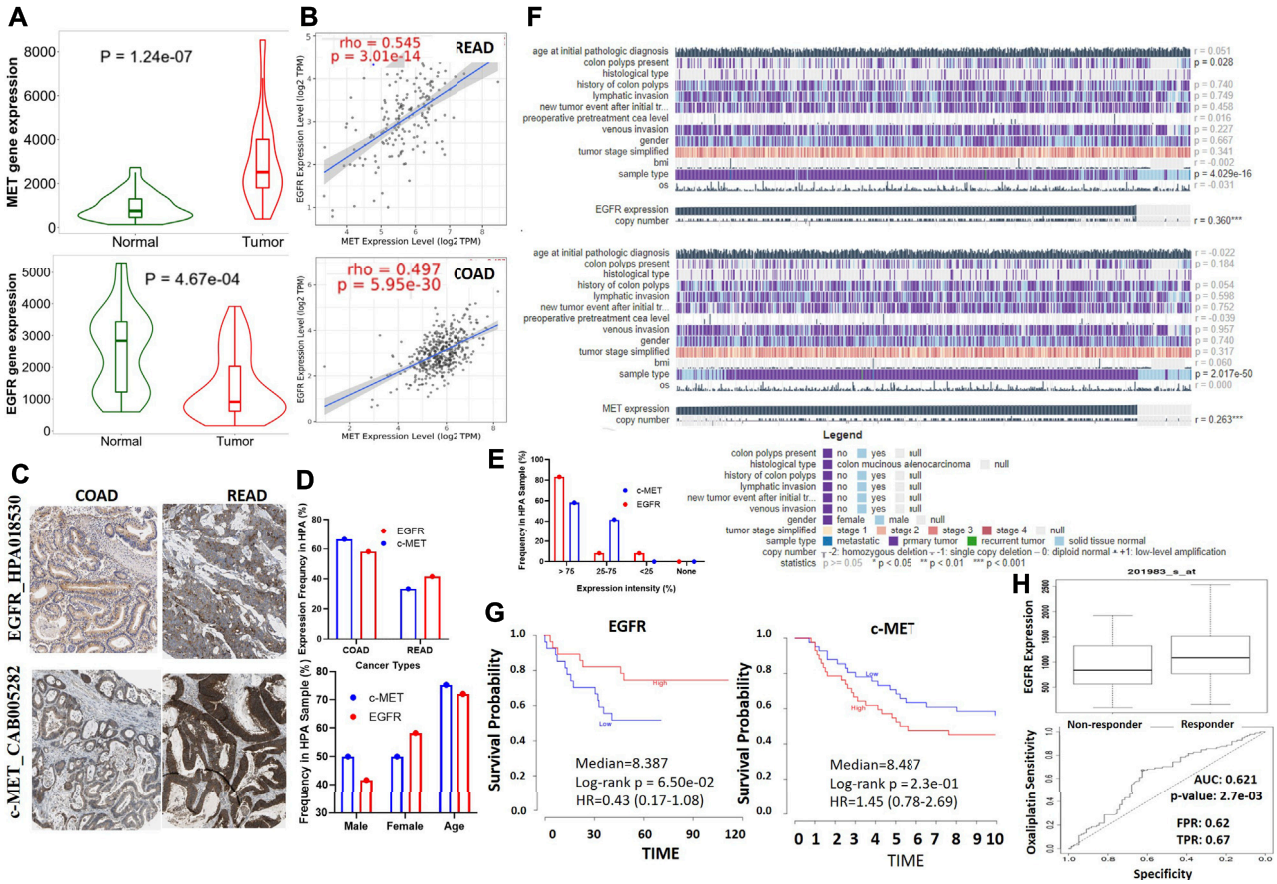


FIGURE 1 | c-Met and epidermal growth factor receptor (EGFR) are overexpressed and are associated with poor prognoses of colorectal cancer patients. **(A)** Violine plots showing differential gene expression levels of c-Met/EGFR between colorectal cancer tumors and adjacent normal tissues. Green labels indicate normal tissues, and red labels indicate tumor samples. **(B)** Expression scatterplots of c-Met correlations with EGFR in COAD and READ. **(C)** Representative IHC staining of c-MET/EGFR protein expressions in colorectal cancer cohorts. **(D)** Frequencies, gender and age differences, and **(E)** expression intensities of EGFR/c-MET in COAD and READ samples. **(F)** Heat map summary of the mechanisms of c-Met/EGFR dysregulation in TCGA colorectal cancer cohorts. **(G)** Kaplan-Meier curve of the survival of colorectal cancer cohorts with high or low c-MET/EGFR expression levels. **(H)** Gene expression profile between oxaliplatin sensitive and resistance colorectal cancer cohorts. The strength of correlations between the genes is reflected by the purity-adjusted partial Spearman's rho value and estimated statistical significance, where a value of $r = 1$ means a perfect positive correlation and a value of $r = -1$ means a perfect negative correlation. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

(**Figure 1D**). Mean ages of patients with high c-Met and EGFR expression profiles were 72.08 and 75.25 years, respectively. Totals of 58.33 and 83.33% of patients had very high intensities (>75%), while 41.66 and 8.33% had medium intensities of c-Met and EGFR expression, respectively (**Figure 1E**). Only 8.33% of patients had a low EGFR expression intensity (**Figure 1E**). Our analysis of the mechanisms of gene dysregulation indicated that EGFR expression is significantly associated with colon polyps ($p = 0.028$) and primary tumors $p = 0.04029$) but less associated with metastasis or recurrent tumor. Furthermore, both c-MET and EGFR expressions show significant correlation with copy number alterations in colorectal cancer (**Figure 1F**). To assess the prognostic relevance of gene signatures, we conducted a survival analysis and found that CRC cohorts with higher expression profiles of c-Met and cohorts with lower expression profiles of EGFR exhibited low survival rates (**Figure 1G**) and resistance to oxaliplatin chemotherapy.

Tyrosine-Protein Kinase/Epidermal Growth Factor Receptor Expressions are Associated With Tumor Infiltration of Various Immune Cells and Cancer Associated Fibroblast, and Poor Prognoses of Colorectal Cancer Cohorts

We explored associations of C-met/EGFR expressions with infiltrating immune cells ($CD4^+$ T cells, B cells, $CD8^+$ T cells, neutrophils, dendritic cells, and macrophages) in CRC tissues (**Figure 2A**, **Supplementary Table 1**). Our results revealed positive correlations of EGFR expression with infiltration of macrophages, dendritic cells, $CD4^+$ T cells, $CD8^+$ T cells, neutrophils, and B cells. The correlation values were $r = 0.173–0.537$, $p < 0.0001$ in COAD and $r = 0.191–0.3860$, $p < 0.01$ in READ. c-MET was positively correlated with various immune cells; $r = 0.185–0.259$, $p < 0.001$ in COAD and $r = 0.090–0.325$ $p < 0.05$ in READ. Furthermore, EGFR

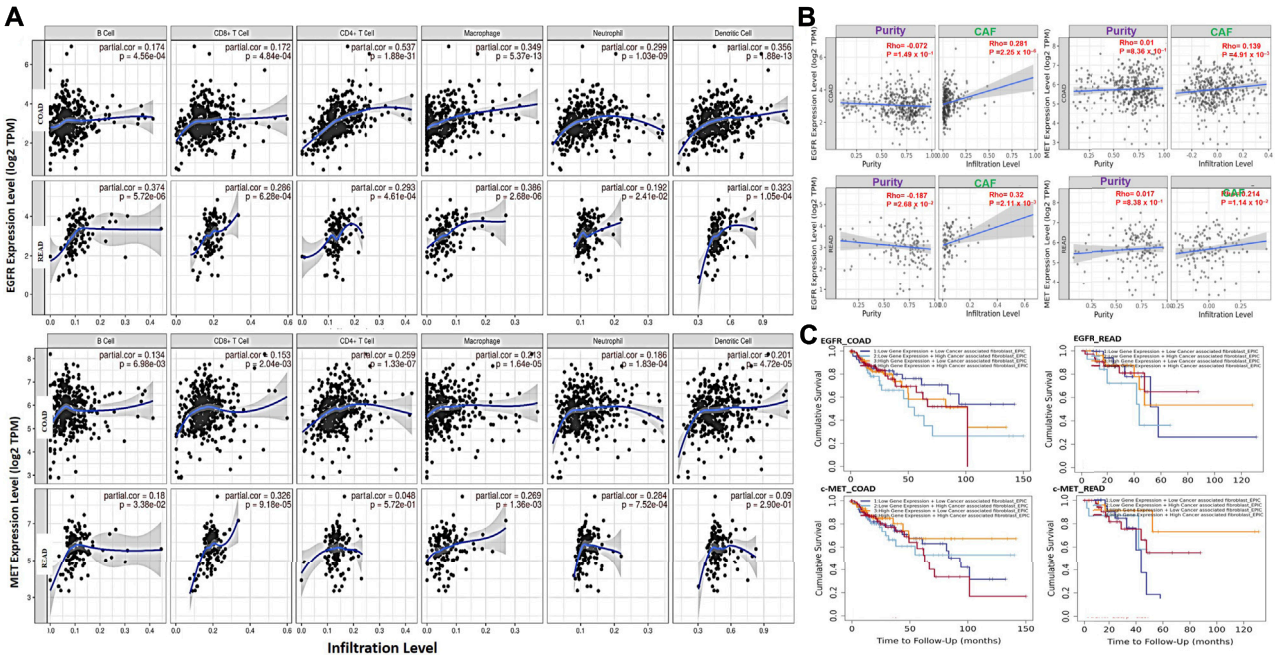


FIGURE 2 | c-MET/epidermal growth factor receptor (EGFR) expressions are associated with tumor immune infiltration and poor prognoses of colorectal cancer cohorts. **(A)** Scatterplots showing correlations of c-MET/EGFR expressions with immune infiltration levels in colon adenocarcinoma (COAD) and rectum adenocarcinoma (READ). **(B)** Scatterplot showing purity-adjusted correlations of c-MET/EGFR expressions and CAF infiltration colon adenocarcinoma (COAD) and rectal adenocarcinoma (READ). **(C)** Kaplan-Meier curve of cumulative survival of COAD and READ cohorts in different categories of EGFR and c-MET associated with CAF infiltration. The ^{high}CAF+^{high}c-Met/EGFR cohort had a low overall survival rate.

TABLE 1 | Multivariate Cox regression analysis of immune infiltration cells in COAD and READ.

	Colon adenocarcinoma				
Imm. Cell	**Coef**	**HR**	**95% CI_l**	**95% CI_u**	***p* value**
B_cell	1.081	2.947	0.031	279.353	0.642
CD8_Tcell	−4.829	0.008	0.000	0.394	0.015
CD4_Tcell	−2.186	0.112	0.001	15.002	0.381
Macrophage	3.597	36.500	0.437	3,049.385	0.111
Neutrophil	−1.915	0.147	0.000	204.039	0.604
Dendritic	1.538	4.654	0.262	82.724	0.295
EGFR	0.269	1.309	0.955	1.793	0.094
MET	−0.136	0.873	0.644	1.183	0.380
	Rectum Adenocarcinoma				
	Coef	**HR**	**95% CI_l**	**95% CI_u**	***p* value**
B_cell	1.352	3.865	0.000	7,5468.261	0.789
CD8_Tcell	−8.946	0.000	0.000	7,934.891	0.328
CD4_Tcell	−4.442	0.012	0.000	3,042,847.483	0.653
Macrophage	4.165	64.385	0.000	38,262,895.774	0.539
Neutrophil	−5.099	0.006	0.000	99,534,652.430	0.671
Dendritic	5.968	390.575	0.042	3,600,096.976	0.200
EGFR	−0.488	0.614	0.289	1.305	0.205
MET	−0.065	0.938	0.489	1.796	0.846

was negatively correlated with tumor purity in COAD (r = -0.0716) and READ (r = -0.18717, p = 0.026802), while MET exhibited no significant correlations with tumor purity in COAD (r = 0.01029, p = 0.836045) and READ (r = 0.017459, p = 0.83777). Collectively, the above results indicate that c-MET/EGFR expressions were correlated with immune infiltration in COAD and READ (**Figure 2A**). Furthermore, we used the abundances of six immune cells and expression levels of c-MET/EGFR to construct univariate and multivariate Cox regression models. The univariate analysis indicated that higher infiltration levels of $CD4^+$ T cell, macrophages, neutrophils, and dendritic cells were correlated with poor survival outcomes in COAD (**Supplementary Figure 1**), while low infiltration levels of $CD8^+$ T cell, B cells, macrophages, neutrophils, and dendritic cells were correlated with poor survival outcomes in READ. A multivariate Cox regression model revealed that lower infiltration levels of $CD4^+$ T cells (hazard ratio (HR) = 0.008, p = 0.015) were risk factors for COAD (**Table 1**).

We analyzed CAF correlations with expression profiles of c-MET/EGFR in CRC samples. We found that EGFR expressions were correlated with CAF infiltration in COAD (partial corr. = 0.281, p = 2.25e-06) and READ (partial corr. = 0.32, p = 2.11e-03). Similarly, c-Met expressions were positively correlated with CAF infiltration in COAD (partial corr. = 0.139, p = 4.91e-03) and READ (partial corr. = 0.214, p = 1.14e-02) (**Figure 2B**). To evaluate the prognostic relevance of these associations, we classified the cohorts into four groups; ^{low}CAF+^{low}c-Met/EGFR, ^{low}CAF+^{high}c-Met/EGFR, ^{high}CAF+^{low}c-Met/EGFR, and ^{high}CAF+^{high}c-Met/EGFR, and we found that the ^{high}CAF+^{high}c-Met/EGFR cohort had a low overall survival rate (**Figure 2C**).

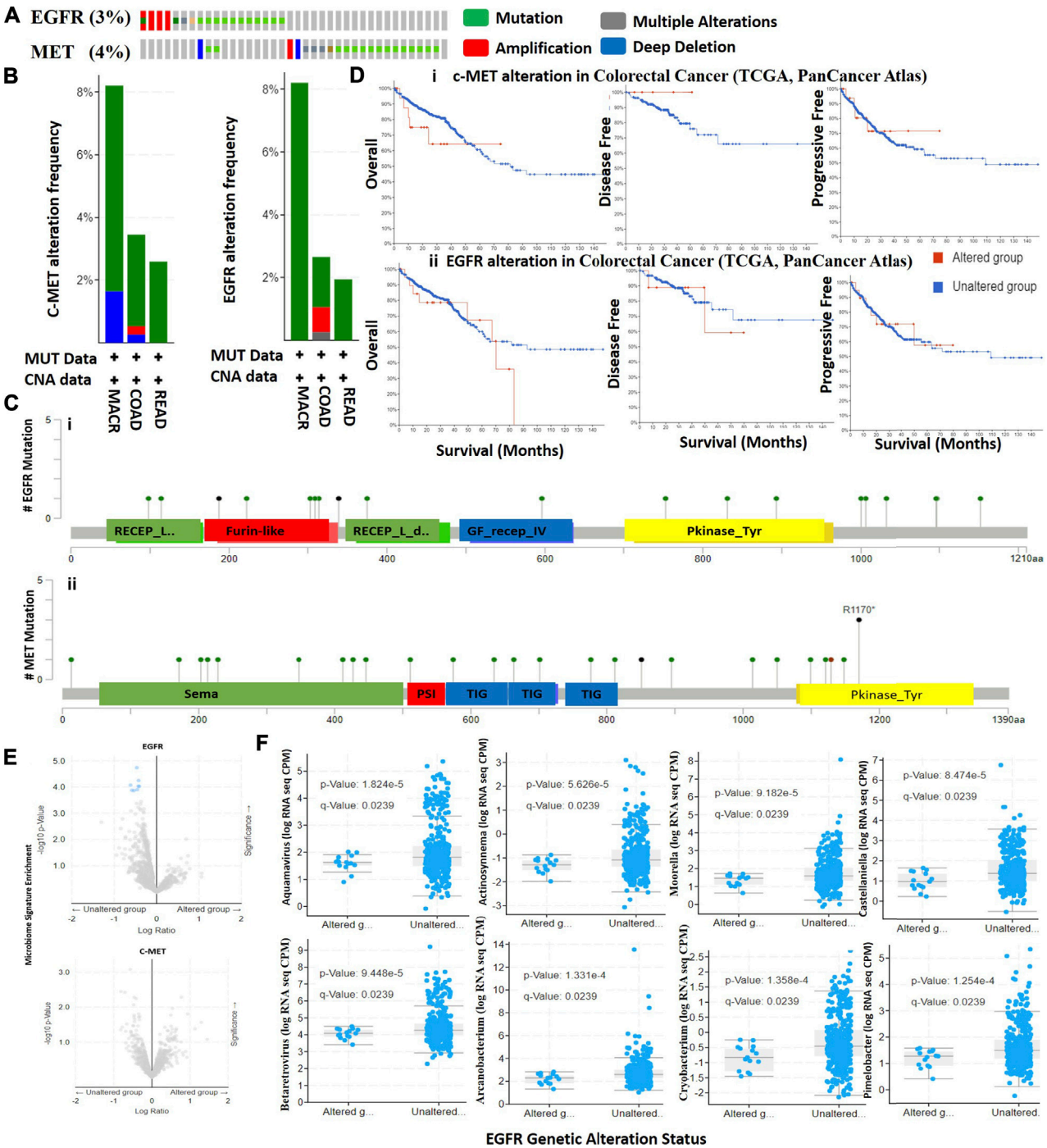


FIGURE 3 | Genetic alterations in c-MET/epidermal growth factor receptor (EGFR) are associated with poor prognoses of colorectal cancer cohorts. **(A)** Total frequency of c-MET/EGFR genetic alterations in colorectal cancer patients. **(B)** Frequencies and types of c-MET/EGFR genetic alterations in colorectal cancer patients. COAD, colon adenocarcinoma; READ, rectum adenocarcinoma; MACR, mucinous adenocarcinoma of the colon and rectum. **(C)** Lollipop plot of c-MET/EGFR mutation types in colorectal cancer patients. Mutations are color-coded as missense, truncating, and in-frame mutations. **(D)** Kaplan-Meier plot of overall survival, disease-free survival, and disease progressive survival of colorectal cancer cohorts with c-MET **(i)** and EGFR **(ii)** genetic alterations. **(E)** Scatterplot of significance levels of enriched microbiome signatures in EGFR **(i)** and c-MET **(ii)** altered and non-altered colorectal cancer cohorts. **(F)** Scatterplot showing differential RNA expressions of microbiome signatures between EGFR altered and non-altered cohorts.

Genetic Alterations of Tyrosine-Protein Kinase/Epidermal Growth Factor Receptor are Associated With Poorer Prognoses of Colorectal Cancer Patients

We mined TCGA PanCancer Atlas dataset for c-MET/EGFR genetic alteration data and evaluated the prognostic relevance of alterations in CRC patients. We found that 3.0 and 4.0% of the 594 CRC patients (63.6% of COAD, 26.1% of READ and 10.3% of mucinous adenocarcinoma (MUAD)) in the dataset respectively harbored genetically altered EGFR and c-MET (**Figure 3A**). Alterations in EGFR were mainly mutations (2.36%), amplifications (0.51%), and multiple alterations (0.17%), while alterations in c-MET were mutations (3.2%), amplifications (0.17%), and deep deletions (0.34%) (**Figure 3B**). By stratifying the mutations, we found 19 mutations consisting of 17 missense and two truncating mutations of EGFR, while c-MET mutations were stratified into 22 missense mutations, four truncations (all nonsense mutations; R1170* and S851*), and one in-frame deletion (I1130del) (**Figure 3C**). A comparative survival analysis indicated that CRC patients with genetic alterations of c-MET/EGFR exhibited shorter overall survival (OS), DFS, and progressive-free survival than cohorts without c-MET/EGFR alterations (**Figure 3D**).

Distinct Microbiome Signatures are Biomarkers of Tyrosine-Protein Kinase/Epidermal Growth Factor Receptor Genetic Alterations in Colorectal Cancer

We analyzed RNA sequencing data of microbiome signatures between CRC cohorts with altered c-MET/EGFR and non-altered c-MET/EGFR. We found significantly higher levels of eight microbiome signatures including Aquamavirus (p = 1.82E-05), Actinosynnema (p = 5.63E-05), Castellaniella (p = 8.47E-05), Moorella (p = 9.18E-05), Betaretrovirus (p = 9.45E-05), Pimelobacter (p = 1.25E-04), Arcanobacterium (p = 1.33E-04), and Cryobacterium (p = 1.36E-04) in EGFR non-altered cohorts compared to altered cohorts (**Figures 3E,F**, **Supplementary Table 2**). However, no significant differences were detected in microbiome signatures between c-MET altered and non-altered cohorts (**Figure 3F**). Collectively, this study suggests that microbiome signatures were lost in CRC cohorts with genetically altered EGFR; thus, they could possibly serve as novel predictors of EGFR alterations in CRC patients.

Genetic Alterations of Tyrosine-Protein Kinase/Epidermal Growth Factor Receptor in CRC Co-Occurred With Other Gene Alterations and are Associated With Overexpression of mRNAs of Some Cancer Hallmark Proteins

We analyzed the frequencies of the co-occurrence of gene alterations with c-MET/EGFR genetic alterations, and found the co-occurrence of genetic alterations in a total of 19,434 genes, enriched in both c-MET/EGFR altered and non-altered cohorts (**Figures 4A, B**). Genes with significantly (all $p < 10^{-4}$) enriched genetic alteration co-occurrences with EGFR alterations included *SMARCD2*, *TRIM7*, *MCMDC2*, *HNRNPUL1*, *BEST3*, *GRB10*, *PDE6C*, and *SNX19*, while *SLC22A2*, *DST*, *AKT3*, and *TAB2* were the only significantly (all $p < 10^{-4}$) enriched gene alterations that co-occurred with c-MET alterations (**Supplementary Table 3**). However, *TTN*, *APC*, *TP53*, *SYNE1*, *DNAH11*, *BRAF*, *PIK3CA*, *COL12A1*, *DNAH3*, and *SRCAP* had the highest mutation frequencies in both EGFR altered and non-altered cohorts, while *TTN*, *APC*, *DST*, *BRAF*, *TP53*, *ZC3H13*, *DNAAH8*, *SYNE1*, *USP34*, and *DNAH9* were the most frequently mutated genes associated with c-MET altered and non-altered cohorts (**Figure 4C**).

Furthermore, we found that genetic alterations of EGFR were associated with overexpressions of mRNAs of a number of proteins in CRC patients. The top ten overexpressed (p = 7.47E-05–2.97E-06) mRNAs in EGFR altered cohorts compared to non-altered cohorts were *TRIM7*, *BARX2*, *CFAP43*, *TPBG*, *HPSE*, *GNB5*, *MARK3*, *FUT8-AS1*, *SS18*, and *KCNK1*, while *SMAD4*, *MBP*, *IER3IP1*, *OXCT1*, *ALKBH5*, *C18ORF25*, *WDR76*, *TYMS*, *LINC00909*, and *GPR3* were overexpressed in c-MET altered CRC cohorts (**Supplementary Table 4**).

We further constructed PPI networks of overexpressed mRNAs in c-MET/EGFR altered cohorts (**Figure 4D**) and performed enrichment analyses. We found that overexpressed mRNAs were enriched in several KEGG pathways associated with CRC development, progression, and drug resistance. The top enriched pathways (with a strength of enrichment (SOE) of >1 and $p < 0.05$) included cell adherent junctions, EGFR tyrosine kinase inhibitor resistance, central carbon metabolism, CRC, pancreatic cancer, hepatocellular carcinoma, gastric cancer, and melanomas (**Figure 4E**), while enriched GOs (with a SOE of >1 and $p < 0.05$) included the c-MET, ERBB2, and EGFR signaling pathways, MAPKK activation, and negative regulation of cellular responses to drugs (**Figure 4F**).

DNA-Methylation and Somatic Copy Number Alterations of Tyrosine-Protein Kinase/Epidermal Growth Factor Receptor are Associated With Immune Infiltration, Dysfunctional T Cell Phenotypes, and Poor Prognoses of Colorectal Cancer Patients

We also analyzed associations of different somatic CNAs, such as arm-level gains, high amplifications, deep deletions, arm-level deletions of c-MET/EGFR and immune cell infiltration in CRC. We found that arm-level gains of EGFR were associated with B cell ($p < 0.005$), $CD8^+$ T cell ($p < 0.001$), neutrophil ($p < 0.005$), and dendritic cell infiltration in COAD, and macrophage ($p < 0.005$) infiltration in READ. Arm-level gains of c-MET were positively correlated with $CD8^+$ T cell, B cell, neutrophil, and dendritic cell infiltration in colon cancer and dendritic cells infiltration in READ, while deep deletions of c-MET were associated with $CD8^+$ T cell and neutrophil infiltration of tumors in READ (**Figure 5A**). Furthermore, CNAs of both c-MET and EGFR were associated with dysfunctional T-cell

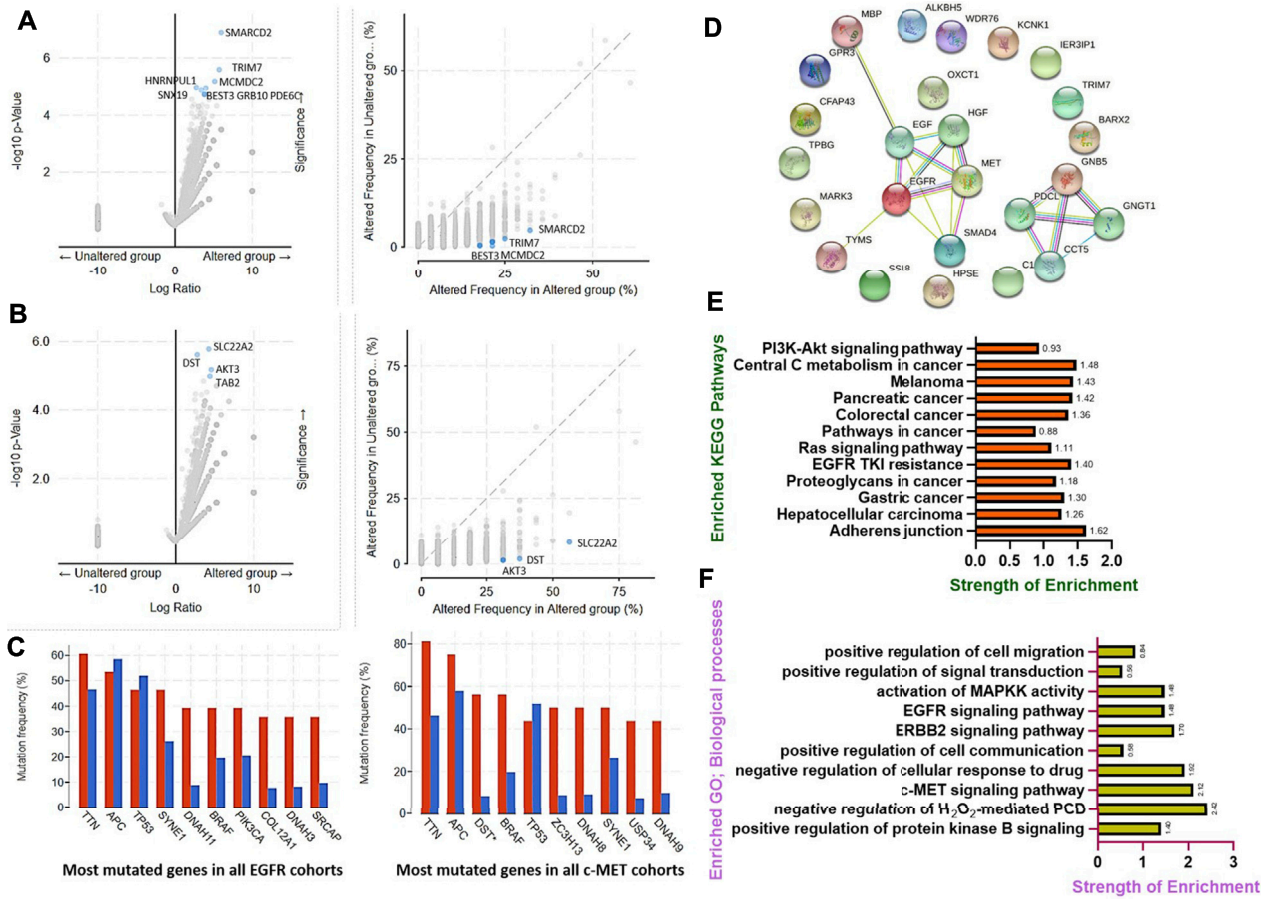


FIGURE 4 | Genetic alterations of c-MET/epidermal growth factor receptor (EGFR) in colorectal cancer co-occurred with other gene alterations and were associated with overexpressions of mRNAs of some cancer hallmark proteins. Enrichment frequencies of gene alteration co-occurrences with c-MET/EGFR alterations in colorectal cancer cohorts. Scatterplot of the significance and frequencies of enriched co-occurring gene alterations in **(A)** EGFR and **(B)** c-MET altered and non-altered cancer cohorts. **(C)** Bar plot showing the top 10 most frequently mutated genes in c-MET/EGFR altered and non-altered cancer cohorts. **(D)** Protein-protein interaction (PPI) network, **(E)** enriched KEGG pathways, and **(F)** biological processes of overexpressed mRNAs in c-MET/EGFR altered colorectal cancer cohorts.

phenotypes (**Figure 5B**) and poor prognoses (**Figure 5C**) of CRC patients. c-MET was found to be hypomethylated, while EGFR was hypermethylated in CRC (**Figure 5D**). These methylation statuses of c-MET and EGFR in CRC were also consistently associated with the tumor stage (**Figure 5D**), dysfunctional T cell phenotypes (**Figure 5E**), and shorter OS of CRC patients (**Figure 5F**). Collectively, these findings indicated that the DNA-methylation and somatic CNAs particularly the arm-level gains of c-MET/EGFR were associated with immune infiltration, dysfunctional T cell phenotypes, and poor prognoses of CRC patients.

NSC777205 and NSC777207 Displayed Desirable Characteristic of a Good Drug Candidate and Exhibited *In Silico* Cytotoxic Activities Against Colon, Brain, Lung, and Bone Cancer Cell Lines

NSC777205 (3-(4-chloro-2-fluorophenyl)-2H-benzo[e][1,3] oxazine-2,4(3H)-dione) and NSC777207 (3-(4-chloro-2-fluorophenyl)-7-methoxy-2H-benzo[e][1,3]-oxazine-2,4(3H)-dione) were obtained at high purity (>95%) via condensation reactions between salicylic acid and the 2,4-disubstituted aniline moieties of gefitinib to produce a salicylanilide which was further cyclized to the 3-phenyl-2H-benzo[e][1,3]-oxazine-2,4(3H)-dione derivatives, NSC777205 and NSC777207, as summarized in **Figure 6A**. Our general *in silico* analysis of drug-like and toxicity characteristics of NSC777205 and NSC777207 suggested that both compounds exhibited suitable physicochemical properties (lipophilicity, polarity, flexibility, solubility, saturation, molecular weight, and other properties) of a good drug candidate. The compounds were both lead-like and satisfied Lipinski's rules for drug likeness candidates. Furthermore, our *in silico* analysis suggested that both compounds can be easily absorbed in the gastrointestinal tract and are well permeant to the blood-brain barrier (BBB) especially NSC777205 which demonstrated 2-fold higher permeation of the BBB than NSC777207 based on *in silico* estimation (**Figure 6B**; **Table 2**). Estimates of the acute toxicity in rats revealed

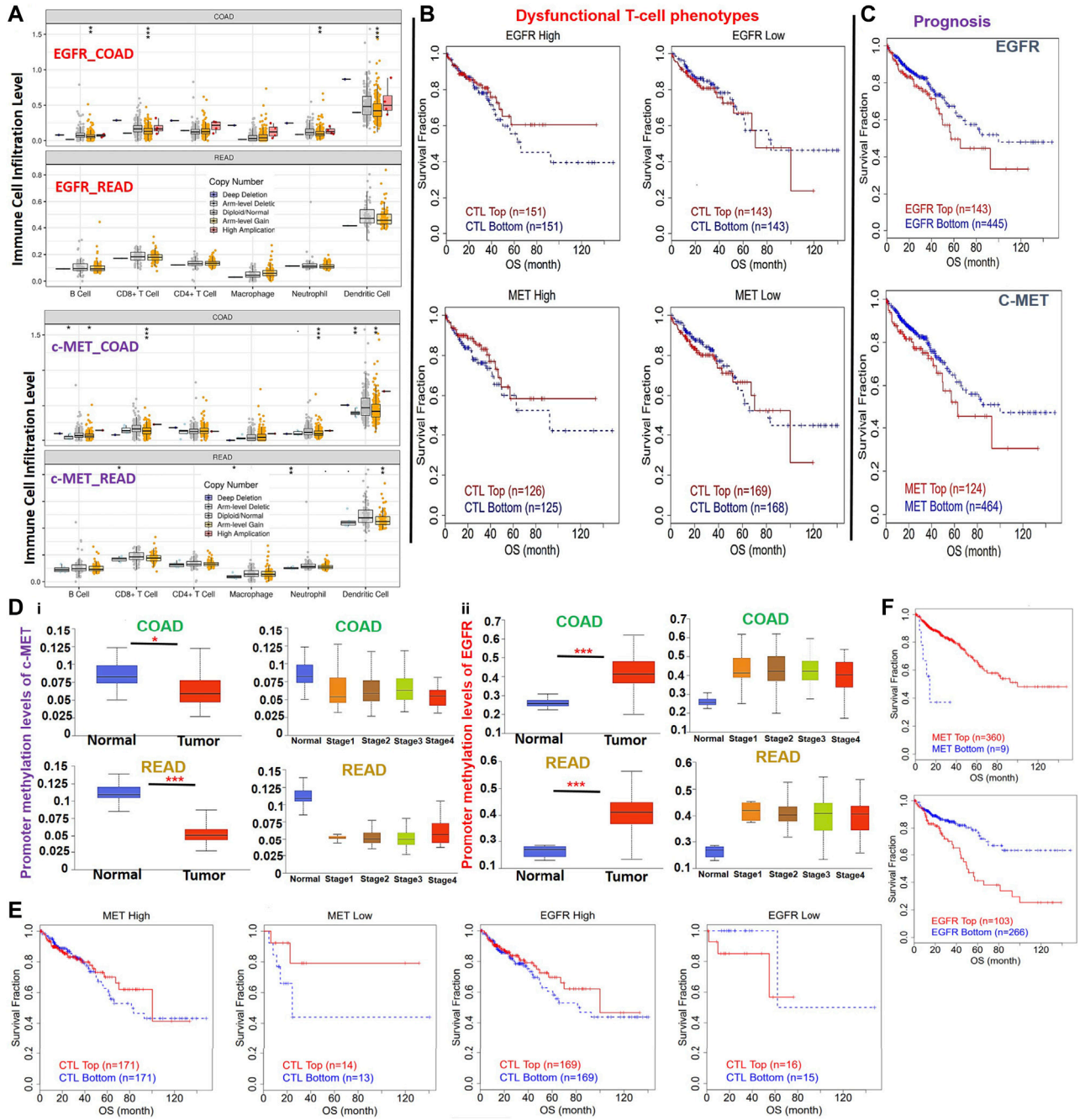
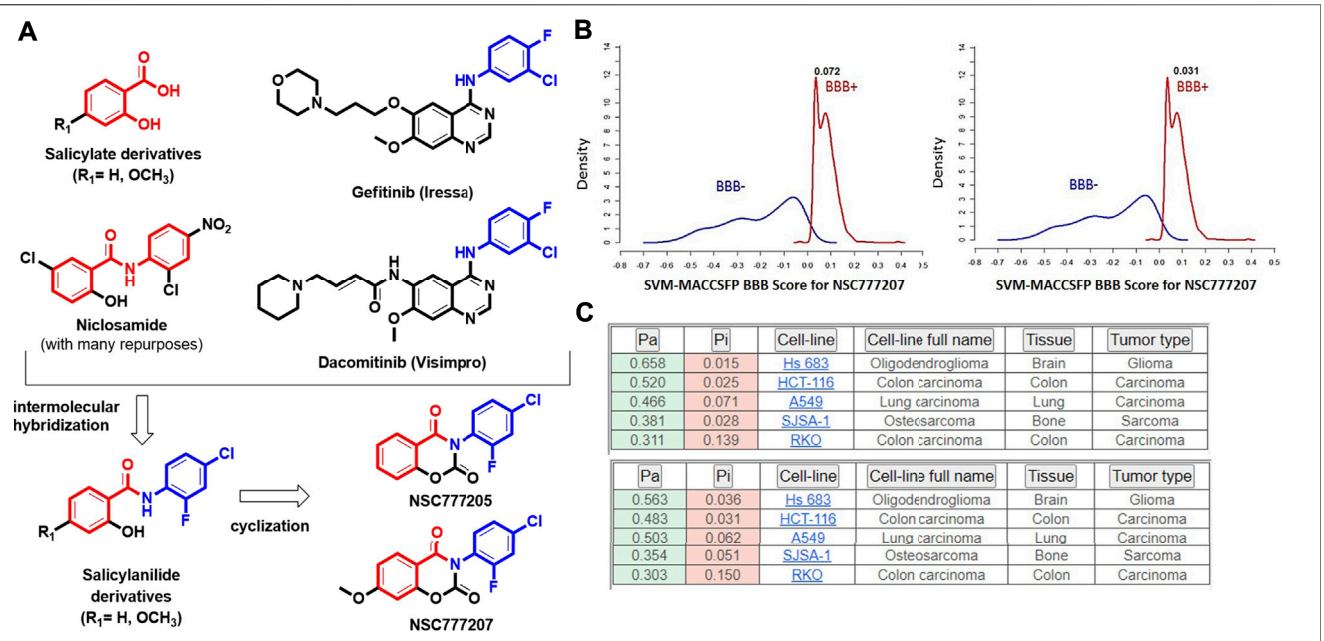


FIGURE 5 | DNA-methylation and copy number alterations (CNAs) of c-MET/epidermal growth factor receptor (EGFR) are associated with dysfunctional T cell phenotypes and poor prognoses of colorectal cancer patients. **(A)** Box plots showing tumor immune infiltration levels in colorectal cancer with different somatic CNAs of c-Met/EGFR. The infiltration abundance in every somatic CNA category was compared to the diploid/normal ratio. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. **(B)** Kaplan-Meier plot of the independent effects of c-MET/EGFR CNAs on dysfunctional T cell phenotypes. **(C)** Kaplan-Meier curve of the overall survival of patient with high or low somatic CNAs of c-MET/EGFR. **(D)** Bar plot showing differential methylation levels of c-MET/EGFR between normal tissues and colorectal tumors **(i)** and different tumor stages **(ii)**. **(E)** Kaplan-Meier plot of the independent effects of c-MET/EGFR methylation on dysfunctional T cell phenotypes. **(F)** Kaplan-Meier curve of the overall survival of patients with a hypo- or hypermethylation status of c-MET/EGFR.

high LD_{50} values for NSC777205 and NSC777207 from oral, intravenous, intraperitoneal, and subcutaneous administration, while bioaccumulation factor (log10) and other markers of eco-toxicity also identified both compounds as having low toxicities (**Table 2**). Collectively, these findings suggested that NSC777205 and NSC777207



Pa	Pi	Cell-line	Cell-line full name	Tissue	Tumor type
0.658	0.015	Hs 683	Oligodendroglioma	Brain	Glioma
0.520	0.025	HCT-116	Colon carcinoma	Colon	Carcinoma
0.466	0.071	A549	Lung carcinoma	Lung	Carcinoma
0.381	0.028	SJSA-1	Osteosarcoma	Bone	Sarcoma
0.311	0.139	RKO	Colon carcinoma	Colon	Carcinoma

Pa	Pi	Cell-line	Cell-line full name	Tissue	Tumor type
0.563	0.036	Hs 683	Oligodendroglioma	Brain	Glioma
0.483	0.031	HCT-116	Colon carcinoma	Colon	Carcinoma
0.503	0.062	A549	Lung carcinoma	Lung	Carcinoma
0.354	0.051	SJSA-1	Osteosarcoma	Bone	Sarcoma
0.303	0.150	RKO	Colon carcinoma	Colon	Carcinoma

FIGURE 6 | (A) Synthesis protocols for NSC777205 (3-(4-chloro-2-fluorophenyl)-2H-benzo[e [1,3]-oxazine-2,4(3H)-dione) and NSC777207 (3-(4-chloro-2-fluorophenyl)-7-methoxy-2H-benzo[e][1,3]-oxazine-2,4(3H)-dione), which were obtained via intermolecular hybridization and condensation reactions between salicylic acid (R_1 = H) or 2-hydroxy-4-methoxybenzoic acid (R_1 = OCH_3) and 2,4-disubstituted aniline moieties of gefitinib to produce salicylanilides which were further respectively cyclized to produce NSC777205 (when R_1 = H) and NSC777207 (when R_1 = OCH_3). Cyclization occurred in the presence of methyl chloroformate and pyridine, while hybridization occurred in the presence of tetrahydrofuran (THF). **(B)** Blood-brain barrier (BBB) permeation curves of NSC777205 and NSC777207 as measured by the support vector machine (SVM) and LiCABEDS algorithms of the BBB prediction server. **(C)** PASS predicted cytotoxic activities of NSC777205 and NSC777207 against various cancer cell lines.

TABLE 2 | Drug-like and toxicity characteristic of NSC777205 and NSC777207.

Properties	NSC777205	NSC777207	Reference value
Formula	$C_{14}H_7ClFNO_3$	$C_{15}H_9ClFNO_4$	—
M.W(g/mol)	291.66	321.69	150–500
R-bonds	1	2	0–9
H-bond ACC.	4	5	0–10
H-bond DON.	0	2	0–5
Molar Refractivity	73.05	79.54	40–130
TPSA (Å^2)	52.21	61.44	20–130
Fraction Csp3	0.00	0.07	0.25 ~ <1
Log Po/w (XLOGP3)		3.46	–0.7 to 5
Consensus Log Po/w	3.15	3.29	≤3.5
Log S (ESOL)	–4.37	–4.42	0–6
Drug-likeness (Lipinski rule)	YES 0 violation	YES 0 violation	MLOGP ≤ 4.15, M.W ≤ 500, H-bond ACC ≤ 10, H-bond DON ≤ 5
Lead-likeness	YES	YES	XLOGP3 ≤ 3.5, M.W ≤ 350, R-bonds ≤ 7
Bioavailability Score	0.55	0.55	>0.1 (10%)
BBB-permeation (LogBB)	High (0.631)	(-0.400)	BBB+ ≥ 0.3, BBB– < –1,
BBB-permeation (SVM_MACCSFP)	0.072	0.031	≥0.02
Synthetic accessibility	3.06	3.08	1 (very easy) to 10 (very difficult).
Route of adm.			
Intraperitoneal	357.300 (OECD:4)	224.500 (OECD:4)	
Intravenous	116.800 (OECD:4)	201.500 (OECD:4)	
Oral	943.300 (OECD:4)	591.800 (OECD:4)	
Subcutaneous	2161.000 (OECD:5)	740.800 (OECD:4)	
Bioaccumulation factor Log10(BCF)	1.462	1.125	<2: low, 2 ≤ 3: Moderate, 3–3.7: High, >3.7: Very high
Daphnia magna LC50 –Log_{10} (mol/L)	6.421	6.599	
Fathead Minnow $LC_{50}Log_{10}$ (mmol/L)	–1.931	–2.108	
Tetrahymena pyriformis IGC50 -Log_{10} (mol/L)	1.401	1.453	

R-bond, Num. rotatable bonds; H-bond ACC, Num. H-bond acceptors; H-bond DON, H-bond donors; TPSA, topological polar surface area; BBB, Blood brain barrier; IP, Intraperitoneal; IV, Intravenous; SC, Subcutaneous.

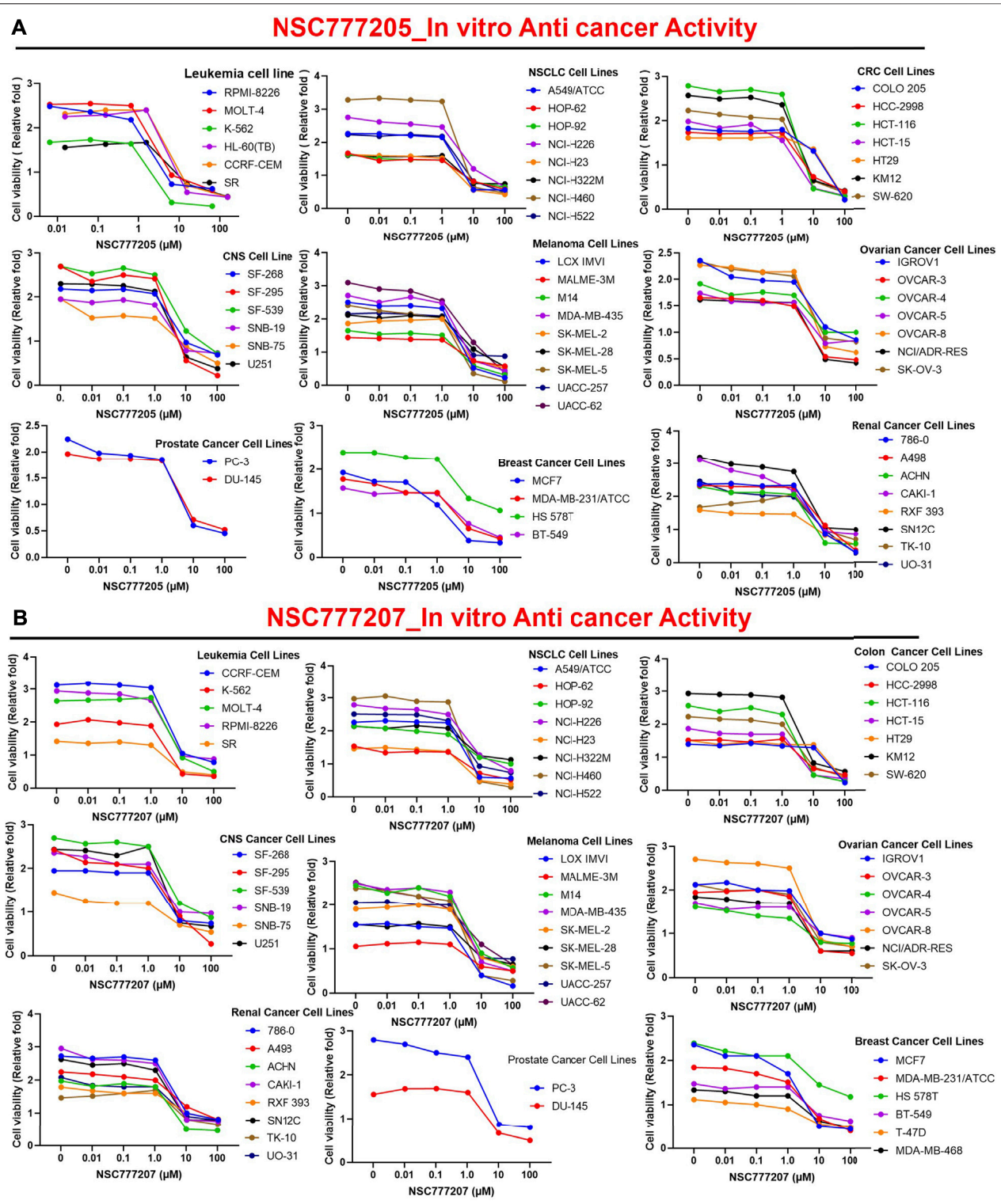


FIGURE 7 | *In vitro* anticancer activities of NSC777205 and NSC777207 against the NCI-60 human cancer cell lines. Dose-response curves of NCI-60 human cancer cell lines to **(A)** NSC777205 and **(B)** NSC777207 treatment.

exhibited desirable characteristic of a drug candidates and can safely be employed for acute administration via oral, intravenous, intraperitoneal, and subcutaneous routes. Our *in silico* cytotoxic analysis suggested that both NSC777205 and NSC777207 exhibited cytotoxic activities against colon (HCT-116 and RKO), brain (Hs683), lung (A549), and bone (SJSA-1) cancer

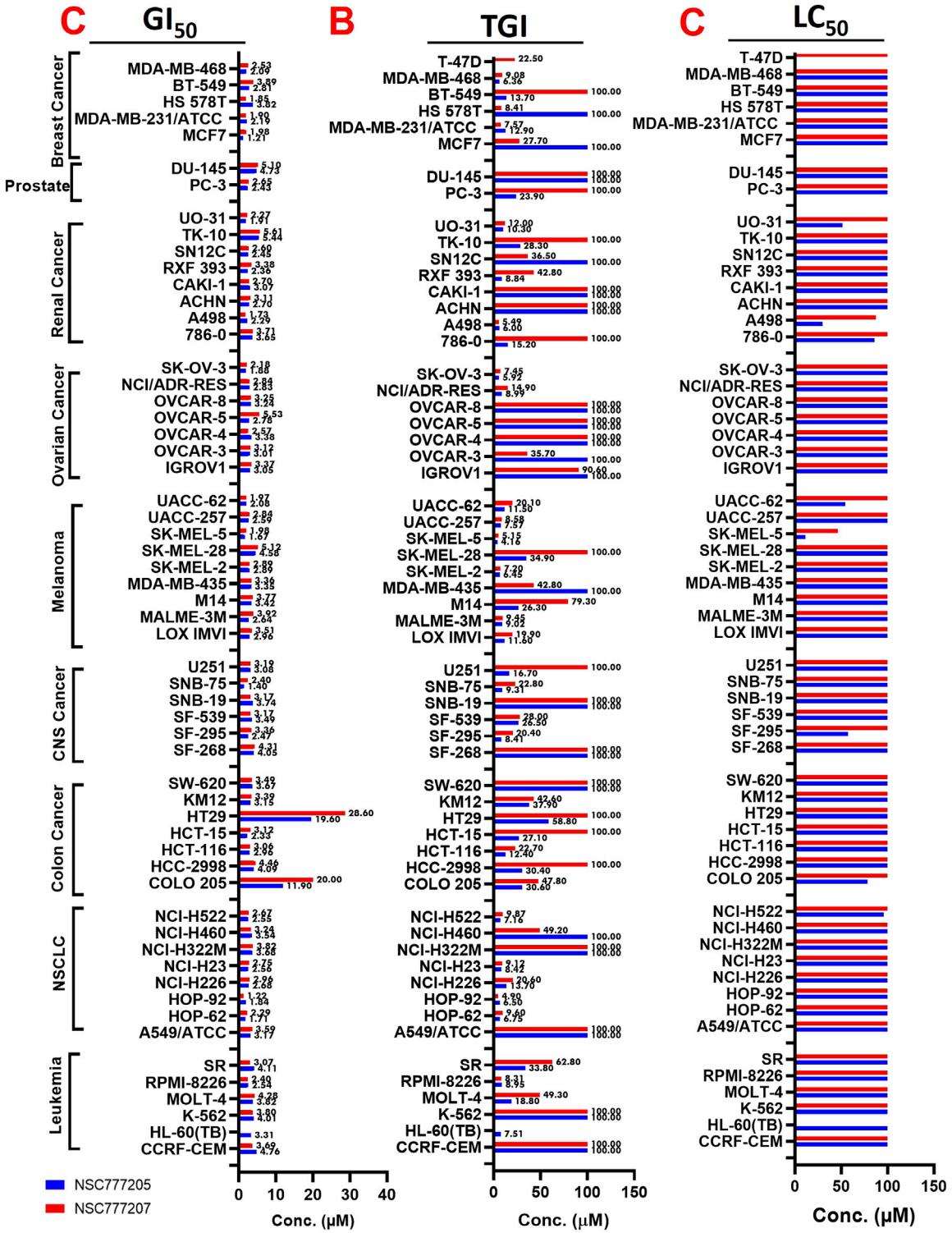


FIGURE 8 | Heat map showing the (C) GI_{50} (D) TGI and (E) LC_{50} value of NSC777205 and NSC777207 against the NCI-60 human cancer cell lines.

cell lines, with NSC777205 demonstrating higher activities (Pa = 0.31–0.658) than NSC777207 (Pa = 0.303–0.565) against all cell lines (**Figure 6C**).

NSC777205 and NSC777207 Exhibited Wide Spectra of Antiproliferative Activities and Selective Cytotoxic Preferences for Melanoma, Renal, Central Nervous System, Colon, and NSCLC Cell Lines *In Vitro*

We initially screened NSC777205 and NSC777207 for anticancer activities against the full NCI-60 panel of human tumor cell lines. Interestingly, we found that with single-dose (10 μM) treatment, both NSC777205 and NSC777207 demonstrated antiproliferative activities against all of the NCI-60 cell line panels of breast, prostate, renal, ovarian, colon, melanoma, CNS, leukemia, and non-small cell lung cancers, with NSC777205 demonstrating higher activities than NSC777207. In addition, most of the leukemia (cell death (CD) = 0.77–10.28%), NSCLC (CD = 4.2–50.81%), CNS (CD = 013.71%), melanoma (CD = 0.57–46.24%) renal (CD = 7.01–30.91%), breast (CD = 10.48%), prostate, and ovarian cancer cell lines demonstrated some cytotoxic response to NSC777205 and NSC777207 single-dose treatment, while panels of colon cancer cell lines were completely insensitive to the cytotoxic activity of the compounds at 10 μM. Collectively, these studies demonstrated that both NSC777205 and NSC777207 exhibited a wide spectrum of anticancer activities but demonstrated selective and exclusive antiproliferative activities against all panels of colon cancer cell lines (**Supplementary Figure 2**).

With five-dose screening, NSC777205 and NSC777207 demonstrated dose-dependent cytotoxic effects against the panel of NCI-60 human tumor cell lines (**Figures 7A, B**). With the exception of HT29 and COLO 205 (GI_{50} = 11.90–20 μM), both NSC777205 and NSC777207 exhibited high anticancer activities with GI_{50} concentrations of <6 μM against all cell line panels (**Figure 8A**). Total cell growth inhibitory (TGI) concentrations of the compounds revealed that NSCLC (1.71–3.68 μM), leukemia (7.51–33.80 μM), CNS (7.51–33.80 μM), melanoma (4.196–34.90 μM), and colon (12.40–58.80 μM) cancer cell lines were the most sensitive to NSC777205 treatment, while a lower but similar trend of anticancer activity against the panel of cell lines was also observed for NSC777207 (**Figure 8B**). Furthermore, as revealed by LC_{50} values, cytotoxic activities of NSC777205 were more pronounced against the 786–0 (LC_{50} = 85.70 μM), A498 (LC_{50} = 29.60 μM), and UO-31 (LC_{50} = 51.10 μM) renal cancer cell lines; the SK-MEL-5 (LC_{50} = 11.30 μM) and UACC-62 (LC_{50} = 54.10 μM) melanoma cell lines; the SF-295 (LC_{50} = 57.40 μM) CNS cell line; the COLO 205 (LC_{50} = 78.40 μM) colon cancer cell line; and the NCI-H522 (LC_{50} = 95.70 μM) NSCLC cell line (**Figure 8C**). Collectively, the anticancer study revealed that NSC777205 and NSC777207 exhibited a wide spectrum of antiproliferative activities and selective cytotoxic preferences for renal cancer, melanoma, CNS cancer, colon cancer, and NSCLC cell lines with NSC777205 demonstrating higher potencies than NSC777207.

Tyrosine-Protein Kinase/Epidermal Growth Factor Receptor/Phosphatidylinositol 3-Kinase/Mammalian Target of Rapamycin Signaling Pathways are Implicated in the Anticancer Activities of NSC777205 and NSC777207

In order to adequately identify potential drug targets of our compounds, we used three different *in silico* drug target identification algorithms and also the DTP-COMPARE algorithm. Using PharmMapper, c-MET and EGFR were both identified as potential targets for NSC777205 and NSC777207. The pharmMapper algorithm identified c-MET as potential targets for NSC777205 and NSC777207 by pharmacophore mapping of one hydrogen bond and four hydrophobic interactions, with normalized fit scores of 0.5876 and 0.5884, while EGFR was mapped with NSC777205 and NSC777207 by two hydrophobic reactions and two hydrogen interactions with normalized fit scores of 0.6981 and 0.6986 (**Supplementary Table 5**). In addition, the PI3K regulatory subunit alpha, several MAPKs, and several serine/threonine-protein kinases were identified as potential targets for NSC777205 and NSC777207 (**Supplementary Table 5**). The PASS prediction server also identified c-MET and EGFR as potential targets for NSC777205 and NSC777207 with pa>0.3 and pi<0.06. In addition, inhibition of MAPK, inositol 1,4,5-trisphosphate 3-kinase, and growth factors among other activities were predicted for NSC777205 and NSC777207 (**Supplementary Table 6**). However, SwissTargetPredition for NSC777207 returned serine/threonine-protein kinases, mTOR, several subunits of PI3K, MAPK, and ErbB-2 among others, while AKT, EGFR, mTOR, several subunits of PI3K, and growth factors were predicted for NSC777205 (**Supplementary Table 7**). Altogether, c-MET, EGFR, PI3K, and mTOR were commonly identified targets of NSC777205 and NSC777207 by the three drug target identification algorithms we employed. Furthermore, results of the DTP's COMPARE algorithm indicated that the anticancer fingerprints of NSC777205 and NSC777207 showed strong correlations (p = 0.58–0.86, common cell line count (CCLC) = 47–58) with fingerprints of some NCI synthetic compounds (**Table 3**). These synthetic compounds are small molecules with molecular weights ranging 277.8–559.5 g/mol. The COMPARE analysis of NSC777205 and NSC777207 anticancer fingerprints with NCI investigational drugs revealed strong associations (CCLC = 55–58, p = 0.3–0.61) with a number of c-MET/EGFR/PI3K/AKT/mTOR inhibitors, including JNJ38877618, MTX211, SGX-523, altiratinib, TAS-6417, voxtalisib, PI-103 HCL, AMG-458 (c-MET), CC115, and others (**Table 4**). In addition, the COMPARE analysis identified MET, EGFR, PIK3R2, RPS6KB1, CDK6, IGHMBP2, and ErbB2 as some of the major targets that were correlated with the anticancer fingerprints of NSC777205 and NSC777207 (**Table 4**). Collectively this study provides a hypothesis implicating C-MET/EGFR/PI3K/AKT/mTOR signaling pathways in the anticancer activities of NSC777205 and NSC777207.

TABLE 3 | NCI synthetic compound correlation with anticancer fingerprint of NSC777205 and NSC777207.

Rank	*P*	CCLC	M.W (g/mol)	NCI-ID	Target descriptor
					NSC777205
1	0.78	55	413.14	777213	3-(2-fluoro-4-iodophenyl)-7-methoxy-2H-benzo[e] [1,3]oxazine-2,4(3H)-dione
2	0.72	56	307.3	50681	3-Hydroxy-2-naphtho-o-phenetidide
3	0.72	56	277.8	37188	2-Naphthalenecarboxamide, 3-hydroxy-N-(2-methylphenyl)-
4	0.72	58	327.12	178296	NICLOSAMIDE(USAN)
5	0.7	58	451.6	766722	(2Z)-3-(1H-Benzimidazol-2-yl)-6-hexyl-2-[(2-methylphenyl)imino]-2H-chromen-7-ol
6	0.68	56	361.29	765598	N-(2,4-difluorophenyl)-2',4'-difluoro-4-hydroxybiphenyl-3-carboxamide
7	0.63	58	317.4	775727	3-Isobutyl-9,10-dimethoxy-1,3,4,6,7,11b-hexahydro-2H-benzo[a]quinolizin-2-one(T2839)
8	0.61	44	310.4	653264	2-[1-(Benzenesulfonyl)indol-3-yl]propanenitrile
9	0.6	58	297.7	12969	2-Naphthalenecarboxamide, N-(3-chlorophenyl)-3-hydroxy-
10	0.59	47	416.4	640519	3'-Carboxypropionyl-(Z)-combretastatin A-4
11	0.47	58	376.31	765690	(4-(6-(2,4-Difluorophenyl)-2,4-dioxo-2H-benzo[e][1,3]-oxazin-3(4H)-yl) benzonitrile
12	0.58	58	372.2	367089	NAPHTHOL AS-BI
13	0.58	56	357.8	50687	5'-Chloro-3-hydroxy-2',4'-dimethoxy-2-naphthanilide
Rank					**NSC777207**
1	0.86	58	327.12	178296	NICLOSAMIDE(USAN)
2	0.84	56	307.3	50681	3-Hydroxy-2-naphtho-o-phenetidide
3	0.81	55	413.14	777213	3-(2-fluoro-4-iodophenyl)-7-methoxy-2H-benzo[e][1,3]oxazine-2,4(3H)-dione
4	0.77	58	451.6	766722	(2Z)-3-(1H-Benzimidazol-2-yl)-6-hexyl-2-[(2-methylphenyl)imino-2H-chromen-7-ol
5	0.75	56	277.8	37188	2-Naphthalenecarboxamide, 3-hydroxy-N-(2-methylphenyl)-
6	0.73	58	317.4	775727	3-Isobutyl-9,10-dimethoxy-1,3,4,6,7,11b-hexahydro-2H-benzo[a]quinolizin-2-one(T2839)
7	0.68	58	290.4	240579	4-(2-(4-(Dimethylamino)phenyl)vinyl)-8-quinolinol
8	0.68	50	382.4	709579	N-(3-Fluorophenyl)-6-hexyl-7-hydroxy-2-iminochromene-3-carboxamide
9	0.68	58	269.12	757391	DICHLOROPHENE
10	0.65	54	559.5	642198	Antineoplastic-642198

P, Pearson's correlation coefficient; CCLC, Common cell lines count; MW, molecular weight.

Comparative Docking Profile of Tyrosine-Protein Kinase/Epidermal Growth Factor Receptor/Phosphatidylinositol 3-Kinase-Mammalian Target of Rapamycin Between NSC777205, NSC777207 and Clinical Inhibitors

We used molecular docking to further understand the possible interactions of NSC777205 and NSC777207 with c-MET/EGFR/PI3K-mTOR. We found that NSC777205, NSC777207 and clinical inhibitors (used for comparison) docked well into the binding cavities of the receptors with respective binding affinities (ΔG) of −6.90, −6.80 and −6.80 Kcal/mol for EGFR; −8.95, 8.96 and −9.56 Kcal/mol for c-MET; −8.60, −7.90, and −9.0 Kcal/mol for PI3K; and −7.1, −7.6 and −9.2 Kcal/mol for mTOR (**Table 5**). The interactions of the ligands with the receptors (c-MET/EGFR/PI3K-mTOR) predominantly involved hydrogen bonds, hydrophobic interactions, various π-interactions, and Van der Waals forces (**Figure 9**; **Supplementary Figure 3**; **Tables 5**),

DISCUSSION

Despite the availability of various treatment strategies, CRC remains among the top ranked most diagnosed and leading causes of cancer deaths globally (Sung et al., 2021). In order to achieve a reasonable shift in the global burden of CRC, the importance of diagnostic and prognostic biomarkers cannot be overemphasized. In the present study, our differential expression and survival analyses indicated that c-MET/EGFR can be an effective biomarker for CRC progression, immune infiltration, CAF infiltration, and poor prognoses, and thus can serve as attractive targets for exploring the development of chemotherapeutic agents against CRC. We found that c-MET and EGFR expression levels were independent biomarkers for clinical prognosis, and their high expressions were correlated with tumor progression and a poor prognosis of CRC. Abnormal expressions of c-MET and EGFR were reported in various human cancers where they support the proliferation and survival of cancer cells (Nicholson et al., 1990; Normanno et al., 2006; Zhang et al., 2018; Chakraborty et al., 2019; Fujiwara et al., 2020; Lee et al., 2020). c-MET and EGFR regulate the synthesis and secretion of several angiogenic growth factors, including basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and interleukin (IL)-8, in tumor cells (De Luca et al., 2008). Taken together, these findings suggest that the c-MET/EGFR system is an important mediator within the tumor microenvironment (TME) that results in enhanced tumor growth.

Furthermore, we explored TCGA clinical database to evaluate the frequency and prognostic consequences of c-MET/EGFR genetic alterations in CRC. We found that 3.0 and 4% of the cohorts that respectively harbored genetically altered EGFR and MET had poor prognoses and shorter survival times compared to cohorts without c-MET/EGFR alterations. Genetic alterations of c-MET and EGFR were previously identified and implicated in the progression of several cancers (Bastien et al., 2015; Piccirillo et al., 2015; Yamaura et al., 2020) and were also implicated in the resistance of most cancers to any generation of EGFR-TKIs including the latest third-generation series (Paez et al., 2004; Pao et al., 2005; Guo et al., 2020a). Of importance, we

TABLE 4 | NCI investigational drugs and molecular targets correlation with anticancer fingerprint of NSC777205 and NSC777207.

Rank	Molecular targets			Investigational drug			
	P	CCLC	Target	*P*	CCLC	Targets	Mechanism of action
				NSC777205			
1	0.35	51	IGHMBP2	0.55	58	PI-103 HCL	PIK3CB inhibitor
2	0.34	55	CG2572	0.50	57	S1038	PI3K inhibitor
3	0.33	56	GRM3	0.48	58	FH535	Wnt/β-catenin inhibitor
4	0.31	57	CDK6	0.48	57	PALOMID-529	TORC1/TORC2 inhibitor
5	0.3	57	MET	0.44	57	BMS986195	inhibitor of BTK,
6	0.31	57	RPS6KB1	0.43	58	CC115	mTOR/PI3K inhibitor
7	0.28	56	CTTN	0.43	55	PNU-74654	CTNNB1-catenin beta 1 inhibitor
8	0.27	54	MOS	0.42	58	CS-1202	PIK3CA inhibitor
9	0.24	57	EGFR	0.41	58	PP242	mTOR inhibitor
10	0.24	54	SLC25A13	0.41	57	TENALISIB	PI3K inhibitor
11	0.24	45	CG2460	0.32	56	TELATINIB	VEGFR/PDGFα/c-Kit Inhibitor
12	0.22	52	PIK3R2	0.32	58	AMG-458 (C-MET)	Met (c-Met) kinase inhibitor
13	0.21	56	ERBB2	0.31	57	JNJ38877618	Met (c-Met) kinase inhibitor
14	0.20	56	TGFB2	0.31	57	TAS120	FGFR Inhibitor
15	0.19	57	CCND1	0.3	58	MTX211	EGFR Inhibitor
				NSC777207			
1	0.43	52	IGHMBP2	0.61	56	ROGARATINIB	FGFRs
2	0.36	56	CTTN	0.53	58	CC115	mTOR inhibitor
3	0.31	57	FGF3	0.52	58	CEP-32496	MEK
4	0.3	57	CCND1	0.49	58	GDC-0349	mTOR/MEK
5	0.24	55	CG2572	0.49	55	ASP-5878	FGFR
6	0.23	57	CDK6	0.49	56	NVP-BEZ235	PI3K/mTOR
7	0.21	57	MET	0.48	58	ALTIRATINIB	c-Met/HGFR inhibitor
8	0.18	57	RPS6KB1	0.47	58	10058-F4	c-Myc inhibitor
9	0.18	55	HBE1	0.46	58	PP242	mTOR/RET
10	0.14	57	BRCA2	0.46	57	VOXTALISIB	PI3K/mTOR Inhibitor
11	0.13	56	TGFB2	0.42	58	NVP-BGT226	PI3K/mTOR
12	0.13	57	EGFR	0.41	58	TAS-6417	EGFR
13	0.13	57	MYCN	0.4	57	PWT33597	PI3K/mTOR
14	0.12	51	RARB	0.38	58	SGX-523	c-Met
15	0.12	52	PIK3R2	0.38	58	MTX211	EGFR

P, Pearson's correlation coefficient; CCLC, Common cell lines count; MW, molecular weight.

analyzed concurrent gene alterations with c-MET/EGFR genetic alterations, and found that EGFR alteration co-occurred with genetic alterations of *SMARCD2*, *TRIM7*, *MCMDC2*, *HNRNPUL1*, *BEST3*, *GRB10*, *PDE6C*, and *SNX19*, while alterations of *SLC22A2*, *DST*, *AKT3*, and *TAB2* co-occurred with c-MET alterations in CRC. Genetic alterations and concurrent alterations of c-MET/EGFR with other genetic alterations have been well reported in lung cancer (Stuart and Sellers, 2009; Tang et al., 2018; Guo et al., 2020b; Gini et al., 2020), but were underreported in other solid cancers especially CRC, and hence our study is significant in this regard. A recent study reported concurrent alterations of p53 (60–65%), RTKs (5–10%), PIK3CA/KRAS (3–23%), Wnt (5–10%), and cell cycle pathways MYC (7–25%) and NKX2-1 (10–15%) in EGFR-altered cohorts (Gini et al., 2020). Another more-recent study also identified concurrent genetic alterations of *EGFE* with *MEK*, *ALK*, *KRAS*, *ROS1*, *TP53*, *PIK3CA*, and *PTEN* in NSCLC (Guo et al., 2020b), while Tang et al. (Tang et al., 2018) reported coexistent genetic alterations involving *ALK*, *RET*, *ROS1*, and *MET* in 15 cases of lung adenocarcinoma (Tang et al., 2018). In addition to concurrent gene alterations in CRC patients, we found that the genetic alteration of EGFR was associated with overexpression of mRNA levels of TRIM7, BARX2, CFAP43, TPBG, HPSE, GNB5, MARK3, FUT8-AS1, SS18 and KCNK1, while genetic alteration of MET was associated with the overexpression of mRNA levels of *SMAD4*, *MBP*, *IER3IP1*, *OXCT1*, *ALKBH5*, *C18ORF25*, *WDR76*, *TYMS*, *LINC00909*, and *GPR3* in CRC cohorts. We also found that these overexpressed mRNAs were enriched in several KEGG pathways including cell adherent junctions, central carbon metabolism, and EGFR TKI resistance, indicating a potential functional role in driving resistance to therapy. We explored the PPI network and pathways of these mRNAs from a different perspective, and we concluded that multiple components and multiple pathways led to the development and progression of CRC. However, we found that c-MET, ERBB2, and EGFR signaling pathways, MAPKK activation, and negative regulation of cellular responses to drugs appeared to play important roles in the development of CRC. Our findings are therefore relevant in predicting and classifying evolutionary trends of genetic alterations during therapy and could guide the selection of combination therapies appropriate for patients with resistance to TKIs in CRC.

TABLE 5 | Comparative docking profile of NSC777205, NSC777207 and clinical inhibitors of C-MET/EGFR/PI3K-mTOR.

	EGFR						c-MET					
	NSC777205	**DIS (Ă)**	**NSC777207**	**DIS (Ă)**	**Gefitinib**	**DIS (Ă)**	**NSC777205**	**DIS (Ă)**	**NSC777207**	**DIS (Ă)**	**Crizotinib**	**DIS (Ă)**
ΔG = (Kcal/mol)	−6.90		−6.80		−6.80		−8.9		−8.9		−9.5	
No. hydrophobic contact	6		4		5		5		6		6	
ConventionalH-bond					MET793	2.32	ARG1227	2.62	ARG1227	2.60		
							ARG1227	2.57	ASP1164	2.75		
C-H bond	LYS846	3.47			ASP800	3.71					ARG1208	3.69
					GLN791	3.50						
Halogen bond	ASP1012	3.56			ASP855	3.09	GLY1163	3.65				
π -anion	ASP1012	3.30					ASP1164	3.58	ASP1164	3.18	ASP1164	3.26
π -cation									ARG1227		ARG1227	
π –sulfur							MET1211					
π -alkyl	LEU792		PRO741		ALA743		VAL1092		LEU1157		ALA1108	
	PRO741		VAL1010		VAL726		ALA1226		VAL1092			
									LYS1110			
									ALA1108		LEU1157	
	PRO794		PRO794						ARG1208		LYS1110	
									TYR1234			
π -π T-shaped	PHE997											
π -π stacked							PHE1089		PHE1223		PHE1089	
							PHE1223					
π-sigma			VAL101		LEU844				MEY1211		PHE1223	
			0		LEU718						VAL1092	
Van der waal forces	VAL1010		VAL101		GLY719		LS1110		ASN1167		ARG1166	
			1ASP10		LYS745				PHE1089		MET1211	
			12		PHE723				GLY1163		ALA1226	
			PHE997,		MET790							
			LEU792		ARG841							
			LYS728		ASN842							
	ASN996		ASN996		THR854							
					LEU792							
					GLY796							
					PRO794							
					CYS797							
	LYS728										GLY1085	
	GLU1015										ILE1084	
	THR847										TYR1234	
Hydrophobic Interactions	PRO741	3.64	PRO794	3.76	LEU718	3.77	PHE1089,	3.75	PHE1089,	3.92	PHE1089	3.99
	, LEU792	3.72	PRO794	3.68	, LEU718	3.70	PHE1089,	3.49	VAL1092	3.64	VAL1092	3.93
	PRO794,	3.45	VAL101	3.60	, LEU718	3.90	VAL1092	3.12	VAL1092	3.92	ALA1108	3.83
	PHE997	3.89	0	3.48	VAL726	3.59	PHE1223	3.70	LYS1110	3.86	LYS1110	3.58
	PHE997,	3.68	ASP101		, VAL726	3.59	ALA1226	3.90	LEU1157	3.56	LEU1157	3.75
	ASP10121	3.48	2						PHE1223	3.69	PHE1223	3.96
	PI3K						mTOR					
	NSC777205_c-pi3k	DIS (Ă)	NSC777207_c-pi3k	DIS (Ă)	COPANLISIB	DIS (Ă)	NSC777205_mTOR	DIS (Ă)	NSC777207_mTOR	DIS (Ă)	DACTOLISIB	
ΔG=(Kcal/mol)	−8.6		−7.9		−9.0		−7.1		−7.6		−9.2	
No. hydrophobic contact	2		5		2		4		3		4	

(Continued on following page)

TABLE 5 | (*Continued*) Comparative docking profile of NSC777205, NSC777207 and clinical inhibitors of C-MET/EGFR/PI3K-mTOR.

	EGFR						c-MET					
	NSC777205	**DIS (Ă)**	**NSC777207**	**DIS (Ă)**	**Gefitinib**	**DIS (Ă)**	**NSC777205**	**DIS (Ă)**	**NSC777207**	**DIS (Ă)**	**Crizotinib**	**DIS (Ă)**
ConventionalH-bond	ARG690	2.40			ARG690	2.61	ARG2224	2.12	ARG2224	2.22	GLN1937	2.19
	ARG690	2.52										
	ARG849	3.36			CYS869	2.28	GLN2200	2.86			ARG2224	2.35
	ARG849	2.94										
C-H bond	GLY868	3.23			TYR787	3.50			GLN2200	3.21		
					GLU852	3.63						
Halogen bond	TYR787	3.01	ASP788	3.45			GLU2196	3.54	GLN2200	3.45		
	ASP788	3.58	TYR787	3.04								
π -anion	GLU880		GLU880								ASP2145	4.82
π -cation	ARG849	3.77	ARG849	3.70	ARG849	3.20						
			ARG690									
π -alkyl	LEU657		PHE698		PRO866		ILE1939		LEU1900		PRO2146	
			LEU657		LYS298		PRO1940		LEU2204		LEU1900	
			LEU660						PRO1940		LEU2204	
π -π T-shaped					TRP201							
π -π stacked	PHE694	3.92	PHE694									
Amide-π stacked											LEU1936	
Van der waal forces	GLN846		GLN846		ASP861		THR2207		GLU2196		ALA1971	
	PHE698		TRP201		HIS295		LEU2204		MET2199		ILE1939	
	CYS869		GLY868		GLY868		LEU1900		GLY2203		GLU2196	
	PRO789		PRO789		MET842		GLY2203		THR2207		MET2199	
	TYR867				ILE870				GLN1937		GLN2200	
	PRO866				PHE898						GLY2203	
	TRP201				GLN846						THR2207	
					LEU865						ASN2147	
					LEU864							
					ASN299							
Hydrophobic Interactions	LEU657	3.46	LEU657	3.43	LYS298	3.88	LEU1900	3.75	LEU1900	3.66	LEU1900	3.31
	PHE698	3.83	LEU660	3.72	LEU657	3.84	GLN1937	3.75	GLN1937	3.44	PRO2146	3.63
			PHE694	3.60			PRO1940	3.73	GLN2200	3.98	GLU2196	3.97
			PHE694	3.57			GLN2200	4.00			GLN2200	3.77
			PHE698	3.62								

π –sulfur, π-electron cloud between the Aromatic rings of ligands and lone pair of electron cloud of sulfur atom in the receptors; π -π stacked, π-electron cloud between the Aromatic rings; π -π T-shaped, T shaped π-electron cloud between the Aromatic rings; π –alkyl, π-electron cloud between the Aromatic ring of ligand and alkyl group of ligand; **Ă**, *Angstrom.*

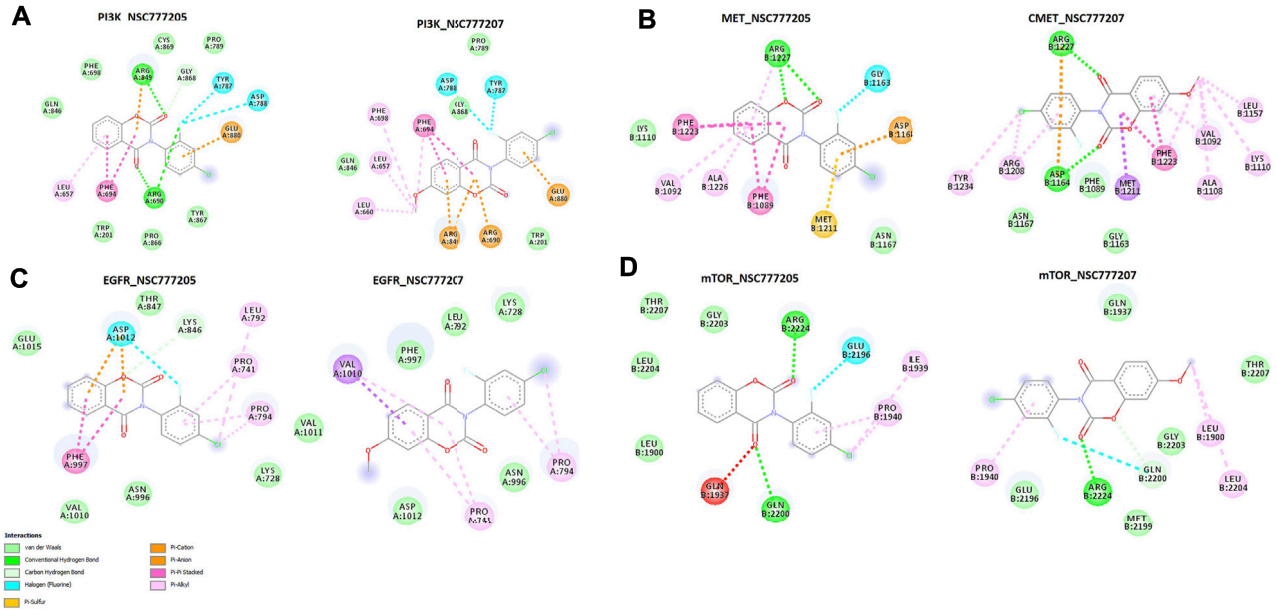


FIGURE 9 | The two dimensional (2D) representations of the docking profiles of NSC777205 and NSC777207 with **(A)** PI3K (PDB:3APC), **(B)** c-MET (PDB: 3RHK), **(C)** EGFR (PDB: 5EDP), and **(D)** mTOR (PDB: 5FLC).

The TME is a complex ecological niche that plays important roles in tumor growth and progression. The TME consists of several components including tumor cells and adjacent non-tumor cells, such as infiltrating immune cells, fibroblasts, signaling molecules, and the extracellular matrix (ECM) (Azizi et al., 2018; Pearce et al., 2018; Zou et al., 2020). Tumor-infiltrating immune cells are well associated with angiogenesis, tumorigenesis, and metastasis of tumor cells, which can in turn regulate the quantity and subsets of immune cells (Taddei et al., 2013). Oncogenic signaling molecules are known to be drivers of immune infiltration and immunotherapeutic responses (Lawal et al., 2021a). Consequently, the present study revealed associations of c-MET/EGFR expressions with infiltration of various immune cells in CRC and predicted poor survival of the cohorts. We also found that both genetic and epigenetic alterations of c-MET/EGFR were associated with immune infiltration, dysfunctional T-cell phenotypes, and poor prognoses of CRC patients, hence adding another layer of prognostic relevance of c-MET/EGFR in CRC.

CAFs are apoptotic-resistant fibroblasts that promote tumorigenic and metastatic properties by secreting cytokines and remodeling the ECM (Erez et al., 2010; Giannoni et al., 2010). We found that associations between c-MET/EGFR expression profiles and CAF infiltration resulted in low cumulative survival of CRC cohorts. These findings could be attributed to the fact that oncogenes induce the CAF phenotype (Lisanti et al., 2013), which in turn inhibits T-cell expansion by mediating the hyper-expression of immune checkpoint molecules in the TME, consequently leading to T-cell allergies (Harryvan et al., 2019), immune therapy failure, aggressive tumor growth, and high death rates in those cohorts. Coherent with our results, a previous study reported that CAFs promote EGFR-TKI resistance via c-MET/IGF-1/ANXA2 signaling in NSCLC (Yi et al., 2018). Another study also reported that CAFs regulate the plasticity of tumor-initiating cells in liver cancer through c-Met/FRA1/HEY1 signaling (Lau et al., 2016), while Lau et al. (2017) reported that CAFs promote the metastasis of ovarian cancer via tumor necrosis factor (TNF)-α/transforming growth factor (TGF)-α/EGFR signaling. Altogether, our study suggested that c-MET/EGFR play important roles in CAF-mediated tumorigenesis and host immune responses to CRC. It is therefore reasonable to conclude that c-MET/EGFR are important biomarker signatures of tumorigenesis, tumor immune invasion, and poor prognoses, and thus can serve as attractive targets for the development of immune/chemotherapeutic strategies against CRC. In addition, our study may be clinically useful in prognosis assessment and follow-up management of immunotherapy of CRC. Immunotherapies are more effective and promising for some tumor patients than others, and our study therefore suggested that c-MET/EGFR could be an important contributor to those less sensitive to CRC immunotherapy compared to other cancer types as reported in a previous study (Banerjea et al., 2005), hence providing a rationale for a combination of c-MET/EGFR antagonists and immunotherapy in CRC. From another perspective, since EGFR aberrations co-occurred with other genetic assaults, developing a potent c-MET/EGFR inhibitor with multi-target potential capable of inhibiting multiple pathways with minimal side effects may open up new windows for long-lasting, multilayer control of CRC. Hence we evaluated NSC777205 and NSC777207, gefitinib-inspired small molecules derivatives of niclosamine, for that purpose.

On the basis of PK studies during the early stages of drug discovery possibly reducing attrition rates (Kola and Landis, 2004), we evaluated NSC777205 and NSC777207 for *in silico* drug-likeness and ADME profiles, and found that

both NSC777205 and NSC777207 possessed good drug-likeness properties and obeyed Lipinski's rule-of-five, a physicochemical-based rule set to evaluate drug candidates for suitability as oral remedy (Pollastri, 2010). The number of rotatable bonds is important in the stereoselectivity of drug molecules for optimal binding with target receptor molecules, while the topological polar surface area (TPSA) and molar refractivity are important for a drug's transport and biodistribution (Veber et al., 2002; Prasanna and Doerksen, 2009). Values of the TPSA, molar refractivity, and other PK parameters observed for NSC777205 and NSC777207 were within the permissible range of a good drug molecule.

The overall analysis of drug-likeness studies strongly suggested that NSC777205 and NSC777207 possessed good drug-likeness properties for optimal drug transport, bioavailability, intestinal availability, membrane permeability, and eventual interactions with receptor molecules and bioactivity. Interestingly both NSC777205 and NSC777207 also demonstrated *in silico* cytotoxic activities against colon cancer, brain, lung, and bone cancer cell lines with NSC777205 demonstrating higher activity (Pa = 0.31–0.658) than NSC777207 (Pa = 0.303–0.565). BBB penetration is an important limiting factor in the use of chemotherapy for treating glioblastomas and other CNS-associated diseases (Moretti et al., 2015). Interestingly, our *in silico* study suggested that both NSC777205 and NSC777207 had good BBB permeation ability, with NSC777205 demonstrating about 2-fold higher ability than NSC777207. Notably, the synthetic accessibilities of these compounds were lower than 3.5, suggesting that they can relatively easily be synthesized. The overall analysis indicated that NSC777205 demonstrated more-favorable drug likeness, ADMET, and cytotoxic properties than NSC777207, thus suggesting better therapeutic prospects of the former than the latter. Considering these promising PK, ADMET, and biological properties, we synthesized these compound by adapting and optimizing our previously established protocols (Chen et al., 2014) and evaluated their anticancer activities against the NCI-60 human tumor cell lines.

To our delight, the *in vitro* anticancer study revealed that both NSC777205 and NSC777207 exhibited wide spectra of antiproliferative activities with GI_{50} values of <6 μM against NCI-60 cell line panels with selective cytotoxic preferences for melanoma, renal, CNS, colon, and NSCLC cell lines, suggesting that the compounds were not generally toxic to growing cells but were selective for melanoma, renal, CNS, colon, and NSCLC cells. This observation corroborates with the *in silico* structure-activity-related cytotoxic predictions for NSC777205 and NSC777207 against cancer cell lines, which indicated that both compounds exhibited cytotoxic activities against colon (HCT-116 and RKO) brain (Hs683), lung (A549) and bone cancer (SJSA-1) cell lines with NSC777205 demonstrating higher activity (Pa = 0.31–0.658) than NSC777207 (Pa = 0.303–0.565). Of importance, among all cell lines, colon cancer cell lines stood out uniquely with all seven cell lines demonstrating strict antiproliferative sensitivity and no cytotoxic response to single-dose (10 μM) treatment of either NSC777205 or NSC777207. This suggests that NSC777205 and NSC777207 would be very useful in growth inhibition and treatment of CRC. Tumors often develop resistance to a wide range of anticancer drugs. Therefore, drug resistance represents an important problem in cancer chemotherapy (Baguley, 2010; Alfarouk et al., 2015). Intriguingly, NSC777205 and NSC777207 demonstrated higher anticancer activities on multidrug resistant NC1-60 human tumor cell lines including the SK-OV-3, OVCAR-3 OVCAR-4, and NCI/ADR-RES cell lines than the corresponding naïve cell lines of the same origin (OVCAR-8 and IGROV1). These cell lines have been well profiled and established to be resistant to multiple clinical drugs (Louie et al., 1985; Lorenzi et al., 2009). It is therefore plausible that since NSC777205 and NSC777207 demonstrated promising activity against these cell lines, they may be useful in treating multidrug-resistant cancers. Furthermore, among the NCI-60 cell lines investigated in this study, the leukemia RPMI-8226 and melanoma SK-MEL-28 cell lines were identified to harbor EGFR mutations which led to their resistance to 12 TKIs, including erlotinib (Ikediobi et al., 2006; Liu et al., 2007). It is therefore noteworthy that both NSC777205 and NSC777207 exhibited high antiproliferative activity against these cell lines, hence suggesting their applicability in treating TKI-resistant tumors. In line with the *in silico* cytotoxic activities and ADMET properties, our *in vitro* anticancer study also indicated that NSC777205 had higher anticancer activities than NSC777207 against the nine cancer types evaluated. These higher activities of NSC777205 could therefore be attributed to its estimated higher BBB permeation and favorable ADME and physicochemical properties that give it better stability and a greater ability to interact with targets than NSC777207. Hence, we evaluated the possible mechanistic properties of these compounds via an *in silico* study and molecular docking simulations of ligand-receptor interactions.

Identification of molecular targets is an important aspect of developing novel small molecules for chemotherapeutic purposes, and a number of computational tools that have been developed and widely employed for these purposes have had translational success in preclinical and clinical practice. However, due to differences in embedded principles for target identification by these tools and also the vast diversity of targets, it is recommended to use multiple computational tools and identify common targets predicted for certain drug candidates (frontiers). In compliance with this recommendation, we used four computational tools including the COMPARE algorithm developed by the USA NCI to identify the most probable targets for NSC7772015 and NSC777207. In the COMPARE analysis, the anticancer fingerprints of NSC777205 and NSC777207 corresponded to anticancer fingerprints of known inhibitors of c-MET/EGFR/mTOR/PI3K pathways. Furthermore, the most highly correlated NCI synthetic compound (p = 0.78) was 3-(2-fluoro-4-iodophenyl)-7-methoxy-2H-benzo[e](Fidler et al., 2016; Sung et al., 2021) oxazine-2,4(3H)-dione, whose detailed mechanistic targets were reported to be PI3K, AKT, mTOR, MEK, and EGFR (Lawal et al., 2021c). In addition, N-(2,4-difluorophenyl)-2',

4'-difluoro-4-hydroxybiphenyl-3-carboxamide and (4-(6-(2,4-difluorophenyl)-2,4-dioxo-2H-benzo[e] (Fidler et al., 2016; Sung et al., 2021)-oxazin-3(4H)-yl) benzonitrile which also showed significant correlations with anticancer fingerprints of NSC777205 and NSC777207 were mechanistically reported, and signal transducer and activator of transcription 3 (STAT3)/cyclin-dependent kinases (CDKs)/PIK3CB/EGFR were identified among other targets (Lawal et al., 2021b). c-MET phosphorylates and triggers intracellular signaling cascades that lead to activation of key molecules such as the extracellular signal-regulated kinase 1/2 (ERK1/2), PI3K/AKT signaling, and STAT3 (Organ and Tsao, 2011; Wood et al., 2021). It is therefore plausible that the COMPARE analysis inferred that both NSC777205 and NSC777207 may also alter the expression capacity of STAT3 in addition to the promising mechanistic target of the c-MET/EGFR/PI3K/mTOR pathways. Collectively, we found that c-MET/EGFR/P13K/mTOR satisfied the target criteria set by the four tools we used. These tools hence suggested that c-MET/EGFR/P13K/mTOR are the most probable targets and mechanisms of action of NSC7772015 and NSC7772017.

Molecular docking aids the identification of drug lead molecules by unraveling possible interactions between a drug candidate and protein targets, providing structure-activity relationships and therapeutic mechanisms of drug candidates prior to *in vitro* or *in vivo* validation. Non-covalent interactions such as hydrogen bonds, hydrophobic, ionic, and π-interactions, and van der Waals forces occurring between ligands and receptors are important for stabilization of a ligand within the binding cavity of a receptor (Zhao and Huang, 2011). Our docking analysis revealed that NSC777205 and NSC777207 docked well into the binding cavities of c-MET, EGFR, mTOR, and P13K by various non-covalent interactions and with binding affinities ranging -6.8~-8.9 kcal/mol. These collective interactions would lead to strong stabilization of NSC777205 and NSC777207 in receptor cavities, which could lead to competitive or non-competitive alterations in the normal expressions and functional properties of the proteins, thus compromising the sustenance and survival of the oncogenic pathways that depend on the proteins for their hyperactivity. Hydrophobic interactions play important roles in the formation of ligand-receptor complexes. Although NSC777205 and NSC777207 shared common interacting residues with the receptors, in most cases, NSC777205 demonstrated more-robust and a higher number of hydrogen bonds, hydrophobic contacts, and van der Waals forces with the receptors than did NSC777207. These higher hydrophobic interactions would enhance the stabilization of the energetically favored NSC777205 in an open conformation surrounding the receptors and further increase its biological activity (Patil et al., 2010; Lawal et al., 2021c). In addition, the higher van der Waals forces would create a strong cohesive environment, that further stabilizes the complex (Arthur and Uzairu, 2019). Both NSC777205 and NSC777207 demonstrated similar tendencies for inhibiting c-MET and EGFR as revealed by the most common interacting residues and similar binding affinities to the binding sites of c-MET and EGFR; however, NSC777205 had higher hydrophobic contacts, H-bonds, halogens, and π-interactions with EGFR than did NSC777207. In addition, the higher number of H-bonds and van der Waals forces around the ligand backbone could be responsible for the higher affinity that NSC777205 had for PI3K than did NSC777207.

Both NSC777205 and NSC777207 exhibited high similarity with gefitinib and crizotinib, known clinical EGFR and c-MET inhibitors, respectively, in terms of common interacting residues with EGFR and c-MET and similar binding affinities with EGFR, suggesting that both NSC777205 and NSC777207 could be as promising as gefitinib and crizotinib at inhibiting EGFR and c-MET, serving as potential dual targets and thus superseding the strength of individual clinical drugs in arresting cancer proliferation and aggressiveness for which EGFR and c-MET are implicated. This multi-target potency of NSC777205 and NSC777207 could be attributed to the observed activities of NSC777205 and NSC777207 against NCI cell lines that are known to be multi-drug resistant including those that are resistant to more than 10 panels of TKIs (Liu et al., 2007).

Collectively, our study revealed that structural hybridization strategies yielded novel and potential c-MET/EGFR/PI3K/mTOR multi-target compounds, NSC777205 and NSC777205, with promising prospects for treating melanoma, renal, CNS, colon, and NSCLC cell lines. Although NSC777205 and NSC777207 share common interacting residues with the receptors, the overall structure-activity relationship study revealed that the addition of an $-OCH_3$ group to the salicylic core of NSC777207 was not favorable, as the added moiety did not contribute significant interactions with the receptor-binding residues and also led to overall less-favorable ADMET, physicochemical, and drug-likeness properties and weaker anticancer activities against the panel of NCI cell lines compared to the properties and activities demonstrated by NSC777205 that has no $-OCH_3$ substituent group. However, further *in vitro* and *in vivo* analyses in tumor-bearing mice are ongoing in our lab to support this claim and to unravel the full therapeutic efficacies of NSC777205 and 777207 in CRC.

CONCLUSION

In conclusion, our results indicated that, c-Met/EGFR are important biomarker signatures of cancer-associated fibroblasts and tumor immune infiltration, and are of clinical relevance in colorectal cancer. Genetic and epigenetic alterations of c-MET/EGFR were associated with dysfunctional T-cell phenotypes, and poor prognoses of the cohorts. c-MET/EGFR/PI3K/mTOR are therapeutic targets for NSC777205 and NSC777207 with consequent selective cytotoxic preferences for melanoma, renal, CNS, colon, and NSCLC cell lines. However, the

removal of an $-OCH_3$ substituent group from the salicylic core of NSC777205 was responsible for more highly favorable drug-likeness properties and anticancer activities, and more-robust interactions with c-MET/EGFR/PI3K/mTOR than those of NSC777207.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

BL wrote the manuscript; helped Y-CW, with data collection and analyses; H-SH synthesized and provided NSC777205 and NSC777207; Y-CW, AW and H-SH designed and oversaw the study. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.691234/full#supplementary-material

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PERSPECTIVE
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Perspective of Human Condensins Involved in Colorectal Cancer

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Although many important roles are played by human condesins in condensation and segregation of mitotic chromosomes, what roles of human condensins play in colorectal cancer are still unclear at present. Recently, abnormal expressions of all eight subunits of human condensins have been found in colorectal cancer and they are expected to become potential biomarkers and therapeutic targets for colorectal cancer in the future. However, there are still no reviews on the significance of abnormal expression of human condensin subunits and colorectal cancer until now. Based on a brief introduction to the discovery and composition of human condensins, the review summarized all abnormally expressed human subunits found in colorectal cancer based on publicly published papers. Moreover, Perspective of application on abnormally expressed human subunits in colorectal cancer is further reviewed.

Keywords: condensin, human condensin, structure of chromosomes protein, abnormal expression, colorecal cancer

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INTRODUCTION

Nowadays colorectal cancer (CRC) is the second leading cause of cancer mortality and the third most commonly diagnosed cancer worldwide (Bray et al., 2018). With the rapid development of the economy, life expectancy correlated positively with the incidence and mortality of CRC both in men and women in China (Gu et al., 2018). Although there has been a clear treatment method for the early colorectal cancer with general surgery and adjuvant chemotherapy, many colorectal cancer patients are in the late stage when they were firstly identified because of the lack of effective biomarkers in early screening of colorectal cancer and they are hard to cure. Therefore, it is of great significance to strengthen the study of molecular mechanism of colorectal cancer and develop effective biomarkers and therapeutic targets for early screening and diagnosis of colorectal cancer. The identification of abnormal expression proteins of colon cancer is pivotal for early detection of colorectal cancer and effective development of biomarkers and therapeutic targets.

Recently, all eight human condensin subunits have been found abnormally expressed in colorectal cancer and they are expected to become potential biomarkers and therapeutic targets for colorectal cancer in the future (Dávalos et al., 2012; Shiheido et al., 2012; Tokunaga et al., 2013; Feng et al., 2014; Je et al., 2014; Jinushi et al., 2014; Yin et al., 2017; Baergen et al., 2019; Montero et al., 2020; Yeh et al., 2020). Therefore, based on the brief introduction of the discovery and composition of human

Abbreviations: CRC, colorectal cancer; SMC, structure maintenance of chromoses; hCAP, human chromosome associate polypeptide; NCAP, non-SMC condensin I complex subunit; MSI-H, high microsatellite instability; GRID2, glutamate ionotropic receptor delta type subunit 2; CBLN1, cerebellin 1 precursor; POU2F1, TF POU class 2 homeobox 1; HPGDS, hematopoietic prostaglandin D synthase.

TABLE 1 | All eight subunits of human condensins involved in colorectal cancer.

Human condensin subunits involved in colorectal cancer	Mutated gene/protein expression	Number of related literature and published year
hCAP-G2(NCAPG2)	unclear/expression of hCAP-G2 is affected by binding of Q15 or Q15 and MIP-2A	Shiheido et al., 2012 Tokunaga et al., 2013
All eight human condensin subunits	gene copy number alterations include deletions, small-scale gains and/or large-scale amplifications/reduced expression	Baergen et al., 2019
SMC2(hCAP-E)	unclear/overexpression frameshift mutation/null not mutated/reduced expression unclear/unclear	Dávalos et al., 2012 Je et al., 2014 Montero et al., 2020 Yeh et al., 2020
SMC4(hCAP-C)	unclear/overexpression unclear/overexpression unclear/overexpression unclear/upregulated	Dávalos et al., 2012 Feng et al., 2014 Jinushi et al., 2014 Zhang et al., 2021
hCAP-H(NCAPH)	missense and frameshift mutation/overexpression	Yin et al., 2017

condensins, this paper reviews the research progress and application perspective of all eight human condensin subunits in colorectal cancer to offer beneficial reference for related research.

Discovery and Composition of Human Condensins

In 2001, human condensin I was firstly purified based on the study of human ortholog of frog condensin from HeLa nuclear extracts (Hirano and Mitchison, 1994; Schmiesing et al., 2000; Kimura et al., 2001). In 2003, Ono et al. (2003) discovered another condensin complex in HeLa nuclear extracts and the two kinds of condesins in human cells are termed as condensin I and II respectively (Kimura et al., 2001).

Both of human condensins are composed of five evolutionally conserved subunits. SMC2 (also named as human chromosome associate polypeptide E, hCAP-E) and SMC4(hCAP-C)are formed heterodimer and shared by the two kinds of human condesins (Schmiesing et al., 1998; Kimura et al., 2001; Ono et al., 2003). The other three non-SMC subunits are hCAP-D2 (NCAPD2), hCAP-H (NCAPH) and hCAP-G (NCAPG) in human condensin I and hCAP-D3 (NCAPD3), hCAP-H2 (NCAPH2) and hCAP-G2 (NCAPG2) in human condensin II (Kimura et al., 2001; Ono et al., 2003). Although the two kinds of human condensins have similar composition and alphabetic structure, they play different roles in the chromosome dynamics during the cell cycle (Hirota et al., 2004; Ono et al., 2004; Onn et al., 2007).

All Eight Subunits of Human Condensins are Abnormally Expressed in Colorectal Cancer

All eight subunits of human condensins have been reported to be abnormally expressed in colorectal cancer until now, as shown in **Table 1** (Dávalos et al., 2012; Shiheido et al., 2012; Tokunaga et al., 2013; Feng et al., 2014; Je et al., 2014; Jinushi et al., 2014; Yin et al., 2017; Baergen et al., 2019; Montero et al., 2020; Yeh et al., 2020; Zhang et al., 2021).

Firstly, expression of hCAP-G2, a non-SMC subunit of human codensin II, is affected by binding of Q15 (an anilinoquinazoline derivatives) and inhibiting proliferation of multiple cultured colorectal cancer cell lines (Shiheido et al., 2012). Furthermore, Q15 can also binds to MIP-2A (MBP-1 interacting protein-2A) and simultaneous targeting of hCAP-G2 and MIP-2A may be a promising strategy for the treatment of intractable colorectal tumors (Tokunaga et al., 2013).

Secondly, hypomorphic expression or no expression of SMC2 (hCAP-E) is implicated in cancer pathogenesis. Concretely, SMC2 gene is frameshift mutated because of mononucleotide repeats and this causes loss of its expression in colorectal cancer with high microsatellite instability (MSI-H) (Je et al., 2014). Likewise, reduced expression of all condensin genes may drive chromosome instability (CIN) and may contribute to colorectal cancer pathogenesis (Baergen et al., 2019). Consistent with this, inhibition of tumorsphere formation in colon cancer cell lines was observed recently when SMC2 was inhibited by intracellular delivery of specific anti-SMC2 antibodies (Ab-SMC2) alone or with anticancer drug by polymeric micelles (PM) (Montero et al., 2020). On the other hand, overexpression of SMC2 has also been found in both colorectal cancer cell lines and samples from CRC patients (Dávalos et al., 2012). The difference between overexpression and low expression of SMC2 may because that it is regulated by different signaling pathways. SMC2 promoter could be driven by WNT signaling and it cause SMC2 abnormal expression correlated with β-catenin levels in CRC cell lines and clinical samples (Dávalos et al., 2012). Similarly, it is reported that the receptor glutamate ionotropic receptor delta type subunit 2 (GRID2) binds ligand cerebellin 1 precursor (CBLN1) to transmit the signal to TF POU class 2 homeobox 1 (POU2F1) through SMC2 (Yeh et al., 2020). This signal upregulates the target gene hematopoietic prostaglandin D synthase (HPGDS) and inhibits the migration and proliferation of CRC cells (Tippin et al., 2012).

These results mentioned above suggest that dysfunction of SMC2 is involved in pathogenesis of colorectal cancer.

Thirdly, SMC4 also shows dysregulations in colorectal cancer cells. For example, overexpression of SMC4 was found in both colorectal cancer cell lines and samples from CRC patients and downregulation of SMC4 plays a suppressive role in the proliferation of colorectal cancer cells and tumor growth (Dávalos et al., 2012; Feng et al., 2014). In addition, it is demonstrated that expression of SMC4 mRNA is downregulated by miR-124-5p (also known as miR-124*) and growth of human colon adenocarcinoma cell is inhibited. Therefore low expression levels of microRNA-124-5p and overexpression of SMC4 is correlated with poor prognosis in colorectal cancer (Jinushi et al., 2014). Consistent with this, recently Zhang et al. (2021) finally identified two molecular subtypes that had been named C1 and C2 based on the cell cycle-related genes in patients with colon cancer and SMC4 were identified to be one of fifty upregulated genes as markers for C2 subtypes.

Finally, NCAPH is overexpressed in colorectal cancer cell lines comparing with normal human colonic epithelial cells. Many NCAPH mutations include missense and frameshift are identified in colorectal cancer patients (Yin et al., 2017). Of note, contrast to SMC4, the patients with NCAPH overexpression in colon cancerous tissues had a significantly better prognosis and survival rate than those with low-expression of NCAPH in tumor tissues.

CONCLUSION AND FUTURE PERSPECTIVES

Taken together, all eight human condensin genes have been found to be abnormally expressed in colorectal cancer until now. Although there are differences in different colorectal cancers with upregulation or downregulation of individual human condensin subunit, it just shows that colorectal cancer has complex signal transduction and genomic and transcriptional heterogeneity (Kyrochristos and Roukos, 2019).

At present, the identification of tumor specific targets or characteristics is of great significance for the treatment of cancer patients (Andre et al., 2014). The development of potential biomarkers to assist individualized therapy and drug discovery is becoming a new trend in the diagnosis and treatment of colorectal cancer (Zhai et al., 2017). The discovery of abnormal expression of human condensin subunits in colorectal cancer opens up a new way for the diagnosis and treatment of colorectal cancer. With the development of evidence-based colorectal cancer precision medicine, all eight condensin subunits could be potential biomarkers and therapeutic targets for colorectal cancer (Dávalos et al., 2012; Shiheido et al., 2012; Feng et al., 2014; Jinushi et al., 2014; Yin et al., 2017; Baergen et al., 2019; Yeh et al., 2020). However, the molecular mechanism of how they function in the process of tumorgenensis remains to be further explored in the future. There are three aspects worthy of attention about the future application research.

Firstly, the molecular mechanism of eight human condensin subunits involved in colorectal cancer needs further research. What roles each human condensin subunit plays in pathology of colorectal cancer are not very unclear. Whether the instability of chromosome is affected by the activity of the whole complex of human condensins or by some unknown signal transduction of each human condensin subunit is also unclear. Recently, it is reported that reduced condensin expression and function may be a significant, yet, underappreciated driver of colorectal cancer (Baergen et al., 2019). On the other hand, according to the idea that the human condensin subunits plays a role by affecting the function of the whole human condensin complex, Palou et al. (2018) proposed a cancer treatment hypothesis based on the overexpression of SMC4, a subunit of human condensin. SMC4 gene is often overexpressed in cancer cells, which exceeds the activity required for chromosome condensation in normal cells. Therefore, some inhibitors that inhibit the activity of condensin may kill cancer cells without damaging normal cells, and appropriately inhibiting the activity of ATPase of human condensin may selectively kill cancer cells without damaging normal cells.

Secondly, the microRNAs that regulate the expression of eight human condensin subunits in colorectal cancer need further research. MicroRNA can be developed as a biomarker for colorectal cancer (Ahmed, 2014; Qin and Liu, 2019; Farace et al., 2020). For example, mir-124-5p plays a role by regulating the expression of SMC4 target gene, which can be used as a biomarker in the diagnosis and treatment of colon cancer patients (Jinushi et al., 2014). The results suggest that microRNAs specifically target the other seven human condensin subunits may also be developed as potential biomarkers and therapeutic targets for colorectal cancer.

Finally, the interaction between the reported biomarkers of colorectal cancer and eight human condensin subunits in colorectal cancer need further research. Until now, multiple biomarkers for colorectal cancer have been found (Ye et al., 2016; Li et al., 2019; Erfani et al., 2020; Harvey et al., 2020; Huang et al., 2020; Lu et al., 2020). Nevertheless, it is unclear whether and/or how these biomarkers interact with the eight human condensin subunits in colorectal cancer.

Moreover, hCAP-D2(NCAPD2) and hCAP-D3(NCAPD3), non-SMC subunit of human condensin I or condensin II respectively, are overexpressed in patients with ulcerative colitis and the overexpression of these two proteins is regulated by IKK/NF-κB signal transduction pathway to promote the release of inflammatory cytokines (Yuan et al., 2019). Similarly, as mentioned above, SMC2 is regulated by WNT signaling and it cause SMC2 abnormal expression in CRC cell lines and clinical samples (Tokunaga et al., 2013). These studies suggested that individual human codensin subunit may have a different signal transduction pathway and the same human codensin subunit may be regulated by different signal transduction pathway in colorectal cancer and related disease.

All in one, it opens up a new way for the diagnosis and treatment of colorectal cancer from the point of exploring

novel function of human condensin subunits in colorectal cancer. Up to now, it is reported that all subunits of human condensins are involved in the tumorgenensis and can be potential biomarkers and therapeutic targets (Dávalos et al., 2012; Shiheido et al., 2012; Feng et al., 2014; Jinushi et al., 2014; Wang et al., 2018; Baergen et al., 2019; Xiao et al., 2020; Yeh et al., 2020; Zhang et al., 2020). With further research on the molecular mechanism of the eight human condensin subunits in colorectal cancer, more attention will be paid to the development and application of these subunits as potential biomarkers and therapeutic targets in the diagnosis and treatment of colorectal cancer in the foreseeable future.

AUTHOR CONTRIBUTIONS

HW and LM raised the concept and design of the research. HW wrote the manuscript. YC, DY, and LM revised the article for important details. All authors have approved the submitted version.

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Akkermansia Muciniphila Potentiates the Antitumor Efficacy of FOLFOX in Colon Cancer

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FOLFOX (oxaliplatin, fluorouracil and calcium folinate) is the first-line chemotherapy regimen for colon cancer therapy in the clinic. It provides superior efficacy than oxaliplatin alone, but the underlying mechanism remains unclear. In the present study, pharmacomicrobiomics integrated with metabolomics was conducted to uncover the role of the gut microbiome behind this. First, *in vivo* study demonstrated that FOLFOX exhibited better efficacy than oxaliplatin alone in colon cancer animal models. Second, 16S rDNA gene sequencing analysis showed that the abundance of *Akkermansia muciniphila* (*A. muciniphila*) remarkably increased in the FOLFOX treated individuals and positively correlated with the therapeutic effect. Third, further exploration confirmed *A. muciniphila* colonization significantly enhanced the anti-cancer efficacy of FOLFOX. Last, metabolomics analysis suggested dipeptides containing branched-chain amino acid (BCAA) might be responsible for gut bacteria mediated FOLFOX efficacy. In conclusion, our study revealed the key role of *A. muciniphila* in mediating FOLFOX efficacy, and manipulating *A. muciniphila* might serve as a novel strategy for colon cancer therapy.

Keywords: FOLFOX, oxaliplatin, colon cancer, Akkermansia muciniphila, pharmacomicrobiomics, metabolomics

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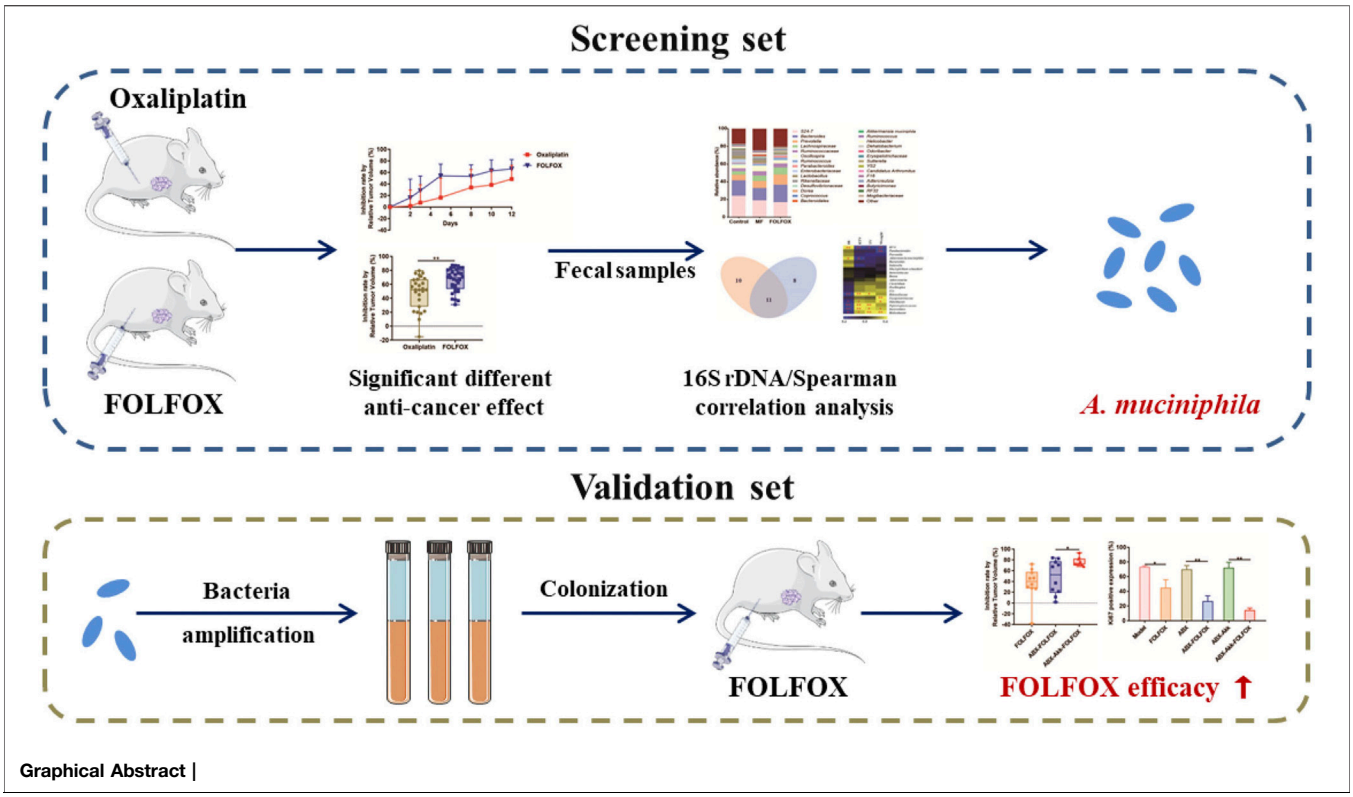
INTRODUCTION

Oxaliplatin is a diamine cyclohexane platinum derivative that shows better tolerance than cisplatin in terms of nephrotoxicity (Yuan et al., 2020). Like other platinum compounds, oxaliplatin acts primarily through binding with inter- and intra-strand cross-links in DNA, forming DNA adducts, and thereby inhibiting cell DNA synthesis (Zimmermann et al., 2020). However, oxaliplatin reached only ~10% response rate when applied alone in clinical practice. Meanwhile, severe peripheral sensory neuropathy occurs in ~10% of patients after six treatment cycles and the rate reaches ~50% after nine cycles, which largely limited its further application (Cvitkovic and Bekradda, 1999; Zhang et al., 2020). Therefore, oxaliplatin is often applied in combination with fluorouracil (5-FU) and calcium folinate in the clinic (i.e., FOLFOX). According to the latest version of the National Comprehensive Cancer Network (NCCN) guidelines (https://www.nccn.org/), FOLFOX is the first-line chemotherapy regimen for advanced colon cancer (Abraham et al., 2020). Compared to oxaliplatin alone, FOLFOX achieves prominently increased efficacy and attenuated toxicity. Recent studies have suggested that gut microbiota, immune regulation, and tyrosine kinase Src might influence the anti-cancer effect of FOLFOX (Parseghian et al., 2017; Dosset et al., 2018; Chang



Graphical Abstract |

et al., 2020). However, the underlying mechanism for the increased efficacy of FOLFOX remains unclear (Machover et al., 1996).

An increasing number of studies suggested that the intrinsic gut microbiome is one of the important factors affecting the efficacy of chemotherapeutics (Liu et al., 2020; Zhang et al., 2020). Pharmacomicrobiomics is an emerging field that investigates the interplay of microbiome variation and drug response and disposition (Doestzada et al., 2018). On the other hand, as the significant role of intestinal flora in mediating drug efficacy is gradually recognized, the application of microflora transplantation alone or in combination with other drugs has achieved surprisingly satisfactory outcomes in the clinic. For example, fecal microbiota transplantation was used in the treatment of pseudomembranous colitis and sepsis, which could overcome traditional drug resistance and reach distinguished therapeutic efficacy (Gupta and Khanna, 2017; Panigrahi et al., 2017). In the case of FOLFOX, we speculated that intestinal flora might also play key roles in mediating the superior efficacy.

In the current study, pharmacomicrobiomics and metabolomics were applied to investigate the involvement of gut microbiota in the efficacy of FOLFOX. First, a colon cancer xenograft model was established to compare the efficacy of oxaliplatin and FOLFOX. Then, 16S rDNA gene sequencing analysis and correlation analysis were conducted to screen differential gut microbiota after oxaliplatin and FOLFOX treatments. Further, bacterial colonization combined with FOLFOX was performed to verify the influence of the focused bacteria on the therapeutic effect. Finally, metabolomics analysis was conducted to discover metabolites derived from gut microbiota.

MATERIALS AND METHODS

Chemicals and Reagents

Oxaliplatin and Calcium Folinate Injection were both obtained from Aosaikang (Jiangsu, China), and 5-FU Injection was obtained from SunRise (Shanghai, China). Oxaliplatin and FOLFOX Injection were prepared according to clinical guidelines as well as existing studies (Iida et al., 2013; Yang et al., 2016). Chemicals including O-Methoxyamine hydrochloride, N-methyl-N-trifluoroacetamide (MSTFA) and cortisone acetate were purchased from Sigma–Aldrich (St.Louis, MO, United States). Vancomycin (MB1260), Ampicillin (MB1507), Neomycin sulfate (MB1716) and Metronidazole (MB2200) were purchased from Meilunbio (Dalian, China).

Colon Cancer Cell

Mice colon cancer cell line CT-26 was purchased from the Cell Bank of the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 (Gibco, Grand Island, United States) supplied with 10% Fetal Bovine Serum (Gibco, Grand Island, United States) in a humidified atmosphere with 5% CO_2 at 37°C.

Colon Cancer Xenograft Model Construction and Sample Collection

Five to six-week-old male BALB/c mice were provided by Beijing Vital River Laboratory Animal Technology Co. Ltd. (License No. SCXK 2019-0001). The mice were housed in a temperature-controlled environment (24 ± 2°C) under a 12/12 h-dark/light cycle. The study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Animals Ethics Committee of China Pharmaceutical University (License No: SYXK 2018-0019).

After acclimation for 1 week, approximately 1×10^6 CT-26 cells were injected subcutaneously into the flank of mice. When the tumors reached to about 100 mm^3, mice were randomly allocated to one of the groups (day 0), i.e., model for oxaliplatin (MO, n = 8), oxaliplatin treatment (Oxaliplatin, n = 27), model for FOLFOX (MF, n = 6), FOLFOX treatment (FOLFOX, n = 33). Based on previous studies, oxaliplatin (10 mg/kg) was intraperitoneally administrated twice a week (Yang et al., 2016), and FOLFOX (oxaliplatin 6mg/kg, 2 h after 5-FU 50 mg/kg and Calcium Folinate 90m g/kg treatment) was intraperitoneally administrated once a week (Robinson et al., 2013; Limani et al., 2016). MO and MF individuals were treated with corresponding vehicles. Individuals in the control group (n = 7) received neither tumor cell injection nor drug treatment. Tumor volume was monitored by a Vernier Caliper throughout the whole experimental period. All the mice were sacrificed at the end of the experiment (day 12), fecal samples were collected for 16S rDNA gene sequencing analysis and non-target metabolomics analysis.

Tumor volume (TV), Relative tumor volume (RTV) and inhibition rate were calculated by the following formulas:

$$TV\ (\mathrm{mm_3}) = \frac{\mathrm{A}}{2} \times \mathrm{B}^2,$$

where A represents the longest diameter of tumor, and B represents the shortest diameter;

$$RTV = \frac{\mathrm{V_t}}{\mathrm{V_0}},$$

where V_0 represents the tumor volume of day 0 (the day of first oxaliplatin administration), V_t represents the tumor volume of day t; Inhibition rate by Relative Tumor Volume (%) = (1–RTV_t /RTV_m) × 100%, where RTV_m represents the RTV of model group, and RTV_t represents the RTV of treatment group.

Bacterial DNA Extraction and Quantification

Total bacterial DNA was isolated from fecal with Stool Genomic DNA Kit (CWBIO, Beijing, China) according to the manufacturer's instructions. DNA quantification was conducted by a NanoDrop 2,000 (Thermo Fisher Scientific, Waltham, United States).

16S rDNA Gene Sequencing Analysis

The DNA integrity was checked by 1% agarose gels electrophoresis. PCR amplification was performed spanning the V3-V4 hypervariable regions of the bacterial 16S ribosomal RNA gene with the conventional barcoded universal bacterial primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′), and sequenced with an Illumina Hiseq 2500 platform (Illumina, San Diego, United States) (Holm et al., 2019; Wan et al., 2019; Chen X. et al., 2020). Raw fastq files were filtered by the Quantitative Insights in the Microbial Ecology software. High-quality sequences were clustered into Operational Taxonomic Units (OTUs) with the similarity threshold of 97% by USEARCH UPARSE (Feng et al., 2019). Then, OTUs were classified into kingdom, phylum, class, order, family, and genus levels referring to the Greengenes database (Shikany et al., 2019), and eventually an OTU table was created. The parameter α-Diversity (Chao1/ Shannon/Simpson) was used to reflect the bacterial gene diversity. Wilcoxon rank-sum test was applied to identify differential taxa between groups (taxa with $p < 0.1$ was screened). Bacteria that existed in less than 50% samples were excluded (Feng et al., 2019). Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was applied to predict the potential function of microbial communities based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database as previously reported (Park et al., 2020). Data concerning the samples included in this study are deposited in the NCBI BioProject database under BioProject accession number PRJNA706146.

Bacteria Culture

Akkermansia muciniphila (*A. muciniphila*, ATCC BAA-835) was purchased from American Type Culture Collection (Manassas, VA, United States), the bacterium was cultured in Brain Heart Infusion Agar at 37°C under anaerobic condition.

Animal Experiment Evaluating *A. muciniphila* on FOLFOX Efficacy

A broad-spectrum antibiotics mixture namely ABX consisting of Vancomycin (100 mg/kg), Neomycin sulfate (200 mg/kg), Metronidazole (200 mg/kg) and Ampicillin (200 mg/kg) was intragastrically administrated to mice every day for five consecutive days (day −14 to −9) to deplete gut microbiota and decrease its α-Diversity (Gong et al., 2019). The mice were then treated with *A. muciniphila* by gavage at 1×10^8 colony forming unit (cfu)/mouse every other day until the end of the experiment (day −7 to 12). Fecal samples were collected at day −7 and 0 for transplantation efficiency verification. CT-26 cells (approximately 1×10^6) were injected subcutaneously into the flank of mice at day −7. When the tumor volumes reached about 100 mm^3, mice were randomly allocated to one of the following groups and the day was marked as day 0: Model (n = 10), FOLFOX (n = 10), ABX (n = 10), ABX-FOLFOX (n = 10), ABX-Akk (n = 10), ABX-Akk-FOLFOX (n = 10). FOLFOX was administrated once a week (day 2 and 9). Tumor volume was monitored by a Vernier Caliper throughout the experiment. All the mice were sacrificed at the end of the experiment (day 12), tumors were removed and prepared for immunohistochemistry analysis.

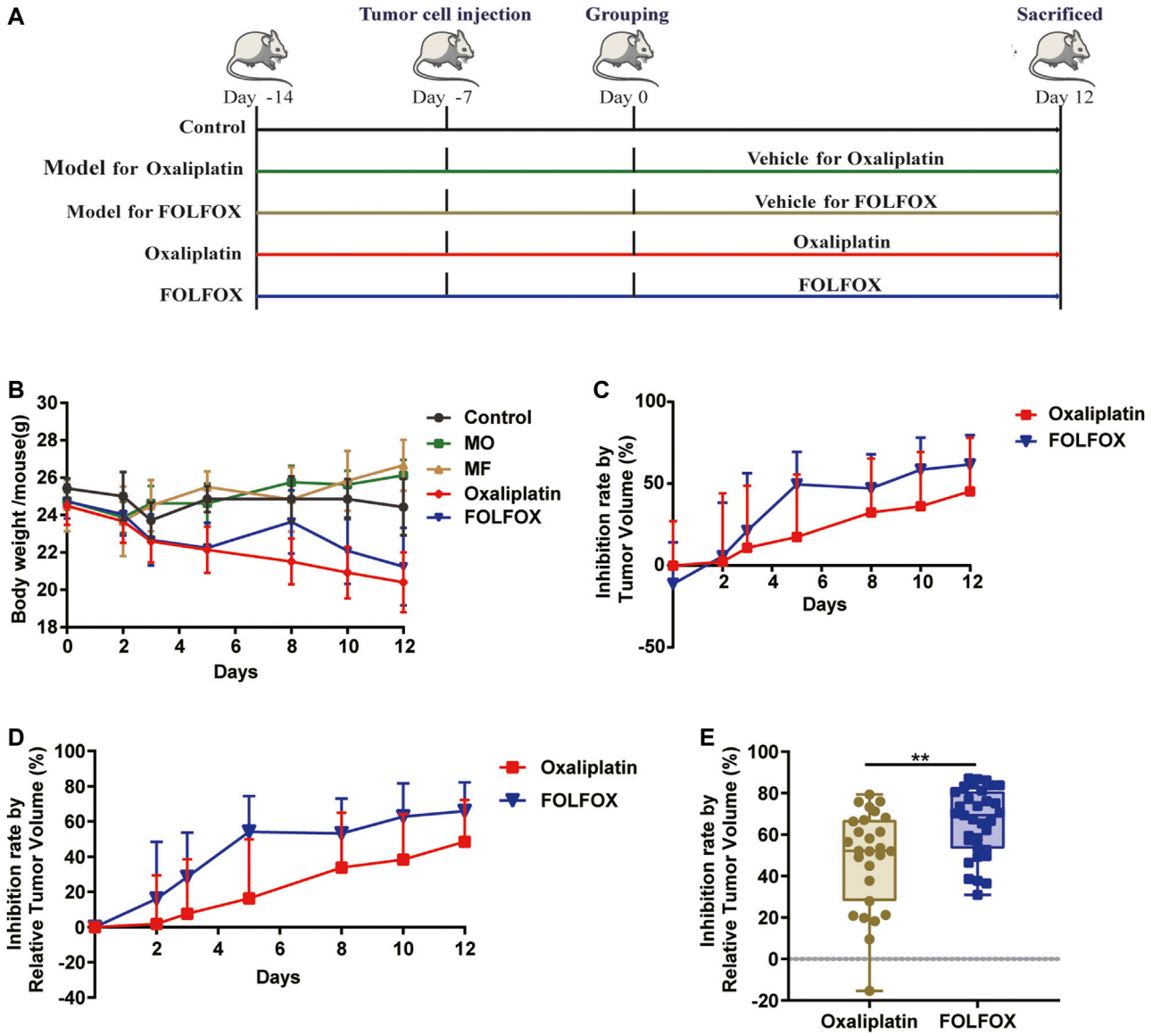


FIGURE 1 | FOLFOX exhibited better chemotherapeutic efficacy in colon cancer xenograft model than oxaliplatin alone. **(A)** Schematic of the pharmacodynamic evaluation of oxaliplatin and FOLFOX in CT-26 colon cancer xenograft model. **(B)** Influence of oxaliplatin and FOLFOX on the body weight of tumor bearing mice. Inhibition rate was calculated by **(C)** TV and **(D)** RTV. **(E)** Inhibition rate calculated by RTV at the end of the experiment (day 12). Data were presented as mean ± SD. The *p*-values < 0.05 were considered statistically significant, **p* <0.05, ***p* < 0.01.

Quantitative Polymerase Chain Reaction

Relative levels of *A. muciniphila* were quantified by qPCR (Tsoi et al., 2017). Total bacterial genome DNA isolation and quantification were conducted as mentioned above. Then, qPCR was performed using SYBR Green I Master (Roche Diagnostics, Basel, Switzerland) on a LightCycler 480 instrument (Roche) following the manufacturer's instructions. The levels of *A. muciniphila* were calculated according to the $2^{-\Delta\Delta CT}$ method (Wang et al., 2021). Information on PCR primers was provided in **Supplementary Table S1**.

Histopathology

Tumors were fixed in formalin and embedded in paraffin. Sections were then subjected for hematoxylin and eosin (HE) staining and Ki67 immunohistochemistry detection as previously reported (Hou et al., 2018).

Non-Target Metabolomics Analysis

Methods for fecal sample non-target metabolomics analysis were presented in our previous studies (Zhang et al., 2017a; Gao et al., 2019). Briefly, gas chromatography-mass spectrometry (GC-MS)

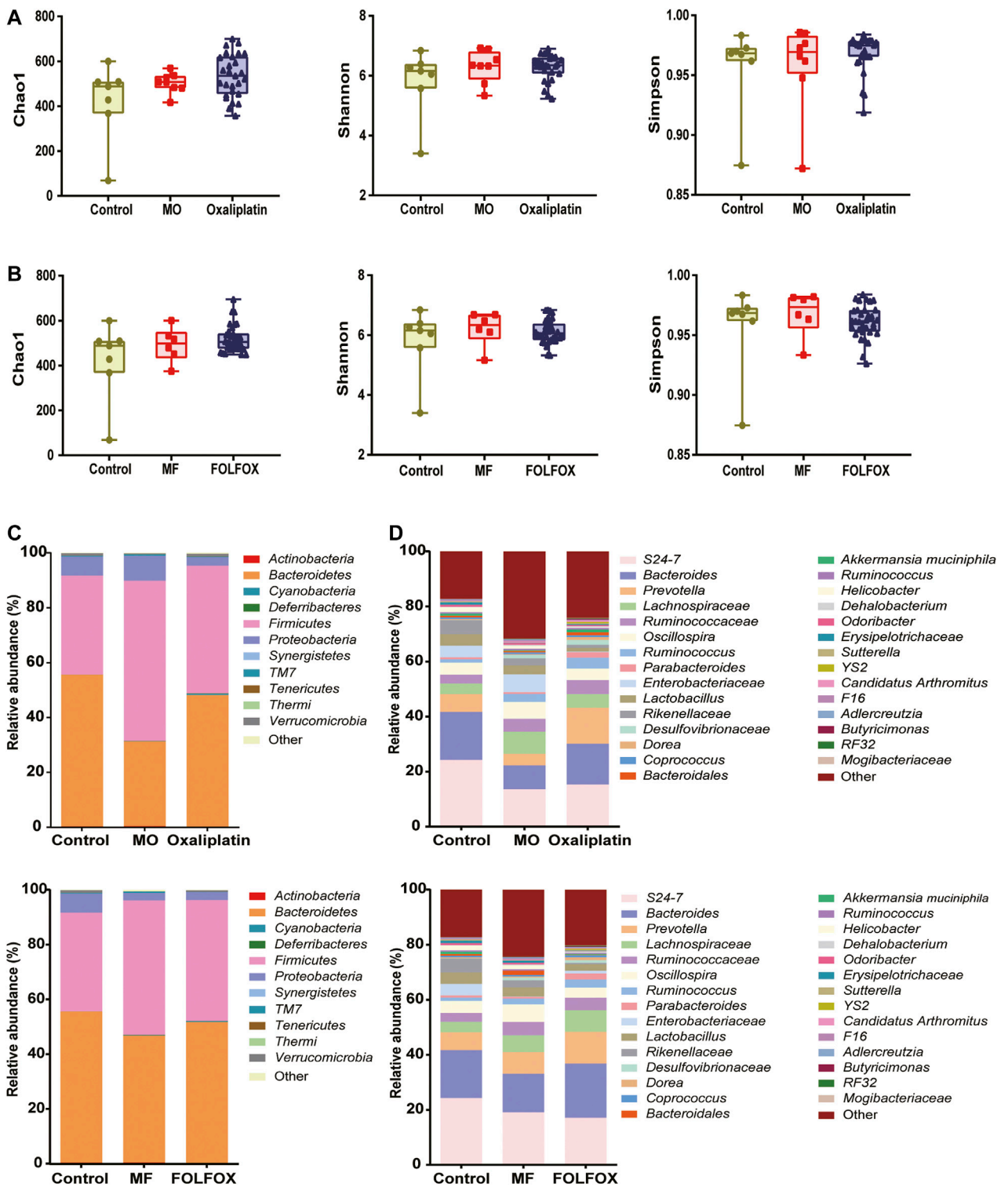


FIGURE 2 | Oxaliplatin and FOLFOX could reverse the disordered distribution of gut microbiota induced by tumor. **(A, B)** Change of α-Diversity estimated by Chao 1, Shannon and Simpson estimator. Taxonomic distributions of bacteria based on fecal 16S rDNA gene sequencing data at **(C)** phylum and **(D)** genus level after oxaliplatin or FOLFOX treatment.

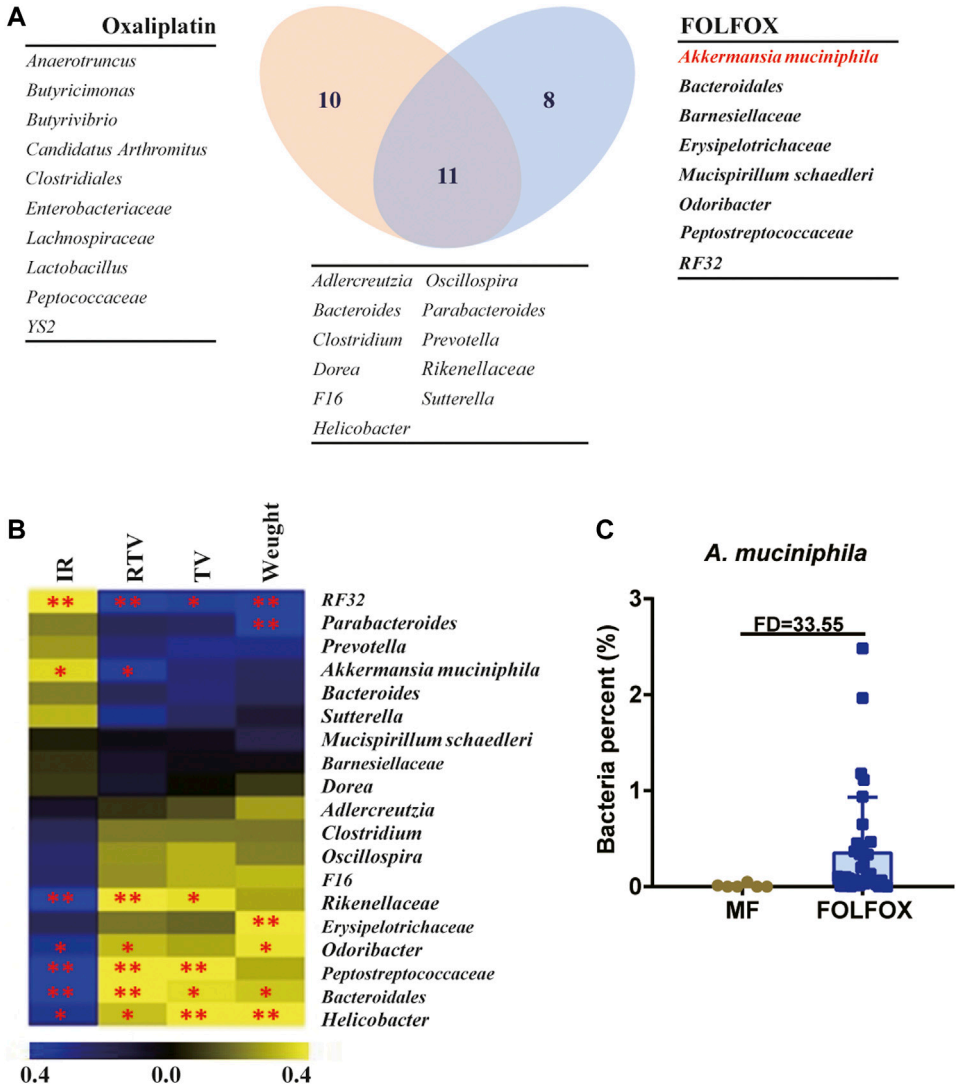


FIGURE 3 | *A. muciniphila* might be the key bacteria accounting for the superior efficacy of FOLFOX. **(A)** Venn diagram illustrating the shared/unique differential gut microbiota OTUs after oxaliplatin and FOLFOX treatment. **(B)** Heatmap of Spearman correlation coefficient between pharmacodynamic indices after FOLFOX treatment and abundance of changed bacterial genera. The intensity of the colors represents the degree of association between the level of pharmacodynamic indices and abundance of changed bacterial genera determined by Spearman's correlations. IR, Inhibition rate by Relative Tumor Volume. **(C)** Relative abundance of *A. muciniphila* in the MF and FOLFOX groups. The *p*-values < 0.05 were considered statistically significant, $^{*}p < 0.05$, $^{**}p < 0.01$.

analysis was performed on a GC-MS-QP2010 Ultra (Shimadzu Inc., Kyoto, Japan) with an Rtx-5MS capillary column (30.0 m × 0.25 mm × 0.25 μm). Liquid chromatography-mass spectrometry (LC-MS) detection was carried out on an ultra-flow liquid chromatography system coupled with ion trap/time-off light hybrid mass spectrometry (UFLC-IT/TOF-MS, Shimadzu Inc., Kyoto, Japan) and compounds were separated by a Phenomenex Kinetex C18 column (100 × 2.1 mm, 2.6 μm). Details on sample preparation, instrument parameters, metabolite annotation, quality control, and data analysis were provided in the supporting information.

Statistical Analysis

Spearman's correlation analysis was applied to test the correlation between fecal bacterial abundance and pharmacodynamic indices (IR, RTV, TV, and body weight) or metabolite intensities. Data analysis and graph plotting were performed by GraphPad Prism 8 software (GraphPad Software Inc., La Jolla, CA, United States). The results were presented as mean ± SD. Independent unpaired two-tailed Student's *t* test was performed to evaluate the differences between two groups, unless elsewhere specified.

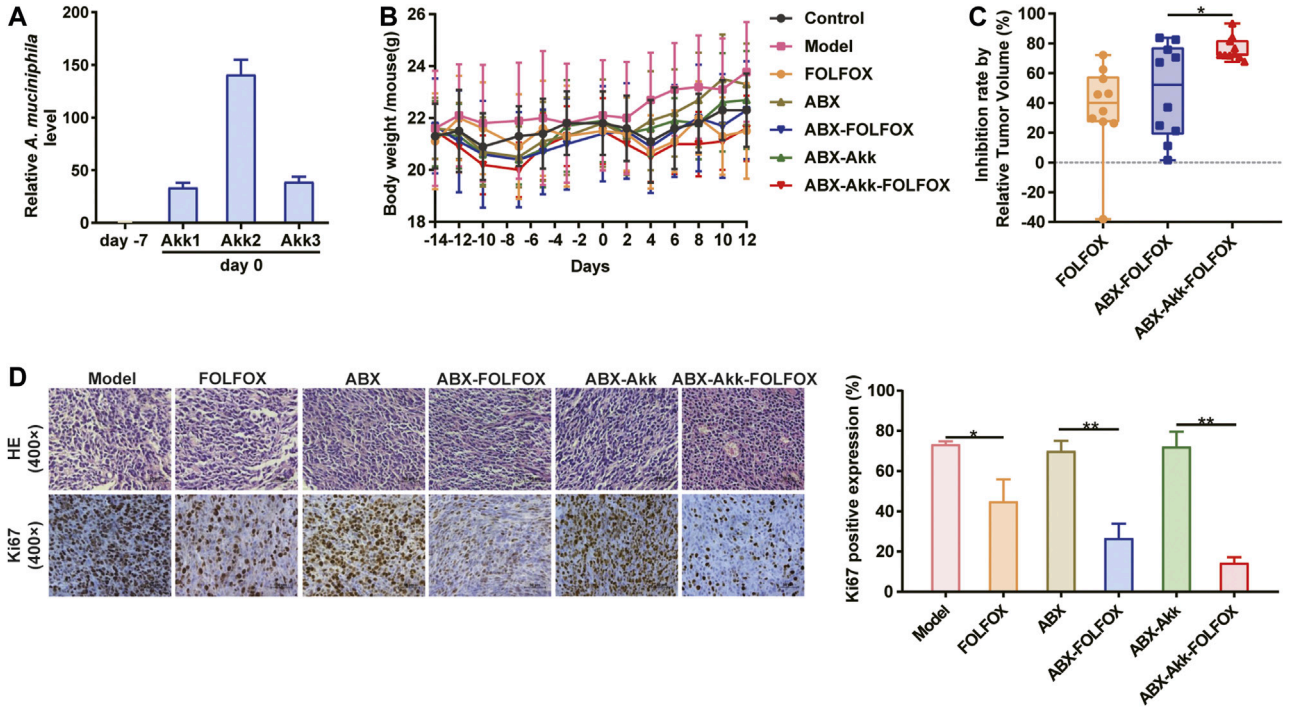


FIGURE 4 | *A. muciniphila* colonization significantly enhanced the anti-cancer effect of FOLFOX. **(A)** Relative abundance of *A. muciniphila* before (day −7) and after (day 0) the transplantation. **(B)** Change of the body weight of tumor-bearing mice across the experiment. **(C)** Inhibition rate by RTV were calculated: Model *vs.* FOLFOX (36%), ABX *vs.* ABX-FOFOX (48%) and ABX-Akk *vs.* ABX-Akk-FOLFOX (76%). **(D)** The percentage of Ki67 positively stained cells. Data were presented as mean ± SD. The *p*-values < 0.05 were considered statistically significant, $^{*}p < 0.05$, $^{**}p < 0.01$.

RESULTS

FOLFOX Exhibited Superior Therapeutic Effect Than Oxaliplatin

In this study, a colon cancer xenograft model was constructed to evaluate the anti-cancer effect of oxaliplatin and FOLFOX (**Figure 1A**). Consistent with clinical practice and existing literature (Cvitkovic and Bekradda, 1999; Hoff et al., 2012), oxaliplatin caused more severe adverse effects than FOLFOX, manifested by a larger body weight reduction (**Figure 1B**). Moreover, the tumor development was more significantly inhibited with FOLFOX treatment compared to oxaliplatin throughout the whole experiment (**Figures 1C,D**). At the end of the experiment (day 12), significant difference in tumor inhibition rate (IR) ($p < 0.01$) was observed between the FOLFOX and oxaliplatin treated individuals (**Figure 1E**). Taken together, FOLFOX exhibited a superior therapeutic effect than oxaliplatin alone in colon cancer xenograft mice.

Oxaliplatin and FOLFOX Showed Different Influence on the Gut Microbiota

Many studies have illustrated important roles of gut microbiota in mediating chemotherapy efficacy (Roy and Trinchieri, 2017), primarily through affecting drug biotransformation directly and interacting with the host indirectly (Vivarelli et al., 2019; Zimmermann et al., 2019). To investigate the underlying mechanism for the superior therapeutic effect of FOLFOX, fecal samples were collected at the end of the experiment and all the samples were subjected to 16S rDNA gene sequencing analysis.

As is shown in **Figures 2A,B**, there was no significant difference in the α-Diversity for the model, FOLFOX, or oxaliplatin individuals compared to the controls indicated by Chao1, Shannon, and Simpson. This suggested that no dramatic changes in the overall microbial community richness were induced by the tumor model construction or the anti-cancer treatment. Notably, both FOLFOX, and oxaliplatin could reverse the disorder of gut microbiota induced by tumor at phylum, class, order, family, or genus level (**Figures 2C,D** and **Supplementary Figure S1**). At phylum level (**Figure 2C**), the control group was dominated by *Bacteroidetes* (55.27%), and *Firmicutes* represented the dominant bacteria in the MO (Model for oxaliplatin, 58.36%) and MF (Model for FOLFOX, 49.09%) groups, while the abundance of *Bacteroidetes* was reversed in the Oxaliplatin (48.00%) and FOLFOX (51.44%) groups. At the genus level, 21 significantly changed bacterial genera were obtained from MO *vs.* Oxaliplatin group (**Supplementary Figure S2**), and 19 bacterial genera from MF *vs.* FOLFOX group (**Supplementary Figure S3**). Moreover, according to LEfSe analysis based on KEGG pathways, oxaliplatin could increase the carbohydrate and nucleotide metabolism of gut microbiota (**Supplementary Figure S4A**), while carbohydrate and lipid metabolism was elevated in the FOLFOX group (**Supplementary Figure S4B**)

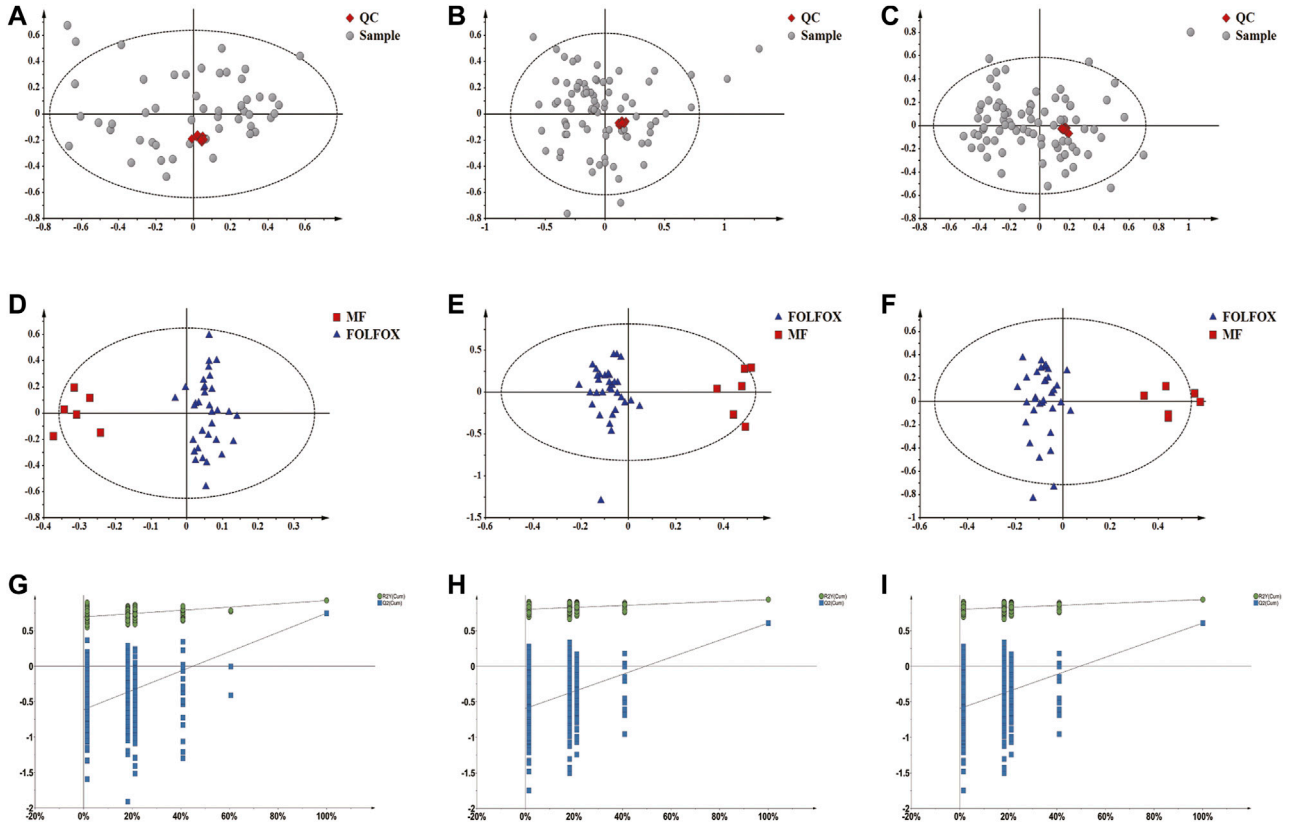


FIGURE 5 | Non-target metabolomics analysis of fecal samples from the MF and FOLFOX groups. **(A–C)** QCs clustered very well in the PCA score plots constructed based on GC-MS, LC-MS (+) and LC-MS (–) data. **(D–F)** OPLS-DA score plots based on GC-MS (R^2X: 0.474; R^2Y: 0.929; Q^2: 0.66), LC-MS (+) (R^2X: 0.449; R^2Y: 0.938; Q^2: 0.607) and LC-MS (–) (R^2X: 0.342; R^2Y: 0.925; Q^2: 0.746) data, respectively. **(G–I)** Permutation test result (500 times) of OPLS-DA models constructed from GC-MS, LC-MS (+) and LC-MS (–) data, respectively.

(LDA score > 2.0 with $p < 0.05$). Taken together, the 16S rDNA gene sequencing analysis suggested an altered gut microbiota composition and function after oxaliplatin and FOLFOX treatment, implicating potential roles of the gut bacterial community in chemotherapy outcome.

Here, changes in the distribution and function of gut microbiota in tumor-bearing mice were observed after oxaliplatin and FOLFOX treatment, but the actual role of gut bacteria in the improved efficacy of FOLFOX requires further exploration. For this purpose, we listed the significantly changed bacteria from the comparisons of MO *vs.* Oxaliplatin and MF *vs.* FOLFOX, respectively. As is shown in the Venn diagram (**Figure 3A**), in total there were 29 bacterial genera, among which 11 were shared by the both treatments, 10 and 8 were unique to oxaliplatin and FOLFOX treatment, respectively. Meanwhile, Spearman correlation analysis was performed to reveal the correlation between differential bacteria and chemotherapeutic efficacy (IR, RTV, TV, and Weight) after FOLFOX treatment (**Figure 3B** and **Supplementary Figure S5**). The abundance of *RF32* and *A. muciniphila* was positively correlated with the IR of FOLFOX ($p < 0.05$). According to existing literature, *A. muciniphila* is known to associate with the improved prognosis of cancer patients and shows beneficial effects on metabolic disorders as well (Everard et al., 2013; Routy et al., 2018). Considering *A. muciniphila* had the highest fold change (33.55) between the MF and FOLFOX groups among all the bacteria (**Figure 3C** and **Supplementary Figure S3**), we conducted further experiments to validate its function.

A. Muciniphila Colonization Significantly Increased FOLFOX Efficacy

To verify the influence of *A. muciniphila* on FOLFOX efficacy, *A. muciniphila* colonization combined with CT-26 colon cancer xenograft model was constructed (**Supplementary Figure S6**). The *A. muciniphila* transplantation was established referring to the published studies and our previous exploration (Gong et al., 2019). Three mice (labeled with Akk1, Akk2, Akk3) were randomly selected to evaluate the bacterial transplantation efficiency. As is shown in **Figure 4A**, the relative abundance of *A. muciniphila* remarkably increased after the colonization, indicating the success of model construction. First of all, FOLFOX treatment caused decreased body weight of tumor bearing mice, while *A. muciniphila* colonization did not influence it (**Figure 4B**). After pharmacodynamic evaluation, we found

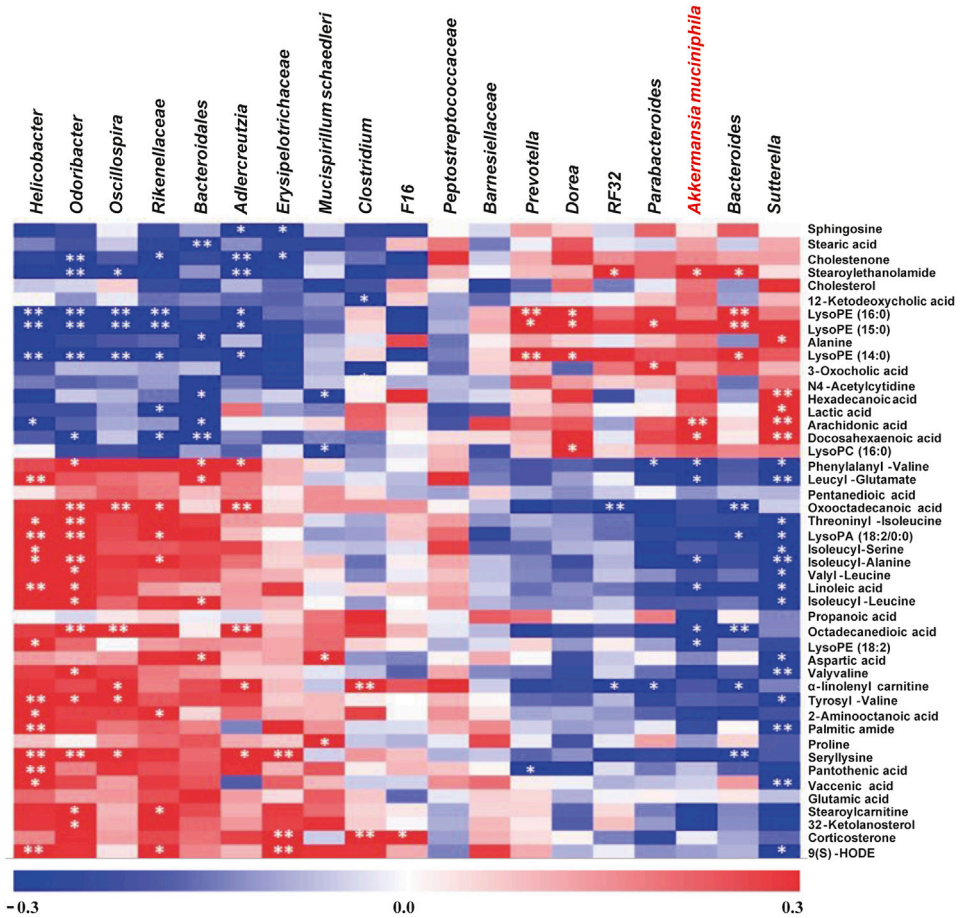


FIGURE 6 | Non-target metabolomics analysis to explore the potential gut microbiota-metabolite axis for FOLFOX efficacy. The intensity of the colors represents the degree of association between the relative level of metabolites and the abundance of changed bacterial genera by Spearman's correlations. The p-values < 0.05 were considered statistically significant, $^{*}p < 0.05$, $^{**}p < 0.01$.

that the anti-cancer effect of FOLFOX was increased from 36 to 48% by ABX pretreatment, suggesting the involvement of gut microbiota in FOLFOX efficacy (**Figure 4C**). More importantly, the inhibition rate of FOLFOX was significantly enhanced (from 48 to 76%) with *A. muciniphila* colonization ($p < 0.05$) (**Figure 4C**). In addition, immunohistochemistry of Ki67 in tumor tissues further supported the above conclusion (**Figure 4D**). To summarize, our study confirmed that *A. muciniphila* transplantation could improve the efficacy of FOLFOX on colon cancer.

Metabolomics Analysis Revealed Potential Gut Microbiota-Metabolite Axis Responsible for FOLFOX Efficacy

It was well known that gut microbiota derived metabolites are important functional readouts of the gut microbiome and play essential roles in the action of chemotherapeutic drugs (Zhang et al., 2017b; Jia et al., 2018). Therefore, fecal samples from MF and FOLFOX groups were further subjected for non-target metabolomics analysis referring to our previous studies (Zhang et al., 2017a; Gao et al., 2019). In metabolomics, LC-MS is the most commonly applied platform. However, GC-MS with chemical derivatization is advantageous in acquiring polar metabolites such as carbohydrates and organic acids that are usually not well retained on a reverse phase column in LC-MS (Beale et al., 2018). Therefore, both GC-MS and LC-MS were utilized in the current study to achieve a wide coverage of metabolites. A tight clustering of the QCs in the PCA score plots was observed (**Figures 5A–C**), indicating good reproducibility of the methods. OPLS-DA models based on GC-MS and LC-MS were established to identify differential features between the two groups (**Figures 5D–F**). Permutation tests with 500 iterations were performed to confirm the OPLS-DA models were not overfitting (**Figures 5G–I**). As a result, 45 significantly changed metabolites were annotated with VIP > 1 and $p < 0.05$ (**Supplementary Table S2**). Spearman correlation analysis was performed to correlate the abundance of differential bacteria and annotated metabolites. As is shown in **Figure 6**, the relative level of stearoylethanolamide, arachidonic acid, and docosahexaenoic acid was positively correlated with the abundance of *A. muciniphila*, whereas phenylalanyl-valine,

leucyl-glutamate, isoleucyl-alanine, linoleic acid, octadecanedioic acid, and lysoPE (18:2) had negative correlations with *A. muciniphila* ($p < 0.05$). Interestingly, we observed three branched-chain amino acid (BCAA) containing dipeptides (i.e., phenylalanyl-valine, leucyl-glutamate, isoleucyl-alanine) which might be potentially important mediators involving in the superior efficacy of FOLFOX.

DISCUSSION

Although oxaliplatin is extensively applied for colon cancer therapy, it was often combined with other chemotherapeutics because of the severe adverse effects and poor prognosis (Kang et al., 2020). In this study, oxaliplatin treatment exhibited a less satisfied anti-cancer effect and decreased body weight compared with FOLFOX, which is consistent with previous reports (Panebianco et al., 2018; Heshiki et al., 2020). FOLFOX is a regimen based on oxaliplatin in combination with 5-FU and calcium folinate, but the mechanism of its high efficacy and low toxicity is still unclear. Meanwhile, a limited response rate of FOLFOX (about 30–50%) existed in clinical practice (Wiseman et al., 1999), which is also confirmed in our study indicated by significantly individualized pharmacodynamic results. Importantly, emerging studies proposed "Pharmacomicrobiomics is the Holy Grail to Variability in Drug Response" (Sharma et al., 2019). Therefore, through the pharmacomicrobiomics approach, the study eventually focused on *A. muciniphila* for further verification.

A. muciniphila was firstly isolated from a sample of healthy human feces by Muriel Derrien in 2004, which is recognized as "beneficial bacteria" for its negative correlation with various diseases (cancer, diabetes, inflammatory bowel disease, autism, etc) (Ottman et al., 2017; Naito et al., 2018; Zhai et al., 2019; Zhang et al., 2019). As previously reported, *A. muciniphila* supplementation could restore the sensitivity of PD-1 inhibitor resistant individuals (Routy et al., 2018). Meanwhile, Chen *et al* also emphasized that *A. muciniphila* significantly improved the anti-tumor effect of cisplatin in Lewis lung cancer mice through immune-regulation (Chen Z. et al., 2020). In the current study, significantly increased efficacy of FOLFOX was observed when combined with *A. muciniphila* transplantation, which confirmed the importance of *A. muciniphila* for FOLFOX response. In brief, our findings may provide new insights into colon cancer therapy.

On the other hand, gut microbiota derived metabolites are important reflections of the distribution and function of the gut microbiome, which play pivotal roles in the interactions between the host and gut microbe (Sharma et al., 2019). In this study, nine metabolites were focused for their significant correlations with the abundance of *A. muciniphila*. Notably, three of them are BCAA containing dipeptides (i.e., phenylalanyl-valine, leucyl-glutamate, and isoleucyl-alanine). Recently, researchers have characterized the increased levels of dipeptides in various cancers (Wu et al., 2013; Li et al., 2019; Ozawa et al., 2020; Stolzenberg-Solomon et al., 2020). More importantly, Li *et al* recruited 3,482 participants for metabolomics analysis, and BCAA contained dipeptide glutamine-leucine was eventually confirmed as potential metabolic markers for early-stage colorectal cancer (Li et al., 2019). Thus, the negative correlations between *A. muciniphila* and these dipeptides observed in our study may reveal the possible gut microbiota-metabolites axis for gut bacteria mediated FOLFOX efficacy. Nevertheless, the relationship between *A. muciniphila* and the dipeptides as well as the underlying signal pathways remain to be elucidated.

Our study suggested the potential role of *A. muciniphila* in FOLFOX response and revealed the possible gut microbiota-metabolites axis which might be responsible for mediating FOLFOX efficacy. However, there are some limitations. First of all, FOLFOX was intraperitoneally administrated to tumor bearing-mice and the period is only 12 days. An extended experimental duration simulating the clinical practice in which several cycles of FOLFOX is applied or analyzing samples from patients might strengthen the clinical guidance. Meanwhile, the effect of *A. muciniphila* on FOLFOX efficacy was confirmed by bacteria colonization in our study, whether a decreased abundance of *A. muciniphila* would have a negative impact needs to be verified. Moreover, whether *A. muciniphila* colonization could influence the efficacy of oxaliplatin as well, further experiments are required. In addition, while up to 19 bacterial genera were initially identified to associate with FOLFOX treatment, we only verified the function of *A. muciniphila* which was the most significantly shifted. Whether the rest were involved in the efficacy of FOLFOX requires further explorations.

In this study, pharmacomicrobiomics approach was applied to investigate the involvement of gut microbiota in the anti-cancer effect of FOLFOX. As a result, *A. muciniphila* was selected for functional verification based on the 16S rDNA gene sequencing and correlation analysis results. The bacterial colonization experiment demonstrated the key role of *A. muciniphila* in FOLFOX efficacy. Metabolomics analysis further revealed a gut microbiota-metabolite axis that might be responsible for FOLFOX efficacy. In a word, this study highlighted the importance of *A. muciniphila* for the therapeutic effect of FOLFOX, providing a novel and effective strategy for clinical colon cancer treatment.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: National Center for Biotechnology Information (NCBI) BioProject database under accession number PRJNA706146.

ETHICS STATEMENT

The animal study was reviewed and approved by Animals Ethics Committee of China Pharmaceutical University (License No: SYXK 2018-0019).

AUTHOR CONTRIBUTIONS

XH and PZ designed the experiments. XH, HD, RS, and SQ performed the experiment and data analysis. XH wrote and commented the manuscript. YT assisted instrument operation and maintenance. WC, ZZ, and FX designed the study, commented the manuscript and study supervision. All authors read and approved the final manuscript.

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Exosome-Derived ADAM17 Promotes Liver Metastasis in Colorectal Cancer

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Exosomes derived from cancer cells are deemed important drivers of pre-metastatic niche formation at distant organs, but the underlying mechanisms of their effects remain largely unknow. Although the role of ADAM17 in cancer cells has been well studied, the secreted ADAM17 effects transported via exosomes are less understood. Herein, we show that the level of exosome-derived ADAM17 is elevated in the serum of patients with metastatic colorectal cancer as well as in metastatic colorectal cancer cells. Furthermore, exosomal ADAM17 was shown to promote the migratory ability of colorectal cancer cells by cleaving the E-cadherin junction. Moreover, exosomal ADAM17 overexpression as well as RNA interference results highlighted its function as a tumor metastasis-promoting factor in colorectal cancer *in vitro* and *in vivo*. Taken together, our current work suggests that exosomal ADAM17 is involved in pre-metastatic niche formation and may be utilized as a blood-based biomarker of colorectal cancer metastasis.

Keywords: exosome, ADAM17, colorectal cancer, liver metastasis, E-cadherin

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HIGHLIGHTS

- Exosome-derived ADAM17 is elevated in the serum of patients with metastatic CRC.
- Exosomal ADAM17 is associated with the metastatic ability of CRC cell lines.
- CRC cell-secreted exosomal ADAM17 increases E-cadherin cleavage and cell migration.
- CRC cell-secreted exosomal ADAM17 promotes liver metastasis *in vivo*.

1 INTRODUCTION

Cancer metastasis is a multi-step, complex process during which tumor cells detach from the primary tumor site and finally seed in target distant organs where they proliferate to form metastatic nodules (Paul et al., 2017; Peinado et al., 2017). Several pre-metastatic niche biomarkers have been identified for cancer diagnosis and prognosis (Zhou et al., 2014; Zhang et al., 2017). Furthermore, targeting the pre-metastatic niche may represent a promising strategy for the prevention of cancer metastasis

Abbreviations: ADMA17, a disintegrin and metalloprotease 17; CM, conditioned medium; CRC, colorectal cancer; HCoEpiC, human colonic epithelial cells; PBS, phosphate-buffered saline; EMT, epithelial-mesenchymal transition.

(Chafe et al., 2015; Zhang et al., 2015). Therefore, the identification of pre-metastatic niche formation-associated biomarkers for the diagnosis, prognosis, and prevention of cancer metastasis is of great relevance.

Exosomes are small vesicles ranging from 30 to 150 nm in size and contain proteins, lipids, and various nucleic acids (Liu et al., 2015). Furthermore, exosomes mediate signal transduction between neighboring and distant cells (Thery et al., 2002; Cocucci et al., 2009). These nano-sized vesicles have also been established as cancer-derived mediators that facilitate pre-metastatic niche formation in distant organs (Costa-Silva et al., 2015; Zhang and Wang, 2015; Becker et al., 2016; Liu et al., 2016). Recently, tumor-secreted exosomes were reported to facilitate cancer-induced vascular permeability and inflammation (Costa-Silva et al., 2015; Hoshino et al., 2015). Importantly, exosomes obtained from the serum of patients with cancer have proven to be reliable markers for cancer diagnosis (Wang et al., 2017; Zhang et al., 2017; Rodrigues et al., 2019). While exosomes are known carriers of miRNAs, mRNA, and long non-coding RNA (Li et al., 2013; Bollati et al., 2015; Chen et al., 2020), the role of exosomal protein, especially membrane-bound proteins, is not yet fully understood. A disintegrin and metalloproteinase 17 (ADAM17), also known as tumor necrosis factor-alpha converting enzyme, is a membrane protein of the ADAM protein family (Scheller et al., 2011). ADAM17 was found to be highly expressed in various types of tumors as well as to affect tumor progression (Zhang et al., 2012). Our previous study revealed that ADAM17 overexpression in digestive tract malignancies was closely associated with tumor proliferation and metastasis (Sun et al., 2017). However, the precise molecular mechanism underlying the role of cancer cell-derived exosomal ADAM17 in metastasis remains unclear.

Herein, we identified that colorectal cancer (CRC) cell-derived exosomal ADAM17 is strongly associated with metastasis in patients with CRC as well as with the metastatic ability of CRC cells. Furthermore, exosomal ADAM17 was shown to effectively enhance the migratory ability of CRC cells by cleaving E-cadherin junctions. Exosomal ADAM17 upregulation and RNA interference *in vivo* and *in vitro* confirmed the function of exosomal ADAM17 as a tumor metastasis-promoting factor in CRC. Further, our clinical data suggested that circulating exosome-derived ADAM17 may be utilized as a blood-based biomarker for the prediction of metastasis in patients with CRC.

2 MATERIALS AND METHODS

2.1 Reagents

GW4869 was obtained from Sigma-Aldrich (St. Louis, MO, United States). Lipofectamine® 3,000 Transfection Reagent was purchased from Thermo Scientific (USA). RIPA lysis buffer was purchased from Beyotime (Jiangsu, China). Primary antibodies for immunoblot analysis included anti-ADAM17 (ab39163), anti-CD81 (ab79559), anti-E-cadherin (ab231303), anti-N-cadherin (ab76011), anti-vimentin (ab92547), anti-snail (ab180714), and anti-β-actin (ab8226) (Abcam, Cambridge, MA, United States). Goat anti-rabbit IgG and goat anti-mouse IgG antibodies were purchased from LI-COR (Lincoln, NE, United States).

2.2 Specimen Collection

Human peripheral blood samples from 20 patients with CRC, with and without liver metastasis, were obtained from the Department of General Surgery of the Changshu No. 1 People's Hospital affiliated to Soochow University. The patients underwent surgical resection in our department of gastrointestinal surgery between 2015 and 2016. The International Union Against Cancer (UICC)/American Joint Committee on Cancer (AJCC) TNM staging modified in 2003 was applied for colorectal cancer staging. The two groups of patients were matched by age and sex, and all cases were pathologically confirmed (**Supplementary Table S1**). Informed consent was provided from all individuals for blood donation using approved institutional protocols. Tubes containing EDTA were applied to collect blood samples and centrifuged at 2,500×g for 10 min to extract the serum for further study (Zeng et al., 2018). Plasma exosomes were isolated as described in Section 2.5 below.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee as well as the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was approved by the Ethics Committee of Changshu No. 1 People's Hospital Affiliated to Soochow University. Informed consent was obtained from all participants included in the study (Sun et al., 2017).

2.3 Animal Models

Six-week-old male athymic BALB/c-nu/nu mice were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd. and maintained in a specific pathogen-free environment. All protocols for animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of Changshu No. 1 People's Hospital Affiliated to Soochow University. For the orthotropic metastasis assay, nude mice were anesthetized, and their ceca were exteriorized by laparotomy. Thereafter, 2×10^6 CRC cells were injected into the mesentery at the tail end of the cecum. To analyze the role of exosomes in tumor metastasis, 10 μg of CRC-derived exosomes were intravenously injected every 3 days after the implantation of CRC cells. After 60 days, the mice were sacrificed, and liver metastases were quantified. To evaluate metastasis, serial sections from the livers were stained with hematoxylin-eosin (HE) and screened for metastatic nodules.

2.4 Cell Culture

Human colorectal carcinoma SW480, SW620, Lovo, DLD-1, and HCT-116 cell lines were purchased from the Cell Bank of the Shanghai Institute of Biochemistry and Cell Biology (China), and human colonic epithelial cells (HCoEpiC) were purchased from ScienCell (USA). Cells were cultured in DMEM (for HCT-116 cells), Ham's F-12K (Kaighn's) medium (for Lovo cells), RPMI-1640 (for DLD-1 cells), L-15 medium (for SW620 and SW480

cells), and colonic epithelial cell medium (for HCoEpiC), supplemented with 10% fetal bovine serum (FBS, except for HCoEpiC), 100 U/mL penicillin, and 100 μg/ml streptomycin (all available from Invitrogen, Grand Island, NY, United States). The FBS used for conditioned medium (CM) collection, exosome isolation, and endothelial cell treatment was depleted of exosomes via overnight centrifugation at 100,000×g and 4°C. All cell lines were cultured under a humidified atmosphere in a 5% CO_2 incubator at 37°C.

2.5 Exosome Isolation, Characterization, and Analyses

Exosomes were purified from CRC cell culture CM or CRC patient serum via ultracentrifugation according to a previous exosome extraction method (Zeng et al., 2018). The amount of exosomal protein was determined using the BCA Protein Assay kit (Beyotime Biotechnology, China). For transmission electron microscopy analysis, exosomes were fixed with 2% paraformaldehyde and observed using a transmission electron microscope (Hitachi H-7500, Japan) (Zeng et al., 2018). The number and size of exosomes were directly tracked using the Nanosight NS 300 system (NanoSight Technology, Malvern, United Kingdom), and data were analyzed with the NTA analytical software (version 2.3).

2.6 Western Blot Analysis and Quantitative Proteomic Analysis

For western blot analysis, total protein was extracted from exosomes, CRC cells, or mouse liver metastasis tissue using RIPA lysis buffer and analyzed as previously described (Fang et al., 2020). The exosomes were labeled with iTRAQ reagents using the iTRAQ multiplex kit (AB Sciex, USA) and followed analyzed according to previous publication (Kawakami et al., 2017). Labeled samples were separated and automatically spotted onto a MALDI plate, and mass spectra were acquired using the AB Sciex TOF/TOF 5800 system. All MS/MS data were analyzed via MASCOT and the Protein Pilot software (version 4.5; AB Sciex) to identify and quantify corresponding proteins in different groups (**Supplementary Table S2**). Protein identification was considered correct, based on the selection criteria (Kawakami et al., 2017).

2.7 DNA Constructs and RNA Interference Studies

Inhibition of ADAM17 expression in CRC cell-derived exosomes was performed using ADAM17-directed siRNAs. Human ADAM17-specific siRNA 5′-TGAGGCAG TCTCTCCTATTC CTGACCAGC-3′ and nonsense siRNA: 5′-TGACCACCCTGA CCTACGGCGTGCAGTGC-3′ were obtained from RiboBio (Guangzhou, China). The pcDNA3.1-ADAM17 plasmid was constructed using standard techniques. Briefly, DNA fragments encoding ADAM17 were generated via high-fidelity PCR and cloned into the pcDNA3.1 vector. All constructs were confirmed via DNA sequencing and purified using an Endofree Plasmid Preparation Kit (QIAGEN, Valencia, CA, United States). Cells were transfected with pcDNA3.1, pcDNA3.1-ADAM17, ADAM17-siRNA, and nonsense (scrambled) control siRNA (NC-siRNA) using a liposome-based method for 48 h, and the cells were harvested for subsequent analyses.

2.8 Wound-Healing Assay and Migration Assay

Cell wound-healing and migration assays were performed as previously described (Wang et al., 2015; Xu et al., 2016).

2.9 Statistical Analysis

The experimental data were analyzed using the Student's t-test and one-way analysis of variance (ANOVA) to determine significant differences between the two groups. By applying a Chi-square test, the relationship between the CirExo-ADAM17 and clinicopathological features (such as gender, age, TNM stage, metastasis, etc.) in colorectal cancer patients who underwent surgical excision were then compared. The Kaplan-Meier survival curve was used to evaluate the relationship between CirExo-ADAM17 and survival outcomes in different patients with colorectal cancer. Significance tests used log-rank test analysis. Univariate survival analysis was further conducted to evaluate the effect of CirExo-ADAM17 on the prognosis of colorectal cancer. Data are presented as the mean ± SD. Western blotting analyses were repeated three times, and the results were quantified using the ImageJ software. Statistical analysis was performed using the SPSS 13.0 software (SPSS Inc., Chicago, IL, United States). Statistical significance was set at $p < 0.05$.

3 RESULTS

3.1 Exosomal ADAM17 is Upregulated in Metastatic Compared to Non-metastatic CRC

Studies have shown that tumor-derived exosomes package specific proteins critical for metastasis into target organs (Peinado et al., 2012; Costa-Silva et al., 2015). Quantitative mass spectrometry comparison of exosomal proteomes revealed that only 24 proteins were differentially expressed in exosomes from liver metastases of patients with CRC when compared to exosomes from patients without metastasis (**Figure 1A**; **Supplementary Table S2**). Combining the protein function in tumorigenesis and development, ADAM17 emerged as a prominent exosomal protein in liver metastasis-derived exosomes, with a low expression in exosomes from patients without metastasis and from healthy donors, suggestive of its specific association with CRC liver metastasis potential (**Figures 1A–D**; **Supplementary Figure S1A**). Moreover, it was observed that CirExo-ADAM17 had a significant impact on the

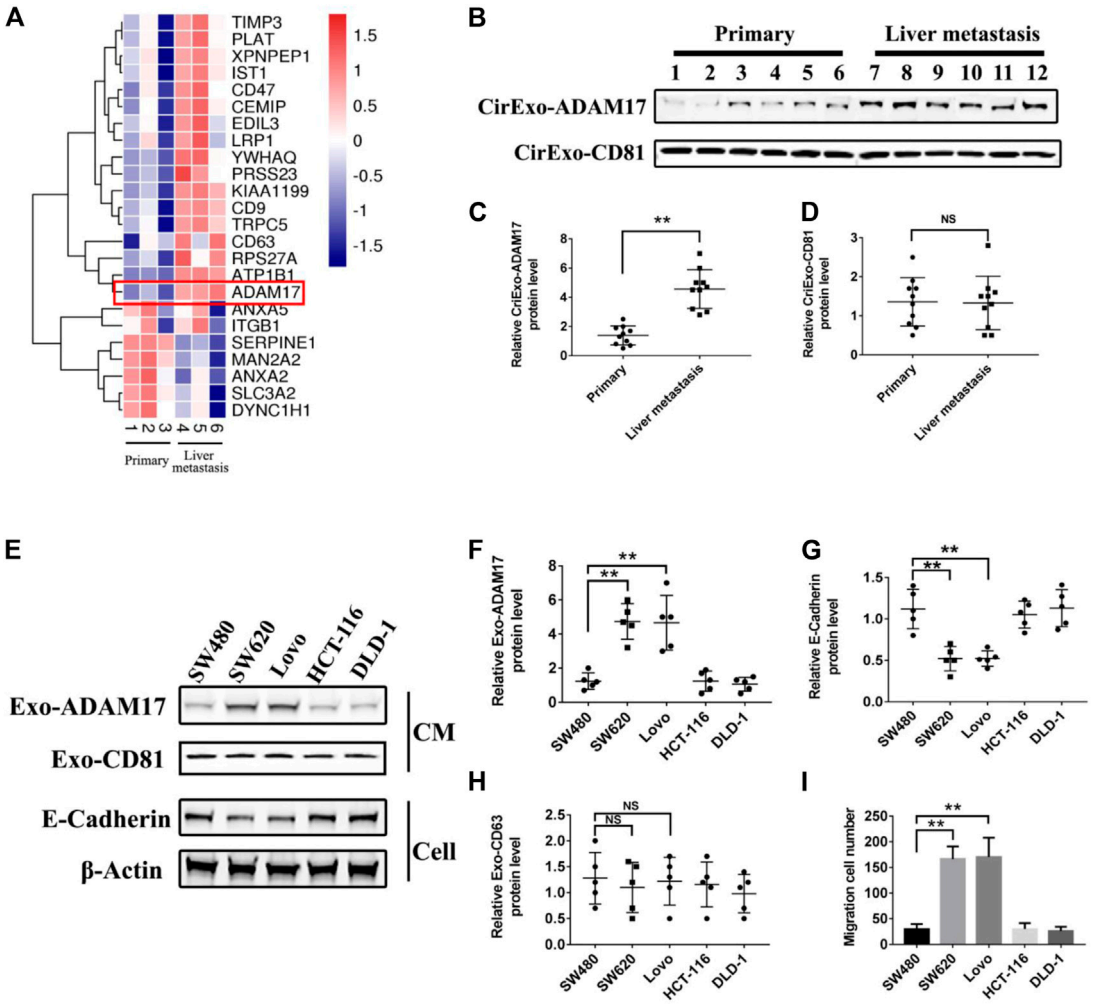

FIGURE 1 | ADAM17 levels in exosomes from CRC patient sera and CRC cell lines. **(A)** Proteomic analysis of serum-derived exosomal protein differentially expressed between non-metastatic (primary) and liver metastastatic patients with CRC. A heatmap of differentially expressed exosomal proteins based on quantitative mass spectrometry values (technical triplicates, * false discovery rate < 0.05 *via* ANOVA). Hierarchical clustering (1 − the sample Spearman's rank correlation coefficient between observations) was performed on protein expression levels ($n = 3$). Exosomal ADAM17 was marked with a red frame as a differentially expressed protein. **(B)** The serum-derived exosomes of non-metastatic (primary) and liver metastatic patients with CRC were collected and lysed for analysis of ADAM17 and CD81 protein levels. **(C,D)** The relative protein levels were further calculated via gray analysis (the total exosomal protein was set as the internal control, $n = 10$). **(E)** SW480, SW620, Lovo, HCT-116, and DLD-1 exosomes and cells were subjected to analysis of ADAM17, CD81, and E-cadherin protein levels (CM, conditioned medium). **(F–H)** The relative protein levels were further calculated via gray analysis (the total exosomal protein was set as the internal control for exosomal protein analysis, β-actin was set as the internal control for cells, $n = 6$). **(I)** Cell migration assessment of SW480, SW620, Lovo, HCT-116, and DLD-1 cells *via* transwell assays ($n = 6$). Data are expressed as means ± SDs. *, $p < 0.05$; **, $p < 0.01$.

postoperative prognosis of patients with colorectal cancer. Kaplan-Meier univariate survival analysis results indicated that CirExo-ADAM17 ($p < 0.05$) was associated with the poor postoperative prognosis (**Supplementary Tables S1, S3**, and **Supplementary Figure S2**). Furthermore, exosomal ADAM17 levels were significantly increased in metastatic CRC cell lines (SW620 and Lovo) relative to those in SW480, DLD-1, HCT 116, and human colonic epithelial cells (HCoEpiC), as determined via western blot analysis (**Figures 1E,F,H**; **Supplementary Figure S1B**). Lower E-cadherin protein levels were detected in SW620 and Lovo cells, in agreement with their high migratory ability (**Figures 1E,G,I**). Taken together, our data identified ADAM17 as a protein upregulated in exosomes from metastatic CRC cells.

3.2 Exosomal ADAM17 Enhanced the Migratory Properties of CRC Cells

To study the role of exosomes in CRC metastasis, we isolated exosomes from two colorectal cancer cell lines with different migratory ability, SW480 and SW620. SW480- and SW620-derived exosomes were 107.2 nm (SW480-Exo) and 115.1 nm (SW620-Exo) in size (**Figures 2A,B**). SW620-Exo, which had higher ADAM17 levels, stimulated the migration of SW480 cells, reducing E-cadherin

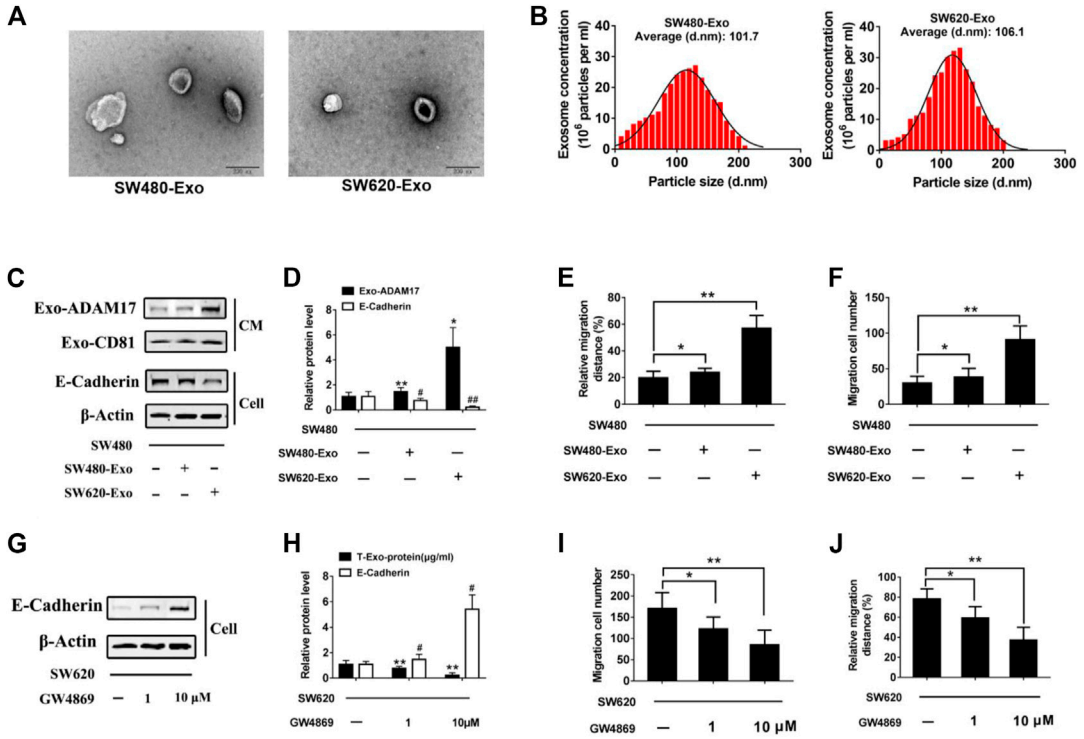

FIGURE 2 | Exosomal ADAM17 enhances the migratory properties of CRC cells. Characterization of CRC cell-derived exosomes. **(A)** Electron microscope images of exosomes isolated from the CM of cultured SW480 and SW620 cells. Scale bar, 200 nm. **(B)** Nanoparticle tracking analysis of isolated exosomes. **(C,D)** SW480 cells were treated with SW480-derived exosomes (SW480-Exo) and SW620-derived exosomes (SW620-Exo) at 50 μg (10 μg/ml) per 1×10^5 cultured cells. Exosomes were collected, cells were lysed, and protein levels were further calculated via gray analysis (the total exosomal protein was set as the internal control for exosomal protein analysis, β-actin was set as the internal control for cells, $n = 6$). **(E,F)** Changes in migration were measured via transwell and wound scratch assays using SW480 cells treated with SW480-Exo and SW620-Exo ($n = 6$). **(G,H)** SW620 cells were treated with different concentration GW4869, and the total exosomal protein as well as cellular E-cadherin levels were calculated ($n = 6$). **(I,J)** Changes in migration were measured via transwell and wound scratch assays using SW620 cells treated with GW4869 ($n = 6$). Data are expressed as means ± SDs. * or #, $p < 0.05$; ** or ##, $p < 0.01$.

protein levels relative to those in SW480-Exo-treated cells (**Figures 2C–F**). To further identify the role of exosomes in the SW620-mediated promotion of migration, the exosome biogenesis/release inhibitor GW4869 (Essandoh et al., 2015) was applied to SW620 cells. GW4869 decreased SW620 exosome production in a dose-dependent manner and further decreased the migratory properties of SW620 cells (**Figures 2G–J**). These results demonstrated that CRC-derived exosomes harbored the ADAM17 oncoprotein, which may play an important role in the exosome-mediated stimulation of CRC metastasis.

3.3 Exosomal ADAM17 Increased the Cleavage of E-Cadherin

To further explore the role of exosomal ADAM17 in CRC metastasis, ADAM17-overexpressing SW480-Exo and ADAM17-knockdown SW620-Exo were applied to SW480 cells (**Figures 3A,D,E**). SW480-Exo treatment enhanced the migratory properties of SW480 cells, whereas ADAM17 knockdown in SW620-Exo eliminated their migration-stimulating effect on SW480 cells (**Figures 3B,C**). E-cadherin prevents cancer cell dissemination from the primary lesion to distant organs by decreasing motility, migratory, and invasive properties (Gogali et al., 2010). E-cadherin is an important substrate of ADAM17. Its shedding requires the cleavage of α-secretase in the extracellular membrane, which is catalyzed by ADAM17 (Ma et al., 2020). E-cadherin levels were decreased following treatment with ADAM17-overexpressing SW480-Exo and increased after treatment with ADAM17-knockdown SW620-Exo (**Figures 3D,E**). Epithelial-mesenchymal transition (EMT) plays a critical role in cancer progression and metastasis (Huang et al., 2015), during which considerable morphological transformation occurs, in parallel to the suppression of epithelial markers (Zheng and Kang, 2014). The relative protein levels of N-cadherin, vimentin, and Snail were increased after treatment with ADAM17-overexpressing SW480-Exo and decreased following treatment with the ADAM17-knockdown SW620-Exo (**Figures 3D,E**). Taken together, CRC-derived exosomal ADAM17 stimulated cancer cell migration via cleavage of E-cadherin and the upregulation mesenchymal expression.

3.4 Colorectal Cancer Cell-Secreted Exosomal ADAM17 Promotes Metastasis *In vivo*

To determine whether CRC-derived exosomes play a role in liver metastasis, we established a mouse CRC tumor model. SW480

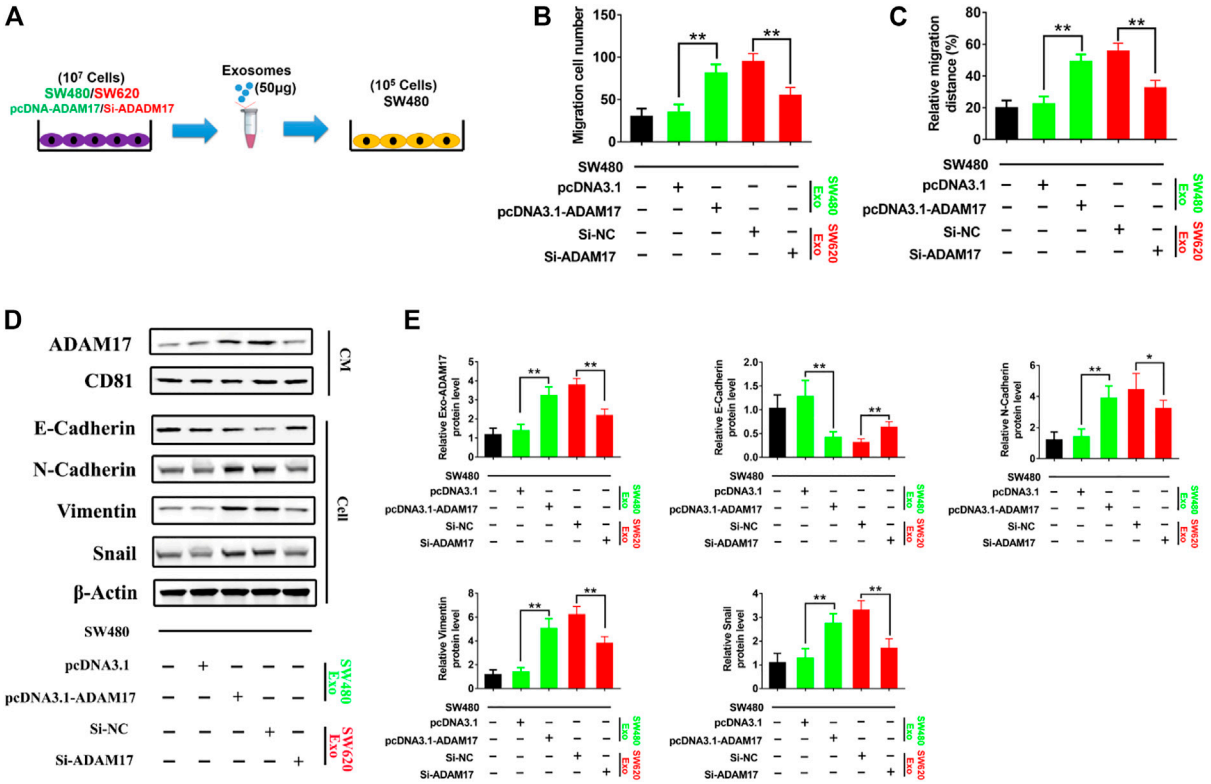

FIGURE 3 | Exosomal ADAM17 enhances the cleavage of E-cadherin. **(A)** Schematic description of the experimental design. The pcDNA3.1-, pcDNA3.1-ADAM17-transfected SW480-derived exosomes and Si-NC-, Si-ADAM17-transfected SW620-derived exosomes were isolated. 50 μg of exosomes were added to 5×10^5 SW480 cells. **(B,C)** Changes in migration were measured via transwell and wound scratch assays using SW480 treated with pcDNA3.1-, pcDNA3.1-ADAM17-transfected SW480-derived exosomes as well as Si-NC-, Si-ADAM17-transfected SW620-derived exosomes (n = 6). **(D,E)** SW480 cells were treated with pcDNA3.1-, pcDNA3.1-ADAM17-transfected SW480-derived exosomes and Si-NC-, Si-ADAM17-transfected SW620-derived exosomes. Exosomes and lysed cells were collected for the assessment of protein levels via gray analysis (the total exosomal protein was set as the internal control for exosomal protein analysis, β-actin was set as the internal control for cells, n = 6). Data are expressed as means ± SDs. *, $p < 0.05$; **, $p < 0.01$.

and SW620 cells were implanted into nude mice separately. The SW620 implanted mice had higher rates of hepatic metastases, and also showed more and larger metastatic nodules in the liver compared with those in the SW480 implanted mice (**Figures 4A,B**). In addition, serum-derived exosomes were isolated, and higher exosomal ADAM17 protein levels were found in SW620 implanted mice compared to those in SW480 (**Figures 4C,D**). Further, to identify the role of exosomal ADAM17 in CRC metastasis, ADAM17 overexpression in SW480-Exo and ADAM17 downregulation in SW620-Exo were applied to SW480 cells implanted in mice (**Figures 5A,D,E**). ADAM17 overexpression in SW480-Exo increased the hepatic metastases rates and showed more and larger metastatic nodules in SW480 cells implanted in the mouse liver, whereas ADAM17 downregulation in SW620-Exo eliminated the SW620-Exo liver metastasis stimulation ability in SW480 cells (**Figures 5B,C**). Furthermore, the E-cadherin level was decreased in ADAM17 overexpression in the SW480-Exo group and increased in ADAM17 downregulation in the SW620-Exo group (**Figures 5D,E**). These findings strongly indicate that colorectal cancer cell-secreted exosomal ADAM17 promotes metastasis *in vivo*.

4 DISCUSSION

CRC-derived exosomes are expected to play a role in the generation of metastatic microenvironments (Shao et al., 2018; Lafitte et al., 2019; Lin et al., 2019), but the underlying molecular mechanisms have not yet been fully determined. This study elucidated the effect of exosomal ADAM17 derived from SW620 cells, a highly liver metastatic CRC cell line, on the promotion of liver metastasis with poorly metastatic SW480 cells. Studies on lung cancer and melanoma have revealed that exosomes are involved in the regulation of angiogenesis and the EMT (Grange et al., 2011). However, as the role of exosomes in cancer metastasis might vary between tumor types, the underlying mechanisms through which exosomes regulate CRC liver metastasis should be elucidated (Wang et al., 2015).

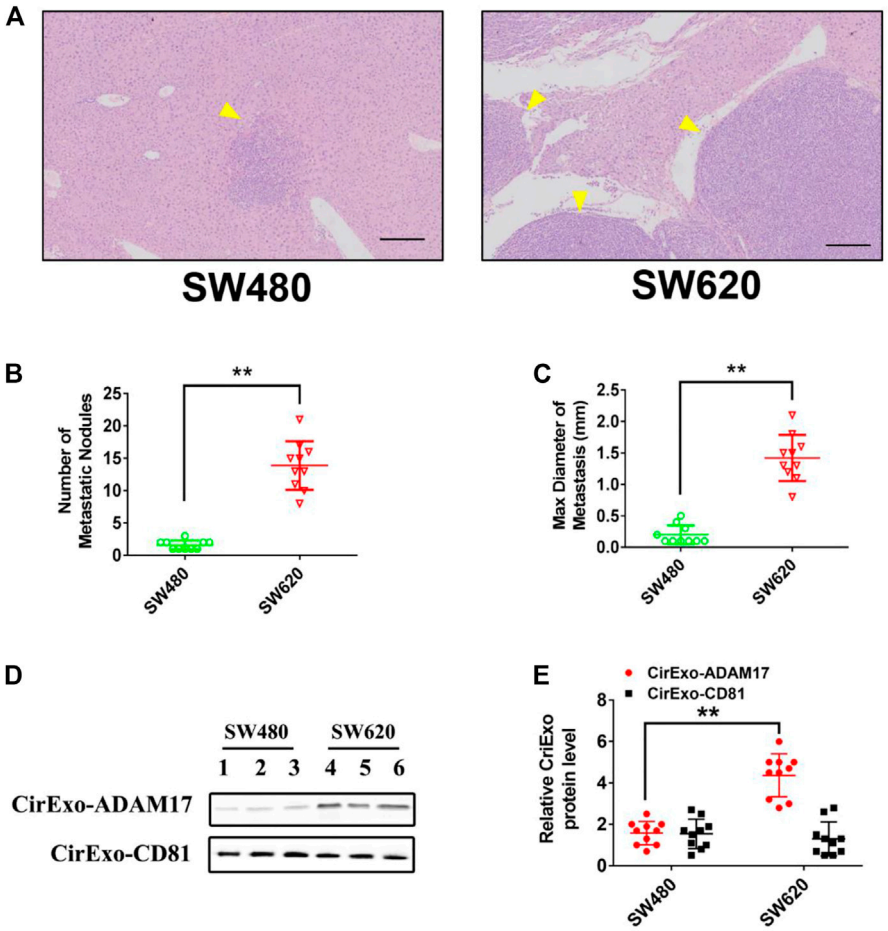

FIGURE 4 | Exosomes derived from CRC cells promote liver metastasis. SW480 and SW620 cells were implanted into the mesentery at the tail end of the cecum. Autopsies were performed, and the presence of metastases was examined macroscopically 60 days after CRC implantation. **(A)** Representative H&E staining results of liver slices from SW480- and SW620-implanted mice. Arrows indicate the tumor nodules. Scale bar, 250 μm. **(B,C)** The number and maximal diameter of metastatic nodules in the livers of SW480- and SW620-implanted mice were calculated and analyzed (n = 10). **(D,E)** The serum-derived exosomes of SW480- and SW620-implanted mice were collected and lysed for analysis of ADAM17 and CD81 protein levels. The relative protein levels were further calculated via gray analysis (the total exosomal protein was set as the internal control, n = 10). Data are expressed as means ± SDs. *, $p < 0.05$; **, $p < 0.01$.

The sheddase enzymes on the exosomal surface have rarely been studied. As a sheddase, ADAM17 cleaves the extracellular domains of transmembrane proteins, thereby releasing them and modulating cell–cell and cell–environment communication (Dusterhoft et al., 2019). ADAM17 plays a profound role in colonic tumorigenesis, and its expression was shown to be increased in tissue samples from patients with CRC (Walkiewicz et al., 2016). As an oncogene, the role of exosome-derived ADAM17 in CRC metastasis remains to be elucidated. Our study identified ADAM17 as a robust serum marker in metastatic CRC patients, in addition to its upregulation in metastatic CRC cells. As a key adhesive molecule in the prevention of tumor progression, E-cadherin is processed via proteolytic modifications (Puisieux et al., 2014; Ma et al., 2020). In particular, E-cadherin shedding requires α-secretase cleavage, which is catalyzed by ADAM17 (Ma et al., 2020). Our study revealed that exosomal ADAM17 effectively enhanced the migratory ability of CRC cells via E-cadherin cleavage. EMT is a central cellular process in cancer metastasis, characterized by the loss of cell–cell junctions and a decrease in the expression of the epithelial marker E-cadherin, in parallel with the upregulation of the mesenchymal marker N-cadherin and cytoskeleton rearrangements intended for enhanced invasive capacity (Tennakoon et al., 2015). Exosomal ADAM17 increased the expression of mesenchymal markers, such as N-cadherin, vimentin, and Snail, in turn promoting EMT in CRC. To verify the direct role of exosomes in CRC metastasis *in vivo*, we established liver metastatic CRC nude mouse models through the implantation of SW620 and SW480 cells, followed by an assessment of liver metastasis frequency and severity. The SW620-implanted mice had higher rates of hepatic metastases, accompanied by high serum exosomal ADAM17 protein levels. Our study further confirmed the role of exosomal ADAM17 in CRC metastasis via ADAM17 overexpression and knockdown in related CRC cell-derived

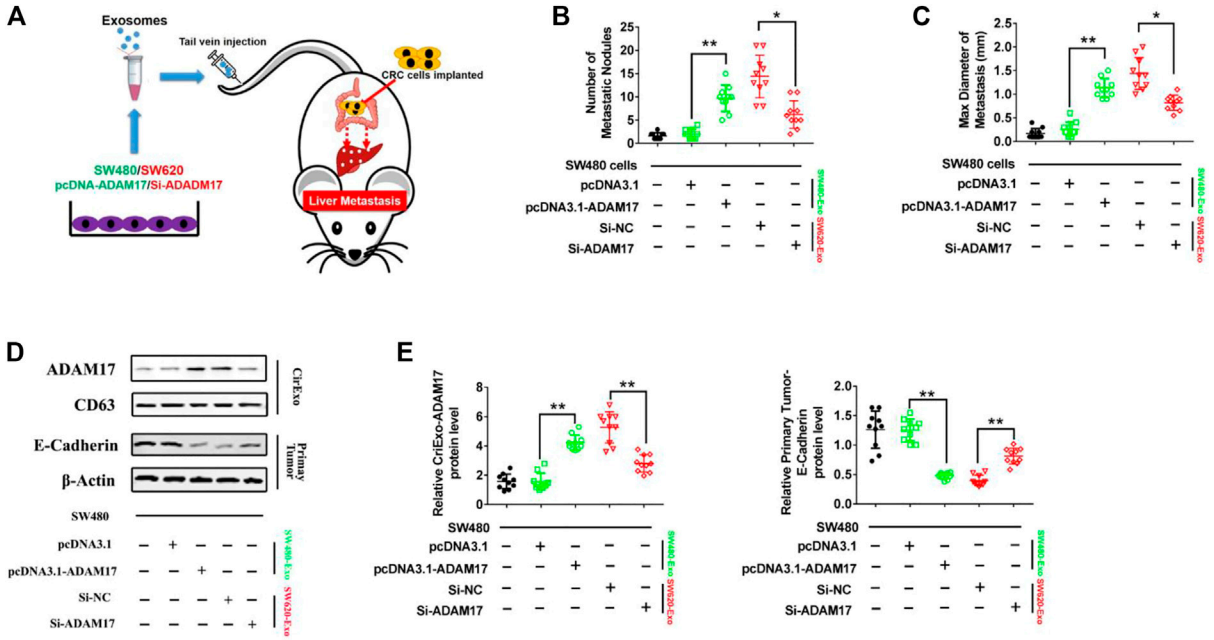

FIGURE 5 | CRC cell-secreted exosomal ADAM17 promotes metastasis *in vivo*. **(A)** Schematic description of the experimental design. The pcDNA3.1-, pcDNA3.1-ADAM17-transfected SW480-derived exosomes and Si-NC-, Si-ADAM17-transfected SW620-derived exosomes were isolated. 10 μg exosomes were used to intravenously inject mice every 3 days for 2 months after the implantation of SW480 cells. **(B,C)** The number and maximal diameter of metastatic nodules in the livers of SW480-implanted mice treated with pcDNA3.1-, pcDNA3.1-ADAM17-transfected SW480-derived exosomes and Si-NC-, Si-ADAM17-transfected SW620-derived exosomes were calculated and analyzed (n = 10). **(D,E)** The serum-derived exosomes and primary tumors of SW480-implanted mice treated with pcDNA3.1-, pcDNA3.1-ADAM17-transfected SW480-derived exosomes and Si-NC-, Si-ADAM17-transfected SW620-derived exosomes were collected and lysed for the assessment of ADAM17, CD81, and E-cadherin protein levels. The relative protein levels were determined via gray analysis (the total exosomal protein was set as the internal control for exosomal protein analysis, β-actin was set as the internal control for cells, n = 6). Data are expressed as means ± SDs. *, $p < 0.05$; **, $p < 0.01$.

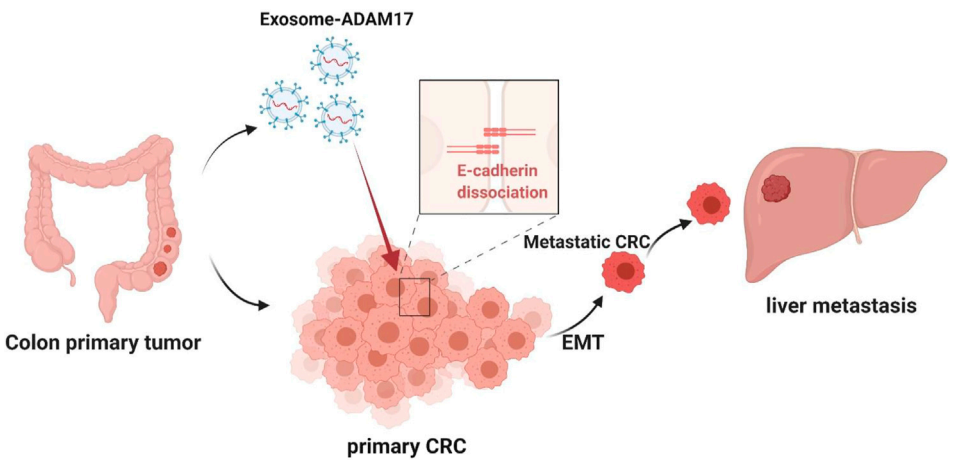

FIGURE 6 | Proposed mechanism for exosomal ADAM17-mediated promotion of CRC metastasis. The CRC-derived exosomal ADAM17 promoted the E-cadherin cleavage, enhancing the migratory properties of CRC cells, which in turn promoted hepatic metastasis *in vivo*.

exosomes. The E-cadherin level in primary CRC tumors was decreased following treatment with ADAM17-overexpressing exosomes, in parallel with an increased rate of hepatic metastasis, as well as more and larger metastatic nodules in the livers of CRC cell-implanted mice. These results highlighted the importance of exosomal ADAM17 in CRC liver metastasis.

In the present study, we demonstrated the pivotal role of exosomal ADAM17 in CRC liver metastasis, suggesting that it might function through the regulation of cellular

migration, thus highlighting its potential as a prognostic biomarker and therapeutic target. We also demonstrated that the effects of exosomal ADAM17 are mediated via E-cadherin cleavage, which promotes cancer cell dissemination from the primary lesion to distant organs by enhancing motility as well as migratory and invasive properties (**Figure 6**). Our research furthers the understanding of how exosomes regulate CRC liver metastasis and provides a basis for the development of associated therapeutics. Future studies should focus on confirming tumor-derived exosomal ADAM17 as a plasma-based biomarker for the non-invasive screening of patients with CRC, as well as on the assessment of targeted ADAM17-based therapeutics against CRC liver metastasis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: A list of SWISS MODEL accessions can be found in the **Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethics Committee of Changshu No. 1 People's Hospital Affiliated to Soochow University. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by The Institutional Animal Care and Use Committee of Changshu No. 1 People's Hospital Affiliated to Soochow University.

AUTHOR CONTRIBUTIONS

JS and JD: study conception and design, data acquisition, analysis and interpretation of data, and writing of the manuscript. ZL and XG: acquisition, analysis, and interpretation of data. WF: analysis and interpretation of data. KL and FX: acquisition, analysis, and interpretation of data, as well as proofreading of the manuscript. JJ, JS, and YY: obtained funding, study conception and design, study supervision, and revision of the manuscript. All authors read and approved the final manuscript.

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Figure 6 was created using BioRender.com (https://biorender.com).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.734351/full#supplementary-material

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REVIEW
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Microbiota and Colorectal Cancer: From Gut to Bedside

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Colorectal cancer (CRC) is a complex condition with heterogeneous aetiology, caused by a combination of various environmental, genetic, and epigenetic factors. The presence of a homeostatic gut microbiota is critical to maintaining host homeostasis and determines the delicate boundary between health and disease. The gut microbiota has been identified as a key environmental player in the pathogenesis of CRC. Perturbations of the gut microbiota structure (loss of equilibrium and homeostasis) are associated with several intestinal diseases including cancer. Such dysbiosis encompasses the loss of beneficial microorganisms, outgrowth of pathogens and pathobionts and a general loss of local microbiota diversity and richness. Notably, several mechanisms have recently been identified how bacteria induce cellular transformation and promote tumour progression. In particular, the formation of biofilms, the production of toxic metabolites or the secretion of genotoxins that lead to DNA damage in intestinal epithelial cells are newly discovered processes by which the microbiota can initiate tumour formation. The gut microbiota has also been implicated in the metabolism of therapeutic drugs (conventional chemotherapy) as well as in the modulation of radiotherapy responses and targeted immunotherapy. These new findings suggest that the efficacy of a given therapy depends on the composition of the host's gut microbiota and may therefore vary from patient to patient. In this review we discuss the role of host-microbiota interactions in cancer with a focus on CRC pathogenesis. Additionally, we show how gut bacteria can be exploited in current therapies and how mechanisms directed by microbiota, such as immune cell boost, probiotics and oncolytic bacteria, can be applied in the development of novel therapies.

Keywords: colorectal cancer, microbiota, dysbiosis, host-microbiota interactions, therapy

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INTRODUCTION

Gut Microbiota: The Neighbours We Need

In an effort to better characterize bacteria in humans, the Human Microbiome Project (HMP) was established in 2008 whose main mission is the generation of a database to allow extensive analysis of the human microbiome and assess its role in health and disease (Human Microbiome Project, 2012). The natural human microbiome consists of a large collection of several microorganisms from viruses to prokaryotes (archaea and bacteria) as well as eukaryotes. In total, a human adult harbours the same number of bacterial cells as its own human cells (Sender et al., 2016). However,

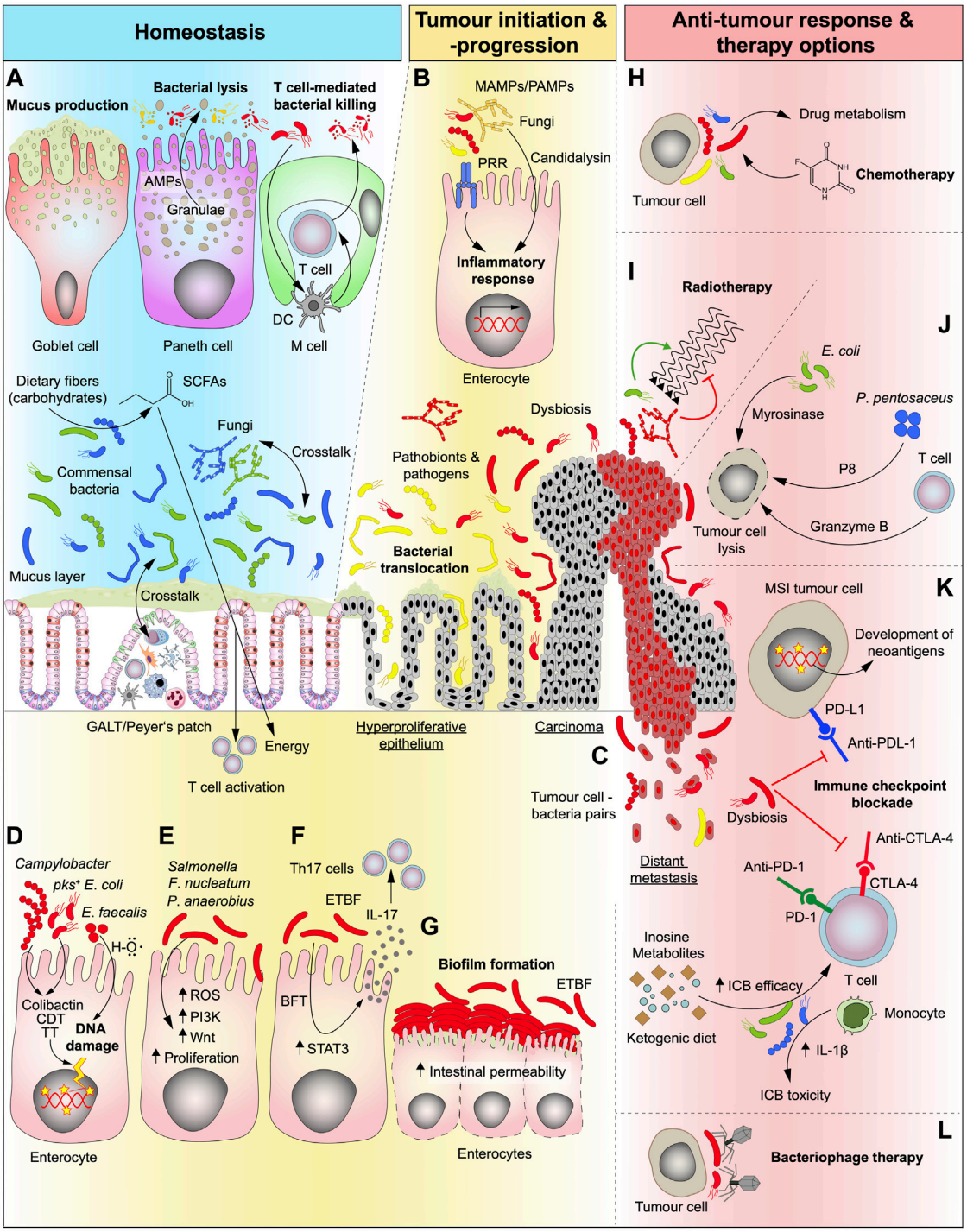

FIGURE 1 | Role of the gut microbiota under homeostatic conditions and in different stages of CRC development as well as its relevance and potential in conventional and future therapy options. **(A)** In the healthy gut, bacteria are essential for the digestive process by breaking down complex foods into metabolites that can be absorbed by the human organism. Carbohydrates are converted by bacteria into short-chain fatty acids (SCFAs) which are an energy source that can be absorbed by the human gut. The intestine is "equipped" with a plethora of mechanisms that synergistically act to help maintain a healthy microbiome composition and to separate fungi and bacteria from the host cells. Goblet cells produce mucus that serve as physical barrier keeping bacteria separated from epithelial cells and Paneth cells kill opportunistic pathogens by the secretion of antimicrobial peptides (AMPs). Microfold cells (M cells) are found in the gut-associated lymphoid tissue (GALT) of the Peyer's patches and can translocate B and T cells to the intestinal lumen in order to kill bacteria. They can also present bacterial antigens to dendritic cells (DC) and elicit an IgA-specific immune response. **(B)** In the transformation of normal to malignant tissue, microbiota is dysregulated (dysbiosis) and activate several cell-intrinsic mechanisms that fuel tumour progression. Microbe- and pathogen-associated-molecular-patterns (MAMPs/PAMPs) activate the innate immune system through

(*Continued*)

FIGURE 1 | pattern-recognition-receptors (PPR) resulting in an inflammatory response in epithelial cells. **(C)** As disease progresses, bacteria were shown to seed together with tumour cells to other organs. **(D)** Genotoxic bacteria produce toxins such as Colibactin, cytolethal distending toxin (CDT) and typhoid toxin (TT) or hydroxyl radicals (H–O) and induce DNA damage and may serve as the initiating event of a malignant transformation. *pks*, polyketide-nonribosomal peptide synthase operon. **(E,F)** Dysregulated microbiota can interact directly with epithelial and immune cells and activate CRC-related pathways, such as phosphatidylinositol-3-kinase (PI3K) and Wnt, involved in proliferation and cell survival **(E)** thereby kickstarting cellular transformation. Additionally, some genotoxins can also upregulate signal transducer and activator of transcription 3 (STAT3) pathway which leads to proliferation and T cell activation and can thereby elicit a Th17 immune response **(F)**. ROS, reactive oxygen species; ETBF, enterotoxigenic *Bacteroides fragilis*; BFT, *Bacteroides fragilis* toxin. **(G)** Accumulation of certain bacteria strains (biofilm formation) can be detected at sites of both normal and tumour tissue which can disrupt the epithelial barrier. **(H,I)** Conventional cancer therapy (radiotherapy and chemotherapy) efficiencies can be modulated by the commensal intestinal bacteria and fungi and the resulting side effects can be, attenuated. Bacterial metabolism of chemotherapeutic drugs impacts both the efficiency and the development of side effects from therapy **(H)**. While bacteria can enhance the effects of radiotherapy, fungi hamper its efficiency **(I)**. **(J)** Gut microbiota can be modulated to halt tumour progression and kill malignant cells, directly, by loading bacteria with cytotoxic cargos, such as P8 or myrosinase, or, indirectly, by stimulating immune cells. **(K)** Immune checkpoint blockade (ICB) drugs are greatly influenced by bacteria (and fungi). The presence of a given bacterial strain can increase therapy efficiency by direct stimulation of immune cells or by production of intermediate metabolites. Dysbiosis can also impair therapy efficiency and at the same time lead to therapy-related toxicity events resulting from the treatment. The presence of a hypermutated phenotype as seen in microsatellite instable (MSI) tumours leads to the generation of neoantigens at the tumour cell surface that are associated with better ICB therapy outcomes, which can additionally be influenced by the gut microbiota. The presence of a dysbiotic microbiota can inhibit the effects of ICB. **(L)** Another way to stop tumour progression could be by targeting harmful tumour-promoting bacterial strains with highly specific (designed) bacteriophages.

the number of genes encoded by these bacteria sums up to a striking number of about 2 million genes (Simon and Gorbach, 1984). The widespread colonization of bacteria in the intestine starts right after birth and results in a gradient along the gastrointestinal tract with increasingly more colony forming units (CFU) from the proximal small intestine to the colon (Sender et al., 2016). Most microbial taxa identified from human stool samples are members of the phyla of *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria* and *Verrucomicrobiota*. Over 90% of them belong to *Firmicutes* or *Bacteroidetes* phyla mostly represented by the genera *Veillonella* or *Bacteroides*, respectively. Although the fraction of these two phyla can differ between individuals, the quantity of genes encoding functionality or metabolic functions remains stable (Human Microbiome Project, 2012). Large scale comparisons including samples from different subjects sampled from different places in the intestine show not only differences between individuals, but also between anatomical sites within the intestine (Eckburg et al., 2005; Human Microbiome Project, 2012).

Bacteria have been shown to play a crucial role in the digestive process taking place in the gut and thus in many metabolic processes responsible for energy production in the human organism (**Figure 1A**). For instance, carbohydrates are fermented and synthesized into short-chain fatty acids (SCFAs) by organisms belonging to the groups of *Bacteroides*, *Bifidobacterium*, *Enterobacteria*, *Fecalibacteria* and *Roseburia* which provide a source of energy to the host (Macfarlane and Macfarlane, 2003). SCFAs can additionally regulate the activity of immune cells by promoting expansion of regulatory T cells (Tregs) and by improving the activity of effector T cells (Luu et al., 2021; Smith et al., 2013) (**Figure 1A**). Gut bacteria are also essential for the transformation of natural compounds present in the human diet. Lignans, for instance, are present in foods such as flaxseeds, vegetables and fruits and its bioconversion by bacteria renders them possible to be digested and absorbed by the human organism, where they were shown to have a protective effect against cancer and other diseases (Landete, 2012; Fuentealba et al., 2014). The conversion of lignans into secoisolariciresinol diglucoside (SDG) and the subsequent production of enterodiol (ED) and enterolactone (EL), which are responsible for the beneficial effects of lignans, involves a very complex series of steps. Eleven bacterial strains were identified to be responsible for these series of processes including several species from the genus *Clostridium* and *Bacteroides* and individual species such as *Eubacterium limosum*, *Peptostreptococcus productus* and *Eggerthella lenta* (Clavel et al., 2006). Isoflavones can be obtained from soy-based foods and are then metabolized in the gut by certain bacterial strains such as *Adlercreutzia equolifaciens*, *Enterorhabdus mucosicola* and *Slackia isoflavoniconvertens* (Vázquez et al., 2020). Also, the resulting metabolite from this interaction (O-desmethylangolensin) has been suggested to bear a protective role in a variety of diseases including cancer, cardiovascular disease and osteoporosis (Atkinson et al., 2005).

Therefore, it is not surprising that the gut microbiota is highly dependent and influenced by dietary preferences (David et al., 2014). In an elegant study from David et al. (2014) the authors were able to address the effects of two very distinct diets: a "plant-based" diet rich in grains, legumes, fruits and vegetables, and an "animal-based" diet, mostly comprising meat, eggs and cheese (David et al., 2014). By administering these diets for 5 consecutive days on human test subjects, the authors reported shifts in microbiota composition that were specifically associated with one diet or the other. For instance, the animal-based diet increased the abundance of bile-tolerant microorganisms (*Alistipes, Bilophila* and *Bacteroides*) and decreased the levels of *Firmicutes* that metabolize dietary plant polysaccharides (*Roseburia*, *Eubacterium rectale* and *Ruminococcus bromii*). Moreover, the former diet led to increased production of bile acids such as deoxycholate (DCA) which were previously described to promote cancer through mitochondrial oxidative stress (Payne et al., 2007; Yoshimoto et al., 2013). More recently, the association between diet and gut microbiome and its effect on disease predisposition was reported in a multi-generational Asian-American immigrant cohort (Vangay et al., 2018). Migrants from rural areas of Asia to the United States were followed up over generations, together with their dietary and microbial changes. Comparison of the microbiome before and after migration showed changes associated with lower phylogenetic diversity and function, reduction of *Prevotella*

strains and particularly of fiber-degrading enzymes. Interestingly, the number of obesity cases increased after migration (Vangay et al., 2018).

Considering the fact that the gut is the organ with the greatest immunological portfolio, the colonization of over 400 different bacterial species along the intestine is quite striking, which suggests an important commensal relationship between the two entities (Simon and Gorbach, 1984). Several studies have shown that the gut microbiota is able to regulate the differentiation and expansion of different types of T cells (IvanovAtarashi et al., 2009; Atarashi et al., 2011; Round and Mazmanian, 2010). Nonetheless, not all bacteria bear a protective or supportive role to the host, which is a reason why the human body has developed several mechanisms to adapt and "learn" to co-exist with bacteria. One of the most important processes in this equilibrium is the formation of a barrier that serves as a physical and chemical wall that separates bacteria from intestinal epithelial cells (Clevers and Bevins, 2013). Paneth and goblet cells are the most important intervening cell types in the mucus barrier formation, by producing granule filled with MUC2, glycosylated mucin glycoproteins, lipids as well as antimicrobial enzymes, and immunoglobulins (**Figure 1A**) (Johansson et al., 2011). The mucus functions as a physical wall that separates intestinal epithelial cells (IECs) from the luminal content of the intestine. Besides protecting intestinal cells against chemical and mechanical insults, it also helps in the removal of debris and bacteria, mediating at the same time the diffusion of small molecules such as ions, water and nutrients to the enterocytes (Paone and Cani, 2020). The gradient of bacteria in the intestine reflects also a gradient of thickness of the barrier along the intestine, increasing from ~120 μm in the jejunum to the ~830 μm in the colon. While the small intestine only harbours a rather loose layer, the colonic mucus barrier comprises a firmly attached and impermeable inner stratified layer. The more dynamic outer layer however, provides loose glycans to nourish microbial habitants (Atuma et al., 2001; Johansson et al., 2011).

For homeostasis to occur in the gut, constant flow of information needs to be assured between bacteria, IECs and immune cells. A rupture in this delicate balance might lead to bacteria invading the mucus layer and reaching direct contact with epithelial cells. Once the mucus layer is conquered by potentially pathogenic bacteria, pattern recognition receptors (PRRs) such as toll-like receptor (TLR) on IECs are activated by microbe-associated molecular patterns (MAMPs) which in turn activate a plethora of innate immune reactions resulting in inflammatory and immunomodulatory responses (**Figure 1B**). At the same time, Paneth cells, which are located in the crypt base of the small intestine, constantly release antimicrobial peptides (AMPs) (**Figure 1A**). These molecules such as defensins, lysozymes and C-type lectins feature an unspecific but effective approach in protecting stem cells in their niche (Sansonetti, 2004). In humans, the most abundant AMPs belong to the group of defensins such as α-defensin, which permeabilize the plasma membrane of Gram-negative and Gram-positive bacteria (Ganz, 2003). Indeed, impaired Paneth cell function was shown to not only result in dysbalanced microbiota but also in chronic inflammation, suggesting that Crohn's disease (CD) is a specific disorder of this cell type (Adolph et al., 2013; Tschurtschenthaler et al., 2014). Additional maintenance of homeostasis is given by the gut-associated lymphoid tissue (GALT) (**Figure 1A**). GALT produce a special type of immunoglobulins (IgA) that are released into the intestinal lumen and neutralizes potential pathogens independent of the complement system (Gutzeit et al., 2014). This process is mainly mediated by M cells which are commonly found overlying organized mucosal lymphoid tissues such as Peyer's patches. M cells, or microfold cells, like the other cells in the intestinal epithelium, derive from stem cells located at the bottom of the crypt, however, display different morphological features. For instance, M cells are devoid of apical microvilli, and present a basolateral pocket harbouring usually a B or T cell that can be translocated to the lumen and direct their action towards pathobionts such as viruses and bacteria (**Figure 1A**). These features are crucial for the mode of action of M cells regarding immunosurveillance (Dillon and Lo, 2019). Moreover, M cells specialize in the uptake and delivery of bacterial or dietary antigens from the intestinal lumen to antigen presenting cells (APCs), such as dendritic cells (DCs), located in the lamina propria. This in turn leads to activation of B and T cells and induces, the already mentioned, antigen-specific IgA production at the mucosa level (**Figure 1A**) (Okumura and Takeda, 2017).

MICROBIAL PERTURBATIONS IN COLORECTAL CANCER DEVELOPMENT

Given the high complexity of biological processes involved in maintaining a healthy environment between bacteria and human tissues, it is not surprising to find that perturbations in microbiota composition (dysbiosis) are seen in several pathological conditions including cancer (Biragyn and Ferrucci, 2018). Dysbiosis relates to any change in the composition of resident gut bacteria and fungi in comparison to the community found in healthy individuals. Moreover, it encompasses the loss of beneficial microorganisms, expansion of harmful microbes (pathobionts and pathogens), a general loss of local microbiota diversity and bacterial translocation across the epithelial barrier (Petersen and Round, 2014; Genua et al., 2021) (**Figure 1B**).

The association of the microbiota with cancer has recently been highlighted in a study that showed that different cancer types present distinct microbial signatures. Strikingly, bacteria were also found to be physically present inside the tumour cells as well as in immune cells of cancer patients (Nejman et al., 2020). This suggests that certain bacteria have a predilection for certain tissue types, likely because these tissues constitute the right environment therefore providing a growth advantage to a given bacterial community. However, the causal nature of these findings remains elusive, since only now we started to understand how bacteria interact with tumour and immune cells to boost tumour growth.

Although no disease-specific microbiota signature has been identified, patients with CRC have shown reduced bacterial diversity and richness compared to healthy individuals.

Firmicutes and *Bacteroidetes* are enriched in CRC (Nejman et al., 2020; Yachida et al., 2019). Moreover, changes in the diversity of bacterial communities in the gut is a common event in colorectal tumours. Specific bacteria, such as *Fusobacterium nucleatum* and several microbial metabolites have been associated with the onset and progression of this disease (Yachida et al., 2019; Castellarin et al., 2012; Kostic et al., 2012; Wu et al., 2021; Feng et al., 2015). Using faecal samples from a large cohort of CRC patients in different stages of disease progression Yachida et al. (2019) conducted the largest metagenomics (n = 616) and metabolomics (n = 406) analysis on human CRC to date (Yachida et al., 2019). The authors were able to pinpoint specific shifts in bacterial composition, bacterial gene abundance and associated metabolites, and map them to different stages of tumour progression. Some elements of the phyla *Firmicutes*, *Fusobacteria* and *Bacteroidetes* are predominantly elevated in carcinoma patients compared to adenomas and healthy controls, whereas species such as *Atopobium parvulum*, *Actinomyces odontolyticus*, *Desulfovibrio longreachensis* and *Phascolarctobacterium succinatutens* are mostly elevated in early stages of disease (Yachida et al., 2019). In another study with a similar approach, the authors performed metabolomic shotgun sequencing on faecal samples from 55 healthy controls, 42 advanced adenoma and 41 carcinoma patients (Feng et al., 2015). Interestingly, there was a significant increase of *Bacteroides* such as *B. dorei* and *B. massiliensis* from healthy to advanced adenoma, and an increase of *B. massiliensis*, *B. ovatus*, *B. vulgatus* and *E. coli* from advanced adenoma to carcinoma. Concomitantly, the lactic acid-producing bacteria *Bifidobacterium animalis*, *Streptococcus mutans* and *Streptococcus thermophilus* were enriched in control faecal samples only. Lactate production was shown to accelerate colon epithelial cell turnover and to maintain gut homeostasis. The decrease in lactic acid-producing commensals caused by dysbiosis, could lead to progression of early to advanced disease stages (Feng et al., 2015). More recently, bacterial communities were also shown to vary their composition during CRC progression (Wu et al., 2021). By comparing the microbiome in stool samples from 306 adenoma patients, 217 CRC subjects and 252 healthy controls, the authors identified 43 bacteria species with distinguishable differential abundances between controls and patients with adenoma. Similarly, 114 differentially abundant bacteria were detected between adenoma and cancer (Wu et al., 2021). Of note, *Fusobacterium nucleatum* was enriched in cancer samples compared to adenoma further confirming previous findings (Yachida et al., 2019). In fact, several studies have suggested a pro-tumorigenic effect of *F. nucleatum* in CRC both *in vitro* and *in vivo* (Yang et al., 2017; Kostic et al., 2013). Using a cohort of human primary CRC and paired hepatic and lymph node metastasis, Bullman et al. (2017) showed the presence of *F. nucleatum* not to be restricted to the primary site of disease (primary tumour) but also at distant sites (metastases). Notably, whole genome sequencing (WGS) analysis and culture of bacteria isolates from tumour-metastasis pairs showed almost complete sequence identity between both sites, strongly indicating that bacteria could indeed migrate with tumour cells to metastatic sites or even facilitate the metastatic process (Bullman et al., 2017) (**Figure 1C**). Interestingly, only *F. nucleatum*-positive tumours derived from CRC patients successfully engrafted in mice (PDX model). Treatment of the latter with metronidazole (a drug known to kill *Fusobacteria*) resulted in decreased tumour growth compared to controls while no effect of the drug was seen in *Fusobacterium*-negative xenografts (Bullman et al., 2017). Although proof of a direct stage-specific functional effect of the microbiota on human tumour lesions remains to be uncovered, it is reasonable to assume that CRC progression can be influenced by the microbiota and its metabolites, thereby adding an extra layer of complexity to the classical adenoma-carcinoma progression model (Fearon and Vogelstein, 1990).

The combined data from the aforementioned studies together with the decrease in the costs of sequencing technologies over time, suggests the possibility of using the gut bacteria for diagnostic assessment of disease. In fact, the idea of using bacteria as a non-invasive way to predict disease has been explored in several recent studies and the results seem indeed promising (Liang et al., 2020; Poore et al., 2020; Wu et al., 2021). Remarkably, by combining the data of the differentially enriched bacteria in different stages of disease together with patient data (such as age, gender and BMI) the authors could successfully distinguish adenoma from healthy control subjects (AUC 0.8) and adenoma from cancer samples (AUC 0.89) (Wu et al., 2021). Concomitantly, metagenomic analysis of the faecal microbiome of a large cohort of normal controls, adenoma and carcinoma samples allowed the identification of a bacterial gene marker (*m3*) from a *Lachnoclostridium sp.* for the diagnosis of colorectal adenoma (Liang et al., 2020). By combining this marker with the presence of *Fusobacterium nucleatum*, *Clostridium hathewayi*, *Bacteroides clarus* and faecal immunochemical test (FIT) the authors reported a sensitivity of 94% for diagnosing CRC (Liang et al., 2020). Notably, by looking at the bacterial sequences of available TCGA data from many distinct cancer types and controls, Poole et al. (2020) developed a machine learning approach which allowed the discrimination between cancer types (n = 32), and from tumour and normal samples in 15 different cancer types. The strength of the pipeline was validated by applying the same procedure on WGS sequencing data of circulating tumour DNA (ctDNA). Once more, the approach proved to be very efficient in differentiating distinct tumour types even for early-stage disease and for cancers that do not show any genomic alterations, thus overcoming one of the main limitations of current existing ctDNA assays (Poore et al., 2020).

We only recently started to unveil the great potential of using bacteria as a diagnostic tool in CRC and other cancer types. Hence, several technical limitations should be taken into account in the process, including the possibility of sample contamination during collection, sample processing, and overall sequencing costs. It is therefore imperative that proper procedural controls are included in the study design in order to minimize the contributions of contaminants to microbial signatures and

prevent interpretation bias of the results (Salter et al., 2014; Eisenhofer et al., 2019).

Besides bacteria, the intestinal tract is also colonized by a variety of fungal species that make up for approximately 0.001–0.1% of the total gut microbiota. The most common phyla that constitute the gut mycobiome belong to *Basidiomycota, Glomeromycota* and *Ascomycota,* and like bacteria, fungal dysbiosis has also been associated with disease (Liguori et al., 2016; Luan et al., 2015). Notably, several studies have reported changes in gut fungi communities in CRC and inflammatory bowel disease (IBD) patients in comparison to healthy individuals (Luan et al., 2015; Gao et al., 2017; Coker et al., 2019). Nonetheless, no disease-specific mycobiome signature has so far been identified. One of the ways by which fungi can contribute to inflammation and ultimately CRC, comes from the observations that *Candida albicans* and *Candida tropicalis* are increased in inflammation and cancer settings. These two fungal species produce a cytosolic peptide called candidalysin, known to promote the disruption of the epithelial barrier function (Qin et al., 2021) (**Figure 1B**). It was suggested that toxic metabolites and other substances produced by fungi could lead to disruption of cell-intrinsic pathways as well as impair immune signalling in order to drive disease. Additionally, the crosstalk between fungi and bacteria adds another perspective to the current state of knowledge in the field, as it has been shown that these two entities can influence each other on different levels (Vallianou et al., 2021) (**Figure 1A**). Further studies are needed, however, to complement the existing information regarding the role of the mycobiome in disease aetiology. The current knowledge on the association of fungi in different cancer types has been recently reviewed elsewhere (Vallianou et al., 2021).

As previously stated, viruses are also a component of the gut microbiota and their role in disease is well elucidated. Viruses are able to infect a plethora of human tissues including the upper respiratory tract and lungs, the colon, liver and blood cells. Upon infection, they are able to induce signalling in host cells and control processes such as growth and survival which are usually altered in cancer (Mesri et al., 2014). Another way through which viruses can induce cell transformation is by inducing the DNA damage response machinery in infected cells causing genomic instability which increases mutation rate (McFadden and Luftig, 2013). Finally, viruses can induce chronic inflammatory reactions which increase the levels of cellular stress and reactive oxygen species (ROS) ultimately leading to the acquisition of mutations (Arzumanyan et al., 2013). It is estimated that almost 10% of cancer cases are caused by viruses, and to date seven viruses have been identified with a strong link to several cancer types (Plummer et al., 2016). Of note, Epstein-Barr virus (EBV) increases the risk of Burkitt lymphoma and other lymphoma types as well as gastric cancer (Young et al., 2016), while hepatitis B (HBV) and C virus (HCV) infections can lead to liver cancer (El-Serag, 2012). Some viruses such as Merkel cell polyomavirus, human herpes virus 8 (HHV-8), human papillomavirus (HPV) and human T-cell leukemia virus type (HTLV-1) can be sexually transmitted and cause several types of cancer including lymphomas and leukaemia, Kaposi sarcoma and carcinomas (Krump and You, 2018).

MECHANISMS OF COLORECTAL CANCER-PROMOTING BACTERIA

Although bacteria are necessary for many biological processes in the organism, they also contribute to different pathological states when dysregulated. New mechanisms have been discovered by which bacteria and their metabolites or toxins can cause direct DNA damage and induce oncogenic mutations. For instance, *E. faecalis* infection leads to increased production of hydroxyl radicals which are known to cause DNA damage (Wang and Huycke, 2007) (**Figure 1D**). On the other hand, *P. anaerobius,* which is increased in human colon tumours, increases ROS levels leading to higher cholesterol levels and increased cell proliferation in colon cancer cells (Tsoi et al., 2017) (**Figure 1E**). Additionally, *P. anaerobius* can also selectively adhere to the CRC mucosa *in vivo* and promote tumour growth in a process mediated by its surface protein, putative cell wall binding repeat 2 (PCWBR2). In this scenario, CRC progression is thought to occur due to the interaction of bacterial PCWBR2 with eukaryotic cells' α2/β1 integrin, which induces the activation of the PI3K–AKT pathway in cancer cells (**Figure 1E**). This in turn leads to increased cell proliferation and NF-κB-driven inflammation. Surprisingly, the resulting effects from this interaction were greatly attenuated or even absent in normal colonic cells (Long et al., 2019). *Salmonella* infection in humans can become chronic if not treated properly and was shown to cause low-grade but persistent inflammation. Infection with an AvrA-expressing *Salmonella* strain of an inflammation-driven CRC mouse model led to increased activation of STAT3 and Wnt signalling pathway thus leading to an increased proliferation and tumourigenesis in these animals (Lu et al., 2016) (**Figure 1E**).

Mounting evidence points to the fact that bacteria can sense stage specific features of cancer cells and use those as signalling cues for their own advantage. In line with this, one of the possible mechanisms by which *Fusobacterium nucleatum* may contribute to the progression of CRC has been reported by Rubinstein et al. (2019). *F. nucleatum* interacts with cancer cells through its adhesin molecule FadA which in turn binds eukaryotic Annexin A1 in a process mediated by E-cadherin. Furthermore, the authors showed that FadA, Annexin A1 and E-cadherin form a protein complex with β-catenin thereby modulating its expression (Rubinstein et al., 2019). β-catenin is a central effector of Wnt pathway which is dysregulated in the majority of CRC (Dekker et al., 2019) (**Figure 1E**).

In addition to the aforementioned mechanisms, bacteria were shown to produce genotoxins such as colibactin, cytolethal distending toxin (CDT) and typhoid toxin (TT) that are able to induce DNA damage *in vitro* and fuel CRC *in vivo* (Cuevas-Ramos et al., 2010; Arthur et al., 2014; Cougnoux et al., 2014; Martin et al., 2019) (**Figure 1D**). For instance, CDT is composed of three subunits CdtA, CdtB and CdtC, and has the ability to induce host DNA damage due to its DNase I-like property (He et al., 2019). The association between the CDT-producing bacteria *Campylobacter* spp. with cancer and inflammation has been reported (Gradel et al., 2009; Allali et al., 2015). More recently *Campylobacter* spp. was also shown to directly promote tumorigenesis in a CRC mouse model, in a process

dependent on the production of CDT (He et al., 2019). On the other hand, colibactin is produced by *E. coli* from a 50-kb hybrid polyketide-nonribosomal peptide synthase operon (*pks*) encoded in its genome. It has been shown that colibactin causes DNA double-strand breaks and activation of the DNA damage checkpoint pathway that can lead to cell death both *in vitro* (Nougayrède et al., 2006) and *in vivo* (Cuevas-Ramos et al., 2010) (**Figure 1D**). Furthermore, an association between this toxin and cancer (Dejea et al., 2018) and IBD (Arthur et al., 2012) has been reported. In an effort to address the effects of pks^+ *E. coli* in the transformation of epithelial cells of the human colon, Pleguezuelos-Manzano and colleagues (Pleguezuelos-Manzano et al., 2020) microinjected this bacteria strain into the lumen of human clonal intestinal organoids. WGS analysis of the latter enabled the identification of a very distinct mutational signature in the infected organoids compared to controls. Surprisingly, this signature was also shown to be present in human cancers (Pleguezuelos-Manzano et al., 2020). These results were recently corroborated in murine organoids (Iftekhar et al., 2021). As such, infection of mouse normal colon organoids with pks^+ *E. coli* leads to increased DNA damage, megalocytosis, formation of multinucleated cells as well as mutations in common cancer-associated genes. Surprisingly, infected organoids lost their dependence on medium Wnt agonists by upregulating Wnt/β-catenin signalling while downregulating differentiation genes such as carbonic anhydrase 4 (*Car4*) and aquaporin 8 (*Aqp8*) (Iftekhar et al., 2021). In line with this, another study showed that targeting the metabolism of malignancy-promoting colibactin-producing *E. coli* strains in the gut microbiota in an AOM/DSS cancer mouse model, reduces the risk of CRC development (Zhu et al., 2019). *Bacteroides fragilis* toxin (BFT) produced by enterotoxigenic *Bacteroides fragilis* (ETBF) is also associated with colitis and CRC (Boleij et al., 2015). In fact, colonizing multiple intestinal neoplasia ($Min^{ApcΔ716/+}$) mice heterozygous for the *Apc* gene, with ETBF, leads to the formation of tumours in the distal colon in a process mediated by IL-17 (**Figure 1F**) (Wu et al., 2009). On a molecular level, bacterial infection led to STAT3 activation in the colon of these animals which in turn leads to a Th17 response characterized by IL-17-secreting $CD3^+CD4^+$ and $CD3^+CD4^-$ cells (**Figure 1F**). The Th17 response is associated with tumour progression through its role in immunosuppression and angiogenesis (Asadzadeh et al., 2017; Salazar et al., 2020). Conversely, blocking IL-17 production in the intestine attenuates tumorigenesis in this mouse model (Wu et al., 2009).

More recently, introduction of a $BRAF^{V600E}$ mutation into the pre-existing $Min^{ApcΔ716/+}$ ETBF mouse model (Wu et al., 2009) caused a shift in the spectrum of colon tumours towards a more proximal location within the colon (DeStefano Shields et al., 2021). In addition, these tumours displayed a serrated-like histopathology and were characterized by a strong IFNλ-driven immune signature. Notably, this signature was associated with a recruitment of PD-L1 expressing myeloid-derived suppressor cells (MDSC) and $CD8^+$ tumour infiltrating T cells. In line with this, treatment of tumour-bearing mice with PD-L1 blockade therapy led to reduced colon tumour numbers in this model (DeStefano Shields et al., 2021). Additionally, loss of membrane-associated E-cadherin, *in vitro* was reported after treatment of a colon cancer cell line with BFT (Wu et al., 2003). However, the exact biological mechanisms supporting the association CRC and ETBF in humans requires further studies.

Another mechanism by which the gut microbiota can promote CRC is the formation of biofilms, which are aggregates of bacteria encased in a polymeric matrix mostly present on right-sided colon tumours (Dejea et al., 2014). Interestingly, biofilms increase the permeability of the intestinal barrier allowing bacterial invasion and at the same time increasing proliferation of the tissue (Dejea et al., 2014). The association between biofilms formation and sporadic human CRC has been reported (Dejea et al., 2014; Johnson et al., 2015). In the first effort to associate bacterial biofilms with CRC, Dejea and colleagues (2014) showed that the presence of invasive polymicrobial bacterial biofilms is associated mostly with right sided tumours in a cohort of CRC patients. Strikingly, patients with biofilm-positive lesions also showed the presence of biofilms in the normal intestinal mucosa far from the tumour (Dejea et al., 2014). In addition, intestinal barrier integrity was compromised in biofilm-positive samples. Although global expression levels of E-cadherin were not altered between biofilm-positive and biofilm-negative samples, there was a shift of the marker expression to the basal pole of the epithelial cells in the former samples, which was consistent with the observation of increased permeability of the epithelial barrier (Dejea et al., 2014). The same group has later suggested that one way by which bacterial biofilms might affect tumour formation and growth is by the production of the polyamine metabolite N1, N12- diacetylspermine which was found to be upregulated in biofilm-positive samples (tumour and healthy tissue) compared to biofilm-negative samples (Johnson et al., 2015). More recently, the association between biofilms and hereditary CRC was also reported (Dejea et al., 2018). In a subset of familial adenomatous polyposis (FAP) patients, biofilms were mainly composed of *Proteobacteria* and *Bacteroides*, with an overrepresentation of *E. coli* and *B. fragilis* species (Dejea et al., 2018). The presence of colibactin and BFT produced by the above-mentioned bacterial strains, respectively, was significantly associated with the mucosa of FAP patients (**Figure 1G**). In order to validate these findings, the authors colonized the colon of two distinct CRC mouse models with *E. coli* and *B. fragilis*, which led to faster tumour growth and increased mortality of the animals (Dejea et al., 2018).

Malignant transformation of eukaryotic cells is a very complex biological process that is influenced mainly by genetic and epigenetic cues that come both from within the cell but also from cell-extrinsic mediators such as stromal and immune cells (Hanahan and Weinberg, 2011). The study of the microbiome in the cancer setting suggests that a new variable must be included in this relationship given the mutagenic abilities of some bacteria that inhabit the human gut. Only recently we started to unravel how bacteria can indeed directly influence cell pathways and how this leads to a disease state. Given the tremendous abundance and diversity of bacteria in the human organism it is likely that many other mechanisms will be identified in the years to come.

THE ROLE OF THE MICROBIOTA IN DRUG METABOLISM

With a ratio of approximately 1:1 of bacteria and cells in the human body, these microorganisms additionally encode for 150-fold more genes than the human genome (Sender et al., 2016). The identification of microbiota-specific metabolic signatures deepens the understanding of the relationship between bacteria and human cells and can help predict the response to a given drug or chemical compound. By using a library of 833 metabolites, Han S. and colleagues (Han et al., 2021) identified the metabolic profiles of 178 gut bacteria through a combination of mass spectrometry and a machine learning pipeline. Studies like the aforementioned are able to comprehensively map genes to metabolic features of bacteria and ultimately link this data to phenotypic bacterial variation which can be greatly explored in the context of therapy. Since all orally administered compounds are primarily absorbed in the gut, together with the liver these are the places where most of the metabolic transformation of therapeutic drugs occurs (Foti et al., 2015). It is important, however, to address the bidirectional relation between drug compounds and microbiota since drugs have a stark effect on microbiota composition and can lead to dysbiosis. On the other hand, it has also been recognized that bacteria have the capacity to metabolize drugs (Maier et al., 2018). Examples of drug-induced toxicity on bacteria are given by anti-diabetics such as metformin (Forslund et al., 2015), proton pump inhibitors (PPIs) (Imhann et al., 2016) and nonsteroidal anti-inflammatory drugs (Rogers and Aronoff, 2016). In an effort to broadly and systematically address these effects, Maier and colleagues (2018) treated 40 different bacteria species with 1,197 drugs belonging to different therapeutic classes, excluding antibiotics (Maier et al., 2018). The authors concluded that nearly 30% of the compounds tested inhibited the growth of at least one bacterial species and speculate that antibiotic resistance might also arise due to microbiota changes when exposed to non-antibiotics (Maier et al., 2018). Distinct additional strategies have been employed to dissect the causes and consequences of drug-microbiota interaction such as gain of function and loss of function genetic screens (Zimmermann et al., 2019a; Zimmermann et al., 2019b) as well as probe enzymatic activity assays in order to identify enzymes responsible for specific drug conversions (Jariwala et al., 2020). To study the effect of the microbiota on drug metabolism of the host, García Gonzalez and colleagues (García-González et al., 2017) used the nematode *C. elegans* as a model. By treating *C. elegans* with 11 different therapeutic drugs while feeding them different bacterial diets they were able to unravel distinct host-microbiota responses to therapy. Dietary *E. coli* and *Comamonas* oppositely affected the response to 5-fluoro-20-deoxyuridine (FUDR), and the topoisomerase I (topo-I) inhibitor camptothecin (CPT) which are two commonly used chemotherapeutics in CRC treatment. Thus, more FUDR-treated animals survived when fed with *Comamonas*, while less survival was reported upon treatment with CPT. The opposite trend for both drugs was reported when *C. elegans* was fed with *E. coli*. Interestingly there were no differences in the efficacy of 5-fluorouracil (5-FU) between the 2 diet regimens evaluated (García-González et al., 2017).

The metabolic capacity of the gut microbiota is a topic of great recent interest as it can help explain the differences in therapy outcomes between patients with similar pathologies treated with the same therapeutic regime. Moreover, the identification of toxic by-products of bacterial drug metabolism could help predict possible side effects in patients under treatment. Given the broad range of effects of compound metabolism by the microbiota that include drug activation (Sousa et al., 2014), inactivation (Haiser et al., 2013) or toxicity (Zimmermann et al., 2019a), the precise identification of the bacteria or bacterial signatures that lead to a certain metabolic outcome poses one of the biggest questions of current disease treatments (**Figure 1H**). For instance, glucuronidation is a phase II transformation that occurs in the liver and inactivates and detoxifies drugs by conjugating them to glucuronic acid (GlcA). These glucuronides are then transported to the intestine where they are excreted from the human body (Li and Jia, 2013). However, once in the intestine, these compounds can also be reactivated by the removal of the GlcA carried out by gut bacterial β-glucuronidases (GUS) enzymes, which leads to local acute toxicity (Jariwala et al., 2020). For instance, irinotecan (CPT-11) is a potent anticancer drug included in different first line therapy regimens to treat several cancer types including CRC (Kelly and Goldberg, 2005). Side effects such as severe diarrhea are common in patients treated with irinotecan. Irinotecan is converted to its active form, human topoisomerase I poison SN-38 in the liver, and later inactivated by DP-glucuronosyl-transferases by adding a GlcA (SN-38-G) conjugate to the original molecule. In the intestine, this inactive conjugate is reactivated by GUS enzymes, which leads to acute toxicity (Wallace et al., 2015). Using a combination of proteomics and bioinformatic analyses on human stool samples, Jariwala et al. identified the GUS enzymes responsible for SN-38 reactivation in the human gut, which is the toxic metabolite of irinotecan (Jariwala et al., 2020). This approach is scalable to other treatment regimens and may therefore be employed to reveal additional biomarkers for prognostic assessment in the era of personalized medicine.

In another effort to characterize the direct metabolic interactions between microbiota and chemical compounds, Javdan and colleagues (2020) developed a tool to identify metabolites generated by microbiome-derived (MDM) enzymes in a set of 23 orally administered drug compounds in human healthy donors. This study encompasses one of the most comprehensive and technically challenging approaches to date, as it involved several different but complementary methodologies including microbial community cultivation, small-molecule structural analysis, quantitative metabolomics, functional genomics and metagenomics, mouse colonization assays, as well a strong bioinformatic component. The authors elegantly showed the feasibility of this system to identify MDM enzymes in a high throughput fashion using drugs from different groups with very distinct modes of action (Javdan et al., 2020). In a similar approach, Zimmermann *et al.* (2019) measured the *in vitro* capacity of 76 naturally occurring bacteria in the human gut

to metabolize 271 orally administered drugs belonging to different groups depending on the mode of action (Zimmermann et al., 2019b). Strikingly, up to two thirds of the drugs examined in this study were shown to be metabolized by at least one of the bacteria included in the study (**Figure 1H**). Moreover, a given bacteria could metabolize up to 95 different drugs. Using a combination of metabolomics, mass spectrometry and DNA sequence analysis, the authors were able to identify specific drug-metabolizing gene products that are responsible for the conversion of drugs into their metabolites (Zimmermann et al., 2019b). Finally, *in silico* tools have also been developed to allow the identification of drugs and respective metabolites by a specific bacteria species (Mallory et al., 2018), and even to predict toxicity events by integrating information regarding bacteria composition, drug activity and diet preferences (Guthrie et al., 2019).

Increasing evidence puts the gut microbiota in the spotlight when discussing drug metabolism in the human organism, as bacteria and their metabolites can impact pharmacokinetics and pharmacodynamics. This becomes particularly important in the context of therapy. The effect of the microbiota in conventional chemotherapy and immune checkpoint blockade therapy (ICB) will be addressed in the next chapter.

MICROBIOTA AS MODULATOR OF CONVENTIONAL COLORECTAL CANCER THERAPY

Chemotherapeutics have been used for decades and are still a common first-line treatment approach to treat a variety of human tumours, including CRC (Dekker et al., 2019). Nonetheless, these drugs are likely to cause treatment-related morbidities and mortality in a high percentage of patients (Dekker et al., 2019). Given the fact that CRC occurs in physical proximity to the gut bacteria, recent studies are focusing on how intestinal microbiota modulates the efficacy and toxicity of current chemotherapeutic drugs (Brandi et al., 2006; Stringer et al., 2009; Lin et al., 2012; Iida et al., 2013). Fluoropyrimidine-based chemotherapy in combination with oxaliplatin and irinotecan are the standard first line treatment regimens for unresectable advanced stage CRC (Dekker et al., 2019). It was shown that the use of conventional chemotherapeutic drugs such as irinotecan (Lin et al., 2012), 5-FU (Stringer et al., 2009) and cyclophosphamide (Viaud et al., 2013) causes changes in the microbiota diversity of mice in preclinical models and in human patients. However, it is still not clear how this impacts prognosis as some studies show opposing results regarding the effects of microbiota in therapy. For instance, germ-free mice tolerate higher doses of irinotecan and exhibit less gastrointestinal damage as a side effect from therapy (Brandi et al., 2006). This can be due to the production of toxic metabolites resulting from bacterial metabolism of the administered compounds. As discussed in the previous chapter, removal of GlcA from SN38-G leads to reactivation of SN38 resulting in adverse side effects to the patient (Wallace et al., 2015). Thus, inhibiting the production of GUS enzymes prevents intestinal toxicity and stabilizes the antitumor efficacy of irinotecan (Bhatt et al., 2020).

Taken together, these results suggest that the presence of some bacteria can lead to increased treatment-related side effects and toxicity. On the other hand, therapy efficacy can also be modulated by the gut microbiota (Taper and Roberfroid, 2005; Iida et al., 2013; Viaud et al., 2013). Interestingly, there seems to be a dual role of bacteria in cancer therapy as some studies report a synergistic effect of microbiota and drug efficacy, while others show the presence of bacteria to compromise therapy. More than 10 years ago, it was shown that supplementation of a diet rich in inulin or oligofructose led to growth inhibition of a transplantable tumour mouse model (Taper and Roberfroid, 2005). Inulin and oligofructose are fructans shown to promote the growth of *Bifidobacteria* in the gut (Gibson et al., 1995). The efficacy of 6 different chemotherapeutic drugs (5-FU, Doxorubicine, Vincristine, Cyclophosphamide, Methotrexate, Cytarabine) was potentiated by the addition of these supplements to the animals' diet, suggesting a prebiotic effect of inulin and oligofructose (Taper and Roberfroid, 2005). In a preclinical model in which mice were injected with CRC cells, tumours showed resistance to gemcitabine treatment. The authors of this study reported that *Gammaproteobacteria* present in the tumour have the capacity to metabolize the drug into its inactive form, rendering the therapy ineffective (Geller et al., 2017). The therapeutic effect of gemcitabine was rescued when mice were treated with the antibiotic ciprofloxacin in combination with gemcitabine (Geller et al., 2017). In another study, by treating CRC cells with Oxaliplatin and 5-FU in combination with *F. nucleatum*, Yu and colleagues (2017) showed that bacteria-positive cells are resistant to the therapy compared to controls. To achieve this, *F. nucleatum* induces autophagy by stimulating the expression of pULK1, ULK1, and ATG7 proteins, rendering the therapy less effective in CRC cells (Yu et al., 2017). In addition, germ-free or antibiotic treated mice showed resistance to cyclophosphamide treatment, and have a poorer anti-tumour response in a sarcoma allograft mouse model in comparison to SPF mice (Viaud et al., 2013). In another study (Iida et al., 2013), mice transplanted subcutaneously with three different tumour cell lines (including a colon cancer cell line) harbouring a normal gut microbiota (WT) or absence of intestinal bacteria (antibiotic treated), were treated with different immunotherapy and chemotherapy regimens. Strikingly, antibiotic treatment impaired both therapies' efficacy leading to higher tumour volumes and decreased survival of the animals when no microbiota is present (Iida et al., 2013). This response was shown to be dependent on TNF production by tumour-associated myeloid cells followed by a $CD8^+$ T cell response. By correlating faecal microbiota composition with TNF production in antibiotic treated mice, the authors identified *A. shahii* species from the genus *Alistipes* to bear the strongest association. Thus, oral administration of *A. shahii* to microbiota-depleted mice reconstituted the ability of tumour-associated myeloid cells to produce TNF leading to an anti-tumour response (Iida et al., 2013). In line with this, a recent study identified two SCFAs (pentanoate and butyrate) as enhancers of adoptive cell therapy in cancer (Luu et al., 2021). By treating mice injected with B16OVA melanoma cells, with

$CD8^+$ T cells previously exposed to butyrate or pentanoate, the authors reported decreased tumour volume and weight compared to non-treated T cells. The mechanism through which SCFAs achieve this effect in T cells is by inhibition of class I histone deacetylases (HDACs) and upregulation of mTOR complex, a key regulator of cell growth and metabolism of immune cells (Luu et al., 2021). In order to test this approach in different treatment settings, the authors used $CD8^+$ CAR T cells that recognize ROR1, a molecule highly expressed in epithelial tumours, to treat mice transplanted with ROR1-expressing pancreatic tumour cells. Treatment of the tumour bearing mice with ROR1-CAR T cells previously exposed to pentanoate led to tumour regression in these animals (Luu et al., 2021).

Faecal Microbiota Transplantation (FMT) was first used in 1958 to treat *Clostridium difficile* infection (CDI) (Eiseman et al., 1958). By helping restoring a beneficial microbiome in infected patients, it was possible to treat up to 80% of all CDI cases. FMT led to effective results in several other conditions such as IBD, diabetes and even autism and therefore became a promising treatment option (Gupta et al., 2016). Given its safety, the advantages of this strategy were also addressed as a way to ameliorate adverse effects from radiotherapy treatment. Radiotherapy is used as first line treatment option in combination with chemotherapy (chemoradiotherapy) for CRC treatment (Dekker et al., 2019). As a result of this treatment, patients may experience various side effects, including bone marrow and gastrointestinal toxicity. Preclinical studies showed that bacteria could ameliorate the side effects of radiotherapy treatment regimens (Cui et al., 2017). FMT from young healthy mice to irradiated mice with ionizing radiation greatly improved the survival of the latter in comparison to non-FMT irradiated controls. Strikingly, the best therapeutic outcomes were achieved when sex-matched donors were used for the FMT strategy (Cui et al., 2017). Moreover, administration of specific bacteria such as *Lactobacillus rhamnosus* to mice undergoing radiotherapy was shown to elicit a protective effect in the intestinal mucosa of test subjects (Ciorba et al., 2012). Clinical studies enrolling cancer patients under radiotherapy have shown that the use of probiotics led to a decrease of radiation-induced gut toxicity, such as diarrhea (Ma et al., 2019). Moreover, the gut microbiome was also recently shown to impact radiotherapy efficacy in distinct preclinical cancer mouse models (Shiao et al., 2021). Interestingly, radiotherapy treatment of antibiotic-treated mice (bacteria specific) failed to delay tumour growth in 2 orthotopic mouse models of breast and melanoma cancer, respectively, in comparison to mice harbouring a normal microbiota (**Figure 1I**). Conversely, ablation of fungal communities in the gut improved survival of animals and elicited a strong anti-tumour response after radiation treatment in the aforementioned cancer models. The authors further concluded that the treatment with antifungal antibiotics prior to treatment with ionizing radiation leads to a reduction of $CD206^+F4/80^+$ suppressive macrophages and a sharp increase of Granzyme B expressing $CD8^+$ T cells. These molecular settings further enhance the immune response against the tumour. Using the same cancer models, this study elegantly highlights the opposing roles of two very distinct organism types (bacteria and fungi) inhabiting the gut in relation to the effectiveness of radiotherapy (Shiao et al., 2021) (**Figure 1I**).

The future of cancer therapy is bound to explore the dual role of gut bacteria in drug responses: on one hand, bacteria, as a result of their metabolism can aggravate the side effects of therapy. On the other hand, the presence of bacteria is crucial for the success of cancer therapy. Recent studies in which the effects of fungi in disease are also addressed (Aykut et al., 2019; Limon et al., 2019; Iliev and Cadwell, 2021; Shiao et al., 2021) further widens the complexity of the relationship between external commensal organisms with host immune cells. These findings raise the possibility that probiotics and other microbiota-modulating compounds could be used as adjuvant therapy for cancer treatment.

BACTERIA IN CANCER IMMUNE CHECKPOINT BLOCKADE THERAPY

The concept of immunotherapy relies on the recognition of tumour cells expressing a specific antigen of the major histocompatibility complex (MHC) by T cells, through its receptors (TCRs) (Waldman et al., 2020). Several molecules are known to regulate this complex biological process. Among them, the cytotoxic T lymphocyte-associated protein 4 (CTLA-4), programmed cell death 1 (PD-1), and PD-1 ligand (PD-L1) received great attention in recent years due to their use in cancer therapy. The function of these molecules is complementary and they act in order to ensure that T cell responses preserve self-tolerance and protect the body from pathogens and neoplasia development (Waldman et al., 2020). Using the immune system to elicit a therapeutic effect against tumour cells is the basis of the concept of immune checkpoint blockade (ICB) therapy. Targeting CTLA-4 (ipilimumab), PD-1 (nivolumab) or its ligand PD-L1 (pembrolizumab) has been shown to be extremely effective in the treatment of a variety of advanced cancers including melanoma and non-small cell lung cancer (Hodi et al., 2010; Brahmer et al., 2012). Many other cancer types however show resistance to this type of compounds thereby compromising the overall therapy efficacy. Given the great potential of ICB therapy in cancer, the identification of the reason (or reasons) leading to the acquisition of resistance upon treatment is of the utmost importance in the field. Recent studies have suggested that the composition of the gut microbiota can predict the effectiveness of ICB therapy in both human patients and animal models (Sivan et al., 2015; Vétizou et al., 2015; Gopalakrishnan et al., 2018; Matson et al., 2018; Routy et al., 2018; Pinato et al., 2019; Tanoue et al., 2019). In fact, antibiotic treatment of patients with a wide variety of solid tumours (including non–small cell lung cancer and melanoma), prior to ICB therapy is associated with a worse treatment response and overall survival (OS) (Pinato et al., 2019). Consistently, dysbiosis was shown to lead to acquired resistance to ICB therapy (Routy et al., 2018). Moreover, the therapeutic efficacy of ipilimumab, an antibody directed towards CTLA-4, was shown to be influenced by the microbiota composition of the host both

in humans and in several preclinical tumour models (Vétizou et al., 2015). In the latter, the authors also reported that germ-free and antibiotic-treated mice did not show response to CTLA-4 blockade therapy. Interestingly, inoculation of specific bacterial species in these mice (*B. fragilis* and/or *B. thetaiotaomicron* and *Burkholderiales*) rescued the therapeutic effect of ipilimumab in a T1H dependant manner (Vétizou et al., 2015). In another seminal study conducted by Sivan et al. (2015) the authors further uncovered the ways through which the microbiome can modulate therapy response in solid tumours (Sivan et al., 2015). When cohousing animals with similar tumour models from different animal facilities, the authors noted that the previously observed differences (before co-housing of the animals) in terms of tumour growth, were then eliminated. FMT experiments from one group of animals to the other showed similar results highlighting the importance of microbiota in tumorigenesis. Additionally, treatment of these animals with antibodies targeting PD-L1 resulted in slower tumour growth and this response was found to be mediated by the increased induction and infiltration of $CD8^+$ T cells. *Bifidobacterium* was identified to be the responsible bacterial community for this effect, that, in combination with anti-PD-L1 therapy, almost abolished tumour growth (Sivan et al., 2015). The possibility of the existence of a protecting and also therapy-prone microbiota is again elegantly supported by the work of Routy et al. (2018) in which the authors used a similar approach to dissect the influence of the microbiota in the efficacy of ICB therapy (Routy et al., 2018). FMT from cancer patients who responded to ICB therapy into germ-free or antibiotic-treated mice, greatly increased the efficacy of PD-1 blockade in different cancer models. When analysing the microbiota of cancer patients who responded to ICB, or did not, one commensal bacteria (*A. muciniphila*) was found to be enriched in responders compared to non-responders and its presence associated with longer patient progression free survival (PFS). Moreover, oral supplementation of mice that were subjected to FMT from patients that did not respond to ICB with *A. muciniphila*, restored the responsiveness of PD-L1 blockade therapy (Routy et al., 2018). In accordance to these findings, treating mice with metformin, a drug primarily used to treat type-2 diabetes which suppresses glucose production in the liver, was found to improve the abundance of *A. muciniphila* in the gut of aged obese mice. Inoculation of obese mice with *A. muciniphila* led to improved body weight and lipid profiles in these animals (Lee et al., 2018). Combined, these findings underlie possible synergistic effect between therapeutic compounds and bacteria in disease treatment. In another study (Matson et al., 2018), using a similar methodology, the authors identified *Bifidobacterium longum*, *Collinsella aerofaciens*, and *Enterococcus faecium* to be more abundant in the stool of melanoma patients that responded to anti PD-L1 therapy compared to non-responders. FMT from responders into germ-free mice induced a T cell-dependant response against tumour cells in an orthotopic melanoma model, further enhancing the efficacy of ICB (Matson et al., 2018). Taken together, these results demonstrate the profound effect of the gut microbiota on the efficacy of cancer immunotherapy *in vivo*.

Several studies suggested a positive therapeutic effect of ketogenic diet (KD) in many diseases including cancer (Branco et al., 2016). KD is characterized by high-fat, moderate-protein content while minimizing the intake of carbohydrates, ultimately leading to an increase of ketone bodies (KB) production. By reducing glucose availability and providing KB as an alternative energy source, it would be possible in theory to counteract the Warburg effect in cancer cells, characterized by a bioenergetic shift from oxidative phosphorylation towards glycolysis (Warburg et al., 1927). Mice kept in a KD showed changes in the microbiota composition (Ferrere et al., 2021). More specifically there was an overrepresentation of the bacteria *Akkermansia muciniphila*, *Ruthenibacterium lactatiformans*, and *Pseudoflavonifractor capillosus*. Strikingly, KD attenuated tumour growth in an orthotopic melanoma mouse model compared to mice fed a normal diet. By combining KD with ICB therapy (anti–PD-1 or anti–CTLA-4) the anti-tumour effects of the treatment were potentiated even in tumours that showed previous resistance to ICB drugs. In combination with ICB, KD regimens induced the upregulation of PD-1 and CTLA-4 on $CD8^+$ T cells, and at the same time prevented the expression of their ligands on splenic macrophages. This in turn leads to a prolonged systemic T cell activation and thus to an increased immune response against the tumour (Ferrere et al., 2021). In summary, these results show how diet can influence the microbiota and how these effects impact on the predisposition of cancer development. Manipulating the microbiota through diet allows concomitant modulation of cancer therapy efficacy, setting the stage for a new perspective on possible cancer treatment options.

The benefits of combined immune checkpoint blockade (CICB) therapy targeting CTLA-4 and PD-1, for the treatment of melanoma has proved highly effective in a subset of patients (Larkin et al., 2015). Nonetheless, a significant proportion of subjects experience immune-related adverse events as a consequence of this treatment (Sznol et al., 2017). A recent study suggests that CICB toxicity may be mediated bacteria that lead to increased production of IL-1β in this cancer model (Andrews et al., 2021). IL-1β is produced by monocytes in response to commensal microbiota and induces inflammation in the intestine (Seo et al., 2015). By analysing the microbiota of 77 melanoma patients that developed immune-related events, the authors identified a higher abundance of *Bacteroides intestinalis* in the faeces of these patients compared to patients that did not show adverse effects. Concomitantly, oral gavage of this bacteria into antibiotic-treated mice was strongly associated with ileal *Il1b* transcription. Interestingly, CICB treatment of these mice led to a stronger overrepresentation of *Bacteroides intestinalis* over other *Bacteroides* species confirming the causal relationship between treatment and bacteria. As a final proof, the authors performed FMT from healthy human donors harbouring high levels of endogenous *Bacteroides intestinalis* into tumour-bearing mice. This led to induction of *Il1b* expression in the intestine following CICB treatment of a melanoma mouse model (Andrews et al., 2021).

Only a small percentage of CRC patients respond to ICB therapy. Surprisingly, combination of conventional

chemotherapeutic agents with ICB have not so far proven superior to chemotherapy alone for the treatment of metastatic disease (Zou et al., 2016). Recent seminal studies using preclinical animal models suggested that ICB therapy efficiency in CRC can be influenced by the gut microbiota (Tanoue et al., 2019; Roberti et al., 2020; Mager et al., 2020). Of note, a consortium of 11 bacterial strains derived from healthy human donors were shown to increase the frequency of colonic IFNγ^+ CD8$^+$ T cells upon inoculation into germ-free mice (Tanoue et al., 2019). IFNγ^+ CD8$^+$ T cells contain subsets that expressed tissue-resident memory T cell marker CD103, and granzyme B (GrB), a key effector molecule of cytotoxic T cells, and are therefore capable of inducing an anti-tumour response (**Figure 1J**). Furthermore, the effect of anti-PD-1 therapy in mice engrafted with MC38 colon adenocarcinoma cells, was markedly ameliorated upon colonization with these 11 bacteria species (Tanoue et al., 2019). Using a similar CRC xenograft mouse model, Roberti at al. (2020) were successful in delaying tumour growth in mice treated with Oxaliplatin plus nivolumab in combination with either the *B. fragilis* or *E. ramosum* bacteria species. These bacteria strongly induced the production of IL-1β and IL-12p70 by DCs in the tumour microenvironment. This in turn drove the differentiation of T cells into Th1 cells, eliciting a response against the tumour (Roberti et al., 2020). In line with these results, another study (Mager et al., 2020) identified further bacterial species involved in the modulation of the efficacy of ICB treatment in CRC. The analysis of the microbiome of mouse AOM/DSS-induced colorectal tumours that responded to ICB, revealed *Bifidobacterium pseudolongum* to be differentially abundant in responders compared to non-responders. The authors then colonized tumour-bearing germ-free mice (injected with MC38 CRC cells) with this bacteria strain and treated them with ICB antibodies. Combinatorial treatment of bacteria and anti-PDL1, or anti-CTLA4, elicited an anti-tumour response shown by reduced tumour size and increased T cell activation in these animals (**Figure 1K**). Surprisingly, replacement of *Bifidobacterium pseudolongum* by inosine, a metabolite produced by this bacterium, in combination with anti-CTLA4 and CpG (a common immunostimulatory anti-cancer compound) led to similar results in this cancer model (Mager et al., 2020) (**Figure 1K**).

Clinically, microsatellite instable (MSI-H) tumours show better response rates to ICB when compared to microsatellite stable (MSS) tumours (Le et al., 2017; Overman et al., 2017). The MSI phenotype is caused by DNA mismatch repair (MMR) deficiencies that trigger the generation of frameshift mutations in several loci of the genome. These mutations can give rise to neoantigens in tumour cells that stimulate immune responses in patients (Kloor and von Knebel Doeberitz, 2016). In line with this, MSI-H tumours present immune-related features, including more immune cell infiltration, upregulation of immune-related genes and higher immunogenicity. It is, therefore, plausible to assume that, given the established relationship between the gut microbiota and immune cells, certain bacteria are likely to interfere in this process by modulating the efficacy of ICB. More studies including the use of MSI-H preclinical cancer models could help explain the low efficacy rate of ICB in MSS tumours and also why only 30–50% of MSI-H tumours show improved therapy responses.

Two recent studies took a leap forward in addressing one of the biggest problems of ICB treatment: acquired therapy resistance (Baruch et al., 2021; Davar et al., 2021). The clinical trial performed by Baruch et al. included previously diagnosed patients with metastatic melanoma who had progressed on at least one line of anti-PD-1 therapy (Baruch et al., 2021). These patients were subsequentially treated with FMT from two donors who had been treated with anti-PD-1 monotherapy for metastatic melanoma, and achieved a complete response for at least 1 year, together with re-induction of anti-PD-1 immunotherapy. Strikingly, 3 out of the 10 patients treated with this combinatorial therapy regime showed clinical responses including two partial and one complete response (Baruch et al., 2021). In another clinical trial with a similar study design, Davar et al. (2021) also showed that anti-PD-1 therapy acquired resistance could be overcome in 40% of patients by FMT treatment from individuals who had previously benefited from the same ICB therapy (Davar et al., 2021). Furthermore, responders showed a boosted immune response, reflected by increased CD8$^+$ T cell activation, and decreased frequency of interleukin-8-expressing myeloid cells (Davar et al., 2021).

It becomes evident that more and more bacteria species will be implicated as modulators of cancer therapy in the years to come, and with that, an improvement in how the best therapeutic outcomes can be achieved. It should be clear, however, that other variables such as diet, geographical location, lifestyle behaviours (such as tobacco smoking) might affect the microbiota composition and its metabolic profile. The concept of a "personalized microbiota" is currently being explored by pharma and biotech companies and several clinical trials using bacteria are ongoing including several cancer models (Zipkin, 2021).

USING BACTERIA TO FIGHT CANCER

Due to their inherent biology, microbes are good at synthesizing active molecules including many therapeutic compounds. In fact, this is a feature currently explored by biotech companies, which commercialize a plethora of biological compounds such as antibiotics, vitamins or antigens produced by genetically engineered microbes (Pedrolli et al., 2019). The basis of this approach resides on the fact that microbes can be used as shuttles or "chassis" to which one can load with specific cargo to elicit a desirable biological effect (Charbonneau et al., 2020). In theory, a suitable chassis such as *E. coli* or *L. lactis* show a high prevalence in the normal human gut but should be non-colonizing, and therefore, cleared shortly after administration (Human Microbiome Project, 2012; Pedrolli et al., 2019).

The use of the microbiota as direct therapy to tackle cancer and other diseases has also been assessed as a possibility and has shown promising results in recent years (Nelson et al., 2021; Braat et al., 2006). With the advent of genetic engineering technologies, it is now possible to use bacteria as a delivery system to selectively

release therapeutic compounds into the tumour *in vivo* (Zhu et al., 2019; Riglar and Silver, 2018; Zhou et al., 2018; An et al., 2019; Chung et al., 2021; Chowdhury et al., 2019; Frahm et al., 2015). Since the first study employing this methodology in cancer (Frahm et al., 2015), several other groups turned their attention to this approach. Of note, oral administration of P8 protein-producing *Pediococcus pentosaceus*, was shown to elicit an anti-tumour effect in two CRC mouse models, to a similar extent as with conventional chemotherapy (Chung et al., 2021) (**Figure 1J**). Furthermore, P8 treatment was shown to also alleviate the change from eubiosis to dysbiosis induced by AOM/DSS in one of the models tested (Chung et al., 2021). In another elegant study, Chowdhury et al. (2019) achieved increased activation of tumour-infiltrating T cells *in vivo* using different tumour models by transforming a non-pathogenic *E. coli* strain with a targeting molecule against CD47 (Chowdhury et al., 2019). As a direct consequence, tumour regression was achieved in all cancer models. CD47 is an anti-phagocytic receptor overexpressed in several human cancers and its blockade not only increases phagocytosis of cancer cells *in vitro* but also promotes activation of T cells against tumours *in vivo* (Chowdhury et al., 2019; Sockolosky et al., 2016). By transforming bacteria with a plasmid that encodes a synchronized lysis circuit (SLC) molecule plus a nanobody antagonist of CD47 (CD47nb), the authors were able to selectively release the therapeutic agent in to tumour cells *in vivo*. This approach avoids common comorbidities associated with systemic CD47 blockade reported in human trials (Chowdhury et al., 2019; Advani et al., 2018). Another seminal example of how one can use bacteria to selectively target CRC cells in an organism comes from the study of Ho et al. (2018). Briefly, by reprogramming commensal *E. coli*, to selectively bind the heparan sulfate proteoglycan (HSPG) located on the surface of cancer cells, the authors could, once again, successfully direct bacteria to the malignant lesion. Once there, bacteria were edited to secrete myrosinase, an enzyme that mediates the conversion of dietary glucosinolate to sulforaphane, and was shown to inhibit growth and promote apoptosis of cancer cells. As a result, tumour clearance was achieved in an AOM/DSS-induced cancer mouse model (Ho et al., 2018) (**Figure 1J**). Interestingly, mice treated with this bacterial system in combination with a cruciferous diet showed the best outcome regarding tumour prevention (Ho et al., 2018), which highlights, once more, the role of diet as a modulator of cancer treatment and prevention.

In line with this, perhaps in a more conventional way, bacteria can also be used as probiotics for therapeutic purposes. Probiotics by definition are microorganisms that confer health benefits when administered in controlled amounts (Geier et al., 2006). Even though the use of probiotics is not sufficient to cure or eradicate disease, its use has shown very convincing results as an adjuvant therapeutic approach in order to ameliorate side effects from both maladies and therapy. In fact, its use was reported to aid in several pathologic states including bacterial infection, by diminishing the colonization of pathogenic bacteria (Geier et al., 2006) and inflammation, by supressing inflammatory pathways or switching the phenotype of macrophages from M1 (pro inflammatory) to M2 (immunosuppressive) (Sichetti et al., 2018). The immunomodulatory properties of probiotics are further highlighted in the work of Chen et al. (2012). The inoculation of the bacterial strain *Lactobacillus acidophilus NCFM* was shown to downregulate MHC class I molecules in tumour cells of CT-26-implanted mouse models, resulting in an increased antitumour T-cell response (Chen et al., 2012). Moreover, certain bacterial species such as *Lactobacillus rhamnosus, Lactobacillus plantarum* and *Escherichia coli* can improve gut barrier function which is often disrupted in CRC (Kumar et al., 2017). The use of probiotics is generally accepted as a safe procedure, nonetheless attention must be placed in subjects with underlying medical conditions for instance immunosuppressed patients. In this setting the translocation of viable bacteria to a donor can lead to infections due to the poor capacity of the immune system to eradicate pathogenic bacteria from the host (Doron and Snydman, 2015). The prolonged use of probiotics could theoretically lead to horizontal gene transfer events where mobile genetic elements are disseminated within bacterial communities and render bacteria resistant to antibiotics (Jacobsen et al., 2007).

Bacteriophages are a class of prokaryotic viruses with the ability to infect host bacterial cells. Once inside the bacteria, bacteriophages replicate and produce endolysins that destroy the bacterial cell wall allowing the release of their viral particles (lytic phages). Lysogenic phages on the other hand, have the ability to integrate the bacterial genome and propagate their genetic information to the next generations. In recent years bacteriophages, given their versatility of use and specificity in infecting a plethora of cell types, have been explored as drug delivery systems for cancer treatment (Yacoby and Benhar, 2008). By employing a biopanning strategy it is possible to scan both *in vivo* and *in vitro* systems for the identification of cell surface-interacting peptides and uncover novel tumour-associated antigens for the design of targeted delivery systems. Several studies showed the feasibility of this strategy, reporting promising treatment approaches in different cancer models, either by conjugation of bacteriophages with therapeutic compounds or nucleic acids (Shadidi and Sioud, 2003; Cai et al., 2008; Du et al., 2010) (**Figure 1L**). By packaging a siRNA against focal adhesion kinase (*FAK*) gene in an EGF-targeting bacteriophage, Cai et al. were able to inhibit cell growth and invasiveness in an EGFR-overexpressing lung carcinoma cell line (Cai et al., 2008). Moreover, after injecting a phage library into a hepatocarcinoma xenograft mouse model, Du and colleagues (Du et al., 2010) identified a phage clone that in conjugation with Doxorubicin elicited a strong anti-tumour activity in the same model. Bacteriophages can also be used in combination with other delivery systems, for instance adeno-associated viruses (AAV) to further increase target specificity and infection efficiency in a safe manner (Hajitou et al., 2006; Hajitou et al., 2008). Only a few examples of the use of bacteriophages as therapeutic tools in cancer are highlighted in this review, since this topic has been extensively reviewed elsewhere (Foglizzo and Marchiò, 2021).

Current advances in understanding the evolution of a tumour as well as its treatment implications have improved the clinical outcome of cancer patients in recent years. Increasing evidence suggests that bacteria play an important role in determining the

effectiveness of therapy. However, there are still concerns about the side effects and drug resistance associated with current treatment programs. Although some side effects are observed in early clinical trials when using live bacteria as therapeutic agents, they still show far less toxicity in comparison to conventional chemotherapy regimens in different cancer types (Toso et al., 2002; Maciag et al., 2009).

CONCLUDING REMARKS

CRC is regarded as a genetic disease, as mutations are the events responsible for the transformation of a normal cell into a cancer cell. Cancer progression further requires a plethora of cell-intrinsic and cell-extrinsic processes that ultimately disguises the tumour from immune regulation, allowing at the same time its proliferation and ultimately invasion to distant organs (Hanahan and Weinberg, 2011). The studies included in this review link bacteria to dysregulation of known cancer pathways, thereby complementing the current knowledge about cancer initiation and progression. Furthermore, they show the importance of the genetic and metabolic features of bacteria, and how these affect human host cells in CRC. Mounting evidence further implies that the gut microbiota determines the effectiveness of conventional and targeted therapy drugs but can also be responsible for its adverse side effects. Thus, we suggest the identification of microbiota-specific features in a given clinical setting to be of the utmost importance for comprehensive disease assessment. Moreover, since these features could potentially be used as biomarkers of disease prognosis and therapy response outcomes, we propose the concept of personalised medicine to be revisited. Clinical management of patients should in the near future include data from both the patient and their associated microbiota when evaluating treatment decisions. We estimate that the use of clinical studies with proper standardisation and grouping according to the genetic and metabolic aspects of microbiota will help explain the discrepancies in therapy efficacies of different patients with similar molecular and histological cancer subtypes.

AUTHOR CONTRIBUTIONS

MS wrote the manuscript together with VB and MT. Figure illustrations were performed by MT together with MS.

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MINI REVIEW
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Inflammatory Bowel Disease and Risk of Colorectal Cancer: An Overview From Pathophysiology to Pharmacological Prevention

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Increased risk of colorectal cancer (CRC) in inflammatory bowel disease (IBD) patients has been attributed to long-standing chronic inflammation, with the contribution of genetic alterations and environmental factors such as the microbiota. Moreover, accumulating data indicate that IBD-associated CRC (IBD-CRC) may initiate and develop through a pathway of tumorigenesis distinct from that of sporadic CRC. This mini-review summarizes the current knowledge of IBD-CRC, focusing on the main mechanisms underlying its pathogenesis, and on the important role of immunomodulators and biologics used to treat IBD patients in interfering with the inflammatory process involved in carcinogenesis.

Keywords: inflammatory bowel deases, colorectal cancer, inflammation, epigenetics, microbiota, immunomodulators

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INTRODUCTION

Inflammatory bowel disease (IBD), comprising ulcerative colitis (UC) and Crohn's disease (CD), is a chronic relapsing inflammatory disorder associated with an increased risk of colorectal cancer (CRC) compared to the general population (Eaden et al., 2001; Bajpai et al., 2019). Interestingly, incidence rates of IBD-associated CRC (IBD-CRC) decreased over the last decades (Castaño-Milla et al., 2014; Choi et al., 2015). Several risk factors have been described, such as disease duration and extension (Ekbom et al., 1990; Eaden et al., 2001), inflammation (Rutter et al., 2004) and primary sclerosing cholangitis (Soetikno et al., 2002). Despite the limited number of studies, younger age at onset and onset of IBD in childhood seem to be associated with an increased incidence of CRC (Jess et al., 2012; Olén et al., 2020). Unlike sporadic CRC (sCRC), in patients with IBD-CRC, long-standing chronic inflammation initiates and drives tumorigenesis and important elucidation of the multiple factors involved in the process of carcinogenesis are emerging (Ullman and Itzkowitz, 2011; Baker et al., 2018). The present mini-review summarizes the recent advances in the pathophysiology of IBD-CRC, including the role of the immunomodulators currently used in the treatment of IBD (**Figure 1**).

PATHOGENESIS OF CRC IN IBD PATIENTS: GENETIC AND EPIGENETIC BASES

CRC tumorigenesis needs a tumor-initiating event that modifies normal intestinal epithelial cells by spontaneous mutation, environmental mutagens or inflammation-induced genetic and epigenetic

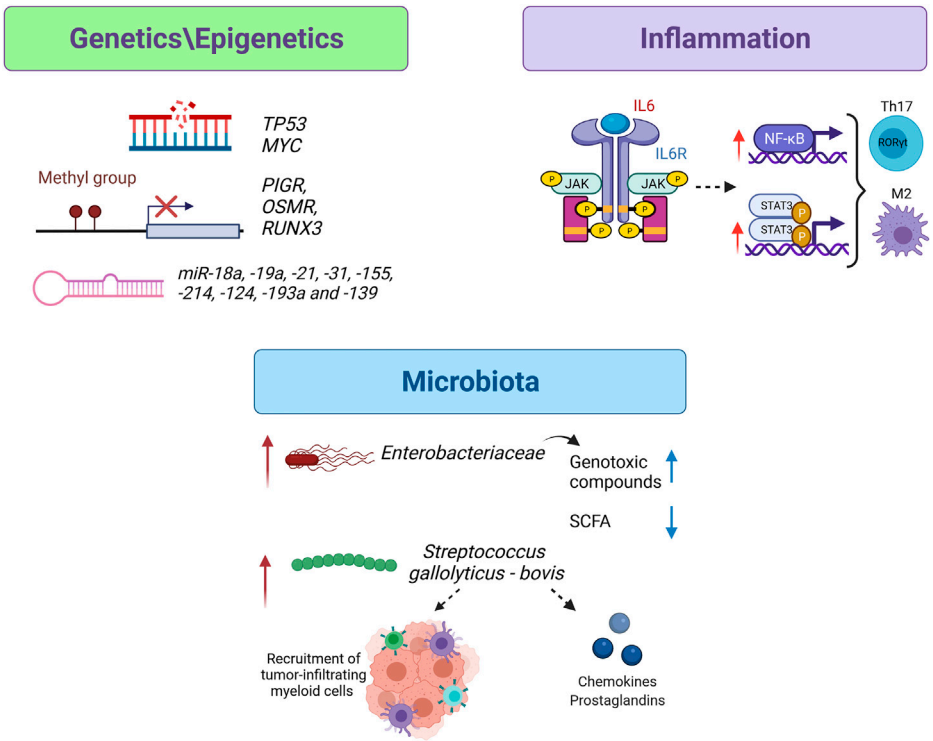

FIGURE 1 | From IBD to CRC: possible key players in carcinogenesis within intestinal mucosa. Intestinal epithelial cells of IBD-CRC patients present a different frequency of somatic mutations, altered DNA methylation sites and deregulated microRNA in comparison to sCRC. The anti-apoptotic role of NF-κB in cancer cells enhanced their proliferation, promoting tumor progression. Moreover, an exacerbated activation of NF-κB `and STAT3 signaling, *via* IL6, drives M2 polarization and Th17 differentiation favouring IBD-CRC carcinogenesis. *Enterobacteriaceae* strains are responsible for the production of genotoxic compounds and for the reduction of intestinal levels of anti-inflammatory SCFA; *Streptococcus* genus could exert its tumorigenic activity through the recruitment of tumor-infiltrating myeloid cells (*S. gallolyticus*) and the release of chemokines and prostaglandins (*S. bovis*). The image was created with BioRender.com.

alterations (Schmitt and Greten, 2021). Several lines of evidence suggest that IBD-CRC can result from a different mechanism of tumorigenesis in comparison to sCRC (Salk et al., 2009; Risques et al., 2011; Baker et al., 2019). The epithelial tumor tissue of IBD-CRC patients presents a lower frequency of somatic adenomatous polyposis coli (*APC)* and Kirsten rat sarcoma virus (*KRAS)* mutations, while tumor protein P53 (*TP53)* mutations and Myc proto-oncogene protein (*MYC*) amplifications are detected earlier during tumor progression in comparison to sCRC (Yaeger et al., 2016; Du et al., 2017; Chatila et al., 2020). Insights on germline alterations of IBD patients with CRC were provided for 25 patients with IBD-CRC, sequencing 39 genes potentially involved in predisposition to cancer (Biscaglia et al., 2021). Six patients (24%) presented pathogenic variants (International Agency for Research on Cancer, IARC class 4 or 5). Of these, four concerned the *APC* region, three the *MLH1* gene, and the remaining ones the *MSH2*, *MSH3*, *MUTYH*, *EPCAM*, *BRCA1*, *CHEK2*, *POLD1*, *POLE*, *CDKN2A* and *PDGFRA* genes. The onset of CRC was significantly earlier in patients carrying these variants than in patients with benign or unidentified variants.

DNA and RNA-sequencing and methylation analysis were performed in 2500 CRC cases, including 31 IBD-CRC (Rajamäki et al., 2021). As expected, somatic mutations in *APC* and *KRAS* were less frequent in IBD-CRC; a significant enrichment of somatic mutations at noncoding 5′UTR of *TP53* in IBD-CRC, resulting in low TP53 expression, was found. Aberrant promoter methylation patterns were detected exclusively in IBD-CRC in two genes related to mucosal immunity, in particular hypermethylation of polymeric immunoglobulin receptor (*PIGR)* and hypomethylation and strong overexpression of oncostatin M receptor (*OSMR*). Interestingly, increased levels of the interleukin-6 (IL-6) family member OSM and its receptor have been already detected in patients with active IBD and their presence was associated with failure of anti-tumor necrosis factor *a* (TNF) therapy (West et al., 2017), suggesting a potential role of the OSMR signaling in the molecular mechanism of IBD-associated tumorigenesis.

Chronic inflammation promotes aberrant DNA methylation in IBD, which in turn may predispose to the development of cancer (Barnicle et al., 2017; Somineni et al., 2019). A progressive increase in the percentage of methylated genes in the WNT signaling pathway from normal colon samples (n = 24) to IBD (n = 25) to IBD-CRC (n = 16) was observed, indicating their potential involvement during cancer development (Dhir et al., 2008). In particular, methylation of *APC1A*, *APC2*, *SFRP1*, and *SFRP2* genes characterized the progression from IBD to IBD-CRC, indicating their potential role as biomarkers for early

TABLE 1 | cytokines and/or pro-inflammatory molecules in IBD-CRC.

Molecule	Function in IBD-CRC	Reference
TNF-α	Activates oncogenic signaling pathways in epithelial cells, such as Wnt and NF-κB, that maintain a pro-inflammatory environment favoring tumor progression and angiogenesis.	Grivennikov and Karin, (2011)
IL-1β	Induces tumor cell proliferation and leads to Wnt signaling pathway activation.	Hnatyszyn et al. (2019)
IL-6	Critical for long-standing inflammation, for the recruitment and activation of Th17 cells and the inhibition of the regulatory T cells functions. The ability of IL-6 to activate STAT3 in epithelial cells is critical for its pro-tumorigenic activity.	Grivennikov et al. (2009)
IL-8	This chemokine was associated with increased metastatic and angiogenic potential in a mouse model of IBD-CRC.	Luo and Zhang, (2017)
IL-21	In mouse intestinal epithelial cells, IL-21 increases the risk of IBD-CRC by enhancing the expression of induced cytidine deaminase gene which deaminates cytosine residues to cause cytosine-to-thymine transitions.	Araki et al. (2019)
IL-22	Induces proliferation of enterocytes and dysplasia in a mouse model of IBD-CRC. IL-22 induces the nitric oxide synthase that leads to nitric oxide production within crypt epithelial cells driving DNA damage and carcinogenesis.	Wang et al. (2017)
COX-2	Overexpression of COX-2 contributes to increased proliferation, angiogenesis and resistance to apoptosis favoring tumour initiation and progression. The release of proinflammatory cytokines induced and maintained COX-2 expression and leads to the transition from acute to chronic inflammation. The use of COX-2 inhibitors reduces IBD-CRC development in mice thanks to the inhibition of cell proliferation, the reduction of β-catenin activation, COX-2 activity and nitric oxide production.	Kohno et al. (2005); Hnatyszyn et al. (2019)
Prostaglandins	Prostaglandin E2/EP favors IBD-CRC development by switching the phenotype of macrophages and neutrophils to pro-tumor, increasing cellular migration through the up-regulation of vascular endothelial growth factor receptor-1 signaling and by activating NF-κB.	Mizuno et al. (2019)

detection of cancer in IBD patients. These results have recently been validated in an additional cohort of UC CRC (Beggs et al., 2019).

The methylation status of 10 candidate genes involved in tumor suppression, cell-cycle regulation, and aging, in UC–CRC tumors and non-neoplastic tissues from both UC-CRC and UC patients (n = 114 for each) was analyzed (Garrity-Park et al., 2010). Methylation of *RUNX3*, *MINT1*, and *COX-2* genes in non-neoplastic tissue was significantly associated with UC-CRC, suggesting their role as potential indicators of carcinogenesis (Garrity-Park et al., 2010). An altered methylation status of *RUNX3* in the non-neoplastic sections of UC-CRC was also observed by Scarpa and others (Scarpa et al., 2016).

Among the epigenetic factors, emerging data have implicated the altered expression of specific microRNAs in IBD-associated tumorigenesis: miR-18a, -19a, -21, -31, -155 and -214 were upregulated in IBD-CRC colon tissues compared to healthy controls while miR-124, -193a and -139 were downregulated (Bocchetti et al., 2021), however, further prospective studies on large cohort of patients are needed.

INFLAMMATION AND TUMORIGENESIS IN IBD-CRC

The innate and adaptive immune system cells play an important role in the onset of IBD-CRC. Crosstalk between these cell types occurs mainly through a network of cytokines that drive and maintain inflammation and contribute to tumorigenesis *via* oxidative stress, epithelial cell proliferation, and angiogenesis (Long et al., 2017). In **Table 1** is reported a brief summary of the effects of different inflammation-related molecules in IBD-CRC.

Among these molecules, the macrophage migration inhibitory factor (MIF), a pleiotropic cytokine that drives cellular proliferation and regulates the migration and activation state of immune cells, seems to be relevant. The pathophysiological role of MIF in a wide range of inflammatory diseases, among which IBD, was already demonstrated (Nishihira and Mitsuyama, 2009). Increased MIF in macrophages in a CRC mouse model was demonstrated, and loss of MIF expression protects mice during tumor initiation (Pacheco-Fernández et al., 2019; Klemke et al., 2021). In cancer cells from CRC patients and in an acute colitis-CRC mouse model, a tumor-specific elevation of MIF expression was demonstrated (Klemke et al., 2021). The heat shock protein 90 (HSP90) chaperone machinery stabilizes and protects MIF from degradation and supports tumor progression *via* macrophage recruitment and angiogenesis.

In the context of inflammation and CRC, the most investigated pathways are the nuclear factor κ light-chain enhancer (NF-κB) and IL-6/signal transducer and activator of transcription 3 (STAT3) and STAT6 signaling pathways (Grivennikov and Karin, 2011).

NF-κB plays an important role in tumorigenic process by several mechanisms: promotes the production of reactive oxygen and nitrogen species which induce DNA damage, causes chromosomal instability and epigenetic changes (Grivennikov and Karin, 2011). Furthermore, by stimulating the production of inflammatory cytokines and growth factors, NF-κB enhances the proliferation of tumor progenitor cells, favoring tumor progression. This effect is also enhanced by the anti-apoptotic role of NF-κB; in fact in a IBD-CRC mouse model, it was demonstrated that NF-κB suppresses apoptosis through the induction of the anti-apoptotic protein B-cell lymphoma-extra large (Bcl-xL) (Greten et al., 2004). The NF-κB pathway results

aberrantly activated in most of colitis-associated tumors and is involved in the expression of pro-inflammatory genes including *COX-2*, *TNF-α* and *IL-6* (Gambhir et al., 2015).

The impaired regulation of NF-κB in tumor cells is also mediated by STAT3 which prompts the retention of NF-κB into the nucleus and hence amplifies its effect during the tumorigenic process and increases the interactions and communication between cancer cells and the microenvironment (Onizawa et al., 2009). The pro-oncogenic effects of STAT3 are mostly evident following inactivation of the negative regulators of IL-6 signaling, such as the suppressor of cytokine signaling 3 (SOCS3), that leads to an increased phosphorylation of protein kinase B (AKT), and NF-κB, initiating the disease process in patients that will progress towards IBD-CRC (Johnson et al., 2018). An increased IL-6/p-STAT3 signaling in dysplasia and colon cancer was demonstrated (Li et al., 2010). SOCS3 expression is reduced during progression from active UC to IBD-CRC and the altered methylation of SOCS3 may be involved in tumor progression increasing STAT3 signaling.

Another mechanism by which STAT3 promotes tumor progression is by favoring immune cell recruitment *via* the sphingosine-1-phosphate (S1P) signaling (Liang et al., 2013). S1P is formed by two related sphingosine kinases, SphK1 and SphK2, and it has already been demonstrated that SphK1 and intracellular S1P maintain a persistent activation of NF-κB and STAT3 pathways that lead to IBD-CRC development (Kawamori et al., 2009; Liang et al., 2013). Interestingly, in mice, the knockout of SphK2 increased SphK1 and S1PR1 expression, providing a pro-inflammatory environment through the secretion of IL-6 and favoring the infiltration of macrophages and T cells into tumor tissues.

Being the most abundant immune cells in tumor microenvironment, tumor-associated macrophages might be critical players in IBD-CRC progression. The role of Wnt5a, a member of the Wnt family, was already assessed in CRC: Wnt5a stimulates macrophages to produce IL-10 through the activation of STAT3 signaling pathways, crucial events for the M2 tumorigenic phenotype (Liu et al., 2020). Wnt signaling has several functions in proliferation, differentiation, migration, and survival and is regulated also by the NF-κB pathway (Du and Geller, 2010). A crosstalk between Wnt/β-catenin and NF-κB signaling pathways can significantly influence the progression of inflammation and the onset of IBD-CRC.

A recent work demonstrated for the first time, using a mouse model of IBD-CRC, that the M2 macrophage polarization could be altered by genetic inactivation of the MAPK-activated protein kinase 2 (MK2), resulting in delayed tumor progression (Suarez-Lopez et al., 2020).

Other cell types involved in cancer-associated inflammation include natural killers, T-helpers, monocytes and regulatory T-cells. During IBD and progression to dysplasia, the regulatory T cells, expressing the Th17-related transcription factor RORγt, increase in the tumor and peripheral blood of individuals with IBD-CRC, releasing pro-inflammatory cytokines (Quandt et al., 2021). Authors linked this phenotype to enhanced Wnt–β-catenin signaling, inducing pro-inflammatory cytokine production and RORγt expression in Treg cells. In particular using a mouse model of IBD-CRC, they demonstrated that the binding of the β-catenin interacting partner, TCF-1, to DNA overlapped with Foxp3 binding at active enhancer regions of pro-inflammatory genes. As a consequence of Wnt–β-catenin activation, new accessible chromatin sites in these pro-inflammatory genes were generated, leading to their upregulated expression. Enhanced β-catenin binding to TCF-1 may alter the TCF-1–Foxp3-dependent regulation of these genes. In particular, pathway enrichment analysis revealed that co-binding of TCF-1 and Foxp3 increases the accessibility and transcription of genes involved in Th17 differentiation and T cell activation pathways such as *IL-17*, *IFN-γ* and *TNF*.

ROLE OF MICROBIOTA IN THE PATHOGENESIS OF IBD-CRC

Although the exact mechanism of inflammation-associated carcinogenesis is still not completely known, the contribution of the gut microbiota, especially of some pathogenic bacterial species, seems to be relevant. There is a general consensus on the relationship between the gut microbiota and the immune system: microbes, through pathogen-associated molecular patterns (PAMPs), are capable to communicate with pattern recognition receptors (PRRs) in the innate immune system, such as Toll-like receptors (TLRs), retinoic acid-inducible gene I-like receptors (RLRs) and nucleotide-binding oligomerization domain-receptors (NLRs), and to trigger the immune response (Mogensen, 2009; Lu et al., 2018). Interestingly, the expression of TLR4 is strongly upregulated in colonic tissues of BALB/c mice treated with azoxymethane/dextran sodium sulphate (AOM/DSS) to induce IBD-CRC, and blocking TLR4 signaling slows the development of the tumor (Pastille et al., 2021). In addition, downregulation of the *TLR2* gene inhibits the proliferation of IBD-CRC. In particular, knocking out the *TLR2* gene in mice treated with 1,2-dimethylhydrazine-dextran sodium sulphate, reduced the shortening of colorectal length, the number and volume of tumors, the pathological score and tumor severity. Furthermore, knocking down the *TLR2* gene in the colorectal cancer cell lines HCT116 and HT29 inhibited their proliferation (Meng et al., 2020). It is noteworthy that also the deficient stimulation of other PRRs, such as NOD2, leads to a higher risk of IBD-CRC: in particular, $NOD2^{-/-}$ mice, treated with AOM to induce IBD-CRC, presented an increase in the number and size of tumors (Couturier-Maillard et al., 2013).

Moreover, upon stimulation with PAMPs, the NF-κB pathway and Wnt signaling, mentioned before for their role in the inflammation and proliferation processes leading to tumorigenesis, can be activated (Santaolalla et al., 2013; Peng et al., 2020).

Growing evidence confirms the association between IBD and the alteration of the composition of gut microbiota, sometimes referred as dysbiosis (Kang and Martin, 2017). Overgrowth of specific bacterial species at the expense of commensals is related not only to IBD but also to the development of CRC (Kang and Martin, 2017; Fan et al., 2021).

Nevertheless, to the author's knowledge, only one study investigated the gut microbiota composition in IBD-CRC patients (Richard et al., 2018), examining the gut microbiota from colonic mucosa of 10 healthy subjects (HS), 10 patients suffering from sCRC and seven patients affected by IBD-CRC. The bacterial microbiota of IBD-CRC patients had a reduced diversity compared to HS and a composition different from that of sCRC patients. In particular, when compared to HS, IBD-CRC patients have a decreased abundance in Firmicutes and Bacteroidetes and an increase in Proteobacteria; instead, *Bradyrhizobiaceae* and *Enterobacteriaceae* families, among Proteobacteria phylum, were overexpressed in the mucosa of IBD-CRC in comparison to sCRC patients (Richard et al., 2018). Interestingly, the *Bradyrhizobiaceae* and *Enterobacteriaceae* families, also abundantly proliferate in the mucosal and luminal gut of IBD patients, suggesting that the predominance of these microorganisms could be due to the pre-existing disease and that they could have a pathogenetic role in the inflammation-associated carcinogenesis (Swidsinski et al., 2002; Kaakoush et al., 2012; Wang et al., 2015).

The proliferation of *Enterobacteriaceae* is also associated with a lower concentration in short chain fatty acids (SCFAs); indeed, these metabolites counteract the competitive edge that O_2 and NO_2 give to this bacterial family during growth (Sorbara et al., 2019). Indeed, SCFAs, such as acetate, propionate and butyrate, are produced through the anaerobic fermentation of non-digestible dietary fibers by specific bacterial species, such as *Faecalibacterium prausnitzii*, *Clostridium leptum*, *Eubacterium rectale* and some *Roseburia* species, belonging to Firmicutes, whose abundance and diversity decreases in the gut microbiota of both IBD and IBD-CRC patients (Tan et al., 2014; Richard et al., 2018; Parada Venegas et al., 2019). SCFAs are a source of energy for colonocytes, elicit anti-inflammatory effects and exert antitumorigenic activity (Tan et al., 2014; Parada Venegas et al., 2019). In particular, SCFAs exert their anti-inflammatory properties binding to their FFAR2 and HCAR2 receptors, expressed on intestinal epithelial and immune cells, and thus inducing neutrophil chemotaxis to inflammatory sites, stimulating intestinal IgA secretion towards pathogenic bacteria and increasing the secretion of IL-18, which promotes gut epithelial integrity, repair and intestinal homeostasis, *via* inflammasome activation and IL-10, which promotes the differentiation of Treg cells (Tian et al., 2018). Instead, the antitumorigenic activity of SCFAs, especially butyrate, has been mainly attributed to the inhibition of the proliferation and the induction of apoptosis in cancer cells achieved through the alteration of gene transcription by inhibiting the activity of histone deacetylase (Tian et al., 2018). The administration of a mixture of SCFAs attenuated colonic inflammation and improved disease activity index, suppressing the expression of the proinflammatory cytokines IL-6, TNFα and IL-17 in BALB/c mice with AOM/DSS-induced CRC; the mixture also reduced the tumor incidence and size (Tian et al., 2018).

Among the *Enterobacteriaceae* family, *Escherichia coli* utilizes virulence factors, such as colibactin, a genotoxic compound, that promotes tumor growth in a xenograft mouse model and in mice with functioning autophagy, who lack for this reason genetic susceptibility for carcinogenesis, after treatment with AOM/DSS to induce CRC (Dalmasso et al., 2014; Salesse et al., 2021). Indeed, colibactin alkylates DNA and induces double-stranded breaks, playing thus a pro-tumorigenic role (Yang et al., 2020). Interestingly, a higher prevalence of colibactin-producing *E. coli* in patients affected by IBD compared to healthy individuals was demonstrated: inflammation could cause the upregulation of the *colibactin* gene and also facilitates the colonization of the mucosa by *E. coli,* leading to an increase in colibactin-induced DNA damage and allowing this bacterial strain to exert its carcinogenic activity (Yang et al., 2020).

Furthermore, a difference in microbial composition between the tumor and tumor-surrounding area, even if less pronounced than in sCRC, was evidenced; indeed, the *Streptococcus* genus was found to be more abundant in the IBD-CRC microbiota compared to the healthy adjacent mucosa (Richard et al., 2018). *Streptococcus* species, representative of the bacterial population of the mucosa and of the lumen of IBD patients, are associated with tumorigenesis (Biarc et al., 2004; Santoru et al., 2017; Zhang et al., 2018; Lo Presti et al., 2019). For instance, *S. gallolyticus* allows the tumor progression in C57BL/6 mice with AOM/DSS-induced CRC through the recruitment of tumor-infiltrating myeloid cells which can inhibit competence of T cells and increase proinflammatory cytokines (Zhang et al., 2018). Moreover, 12 proteins isolated from *S. bovis* are able to trigger the release of chemokines and prostaglandins in both human epithelial colonic Caco-2 cells and in rat colonic mucosa, and to promote pre-neoplastic lesions in AOM-treated rats (Biarc et al., 2004).

Investigating the gut microbiota in the AOM-DSS mouse model of IBD-CRC, similarly to what was encountered for IBD-CRC patients the microbial community was drastically altered by chronic colitis: in particular, in addition to *Lactobacillus hamster*, *Bacteroides uniformis* and *Bacteroides ovatus*, also *Streptococcus luteciae*, belonging to *Streptococcus* genus mentioned above for its pathogenic role, increases (Liang et al., 2014).

CHEMOPREVENTIVE EFFECTS OF THERAPIES FOR IBD

Since several observations support the important role of inflammation in the development of IBD-CRC, the use of anti-inflammatory and immunosuppressant drugs in IBD can reasonably reduce inflammation in the gut and consequently the risk of inflammation-related cancers.

The chemopreventive effect of 5-aminosalicylic acid (5-ASA) in IBD patients has been widely studied even though the results remain conflicting (Terdiman et al., 2007; Bernstein et al., 2011; Carrat et al., 2017). A systematic literature search including 164 studies and meta-analyses to identify all prognostic factors for advanced CRC in patients with IBD (Wijnands et al., 2021),

showed that patients who received 5-ASA had a lower risk of advanced CRC. In a systematic review, a protective role of 5-ASA against CRC in UC patients in clinical-based studies but not in population-based studies was shown (Qiu et al., 2017). In IBD patients, 5-ASA at a dosage ≥1.2 g/day showed higher protective effects against CRC than at dosages <1.2 g/day. Interestingly, a recent observational study provided molecular evidence of changes in genes related to the carcinogenesis pathways such as *CDC25A, CXCL10, IL8, NF-κB*, and *Ki-67* in colonic biopsies of 62 UC patients during long-term 5-ASA maintenance therapy; these changes may contribute to the chemopreventive effects observed in UC patients (Bajpai et al., 2019).

One of the most recent systematic review and meta-analysis, including 11 cohort and 16 case-control studies and involving 95,397 patients, highlighted that the use of thiopurines, azathioprine and mercaptopurine, was associated with a reduced risk of CRC; this chemopreventive effect was confirmed in patients with long disease duration (Beaugerie et al., 2013) but not in those with extensive colitis or primary sclerosing cholangitis (Zhu et al., 2018). Studies conducted on CESAME (19,486 patients) and ENEIDA (831 patients) cohorts confirmed that the risk for CRC is lower among IBD patients receiving thiopurine therapy (Beaugerie et al., 2013; Gordillo et al., 2015). In a well-established murine model, the thiopurine thioguanine inhibits colitis-associated cancer by decreasing β-catenin activation/nuclear translocation, providing important evidence in support of the potential therapeutic utility of this class of drugs (Sheng et al., 2021).

The impact of biological drugs on IBD-CRC development has yet to be definitely confirmed and long-term follow-up studies will be extremely important. Considering the role of TNFα in the initiation and progression of IBD-CRC (Popivanova et al., 2008; Wilson, 2008) the use of anti-TNFα agents may be useful in preventing CRC in patients with IBD (Biancone et al., 2009). A large-scale database study showing the inverse association of CRC with anti-TNFα therapy in the IBD population (225,090 CD and 188,420 UC) was recently published (Alkhayyat et al., 2021). In this study, patients with IBD who received any of the anti-TNFα agents and those who received combined treatment (anti-TNFs plus immunomodulators) had a lower risk of developing CRC. The relationship between anti-TNFα and CRC in IBD is also supported by the results obtained in animal models treated with infliximab in which CRC carcinogenesis associated with chronic colitis was reduced (Kim et al., 2010).

CONCLUSION

In summary, this mini-review summarizes the recent advances in the knowledge of the pathophysiology of IBD-CRC, a complex disease associated with multifactorial causes. Inflammatory pathways seem to be the major drivers of tumorigenesis in IBD patients even if the mechanisms that link inflammation and carcinogenesis remain not well characterized in patients. In this context, development of therapies targeting specific proinflammatory cytokines involved in tumorigenesis can provide a novel approach to prevent tumor initiation or progression. Further studies with large numbers of subjects are needed to address the existing gaps in the knowledge of the role of epigenetics in the process of carcinogenesis and to validate the predictive power and clinical value of the data collected so far. The identification of predictive and prognostic epigenetic markers could favor an early detection of IBD patients with increased risk of CRC. These analyses could also consider purified cellular populations, in particular epithelial cells.

Since the mucosal associated microbiota of IBD-CRC patients is characterized by the overgrowth of bacterial species playing a role in the pathogenesis of IBD-CRC, it could be assumed that increasing the levels of beneficial bacteria with probiotics could enhance the levels of the anti-inflammatory bacterial products SCFAs, restore the equilibrium and possibly ameliorate IBD-CRC condition. In addition, IBD-CRC may be at least partially prevented through mucosal healing of intestinal lesions, and the power of the potential anti-tumor effects of IBD drugs should be evaluated by rigorous prospective studies in the near future.

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ML, DC and MF: Conceptualization and drafting of the initial manuscript. GD and GS: writing review and editing.

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Therapeutic Suppression of FAK-AKT Signaling Overcomes Resistance to SHP2 Inhibition in Colorectal Carcinoma

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SHP2 mediates signaling from multiple receptor tyrosine kinases (RTKs) to extracellular signal-regulated kinase (ERK) and Ser and Thr kinase AKT, and its inhibitors offer an unprecedented opportunity for cancer treatment. Although the ERK signaling variation after SHP2 inhibition has been well investigated, the AKT signaling variation in colorectal carcinoma (CRC) is still unknown. Therefore, we performed immunohistochemistry and bioinformatics analyses to explore the significance of p-SHP2 in CRC. A panel of CRC cell lines with the SHP2 inhibitor, SHP099, was used to assess the effects on viability and signaling. The inhibitors of AKT and focal adhesion kinase (FAK) signaling were examined in combination with SHP099 as potential strategies to enhance the efficacy and overcome resistance. Frequent resistance to the SHP2 inhibitor was observed in CRC cells, even in those without RAS mutations. We observed rapid adaptive reactivation of the AKT pathway in response to SHP2 inhibition, possibly driven by the reactivation of RTKs or released *p*-FAK. High baseline p-FAK may also be associated with CRC cell resistance to SHP2 inhibition. Co-inhibition of FAK abrogated the feedback reactivation of AKT in response to SHP2 inhibition. Moreover, the combined inhibition of SHP2 with AKT or FAK resulted in sustained AKT pathway suppression and improved antitumor efficacy *in vitro* and *in vivo*. Our study found that reactivation of the AKT pathway is a key mechanism of adaptive resistance to SHP2 inhibition, highlighting the potential significance of AKT and FAK inhibition strategies to enhance the efficacy of SHP2 inhibitors in CRC treatment.

Keywords: SHP2, AKT rebound, FAK, drug resistance, colorectal carcinoma

INTRODUCTION

The non-receptor protein tyrosine phosphatase, SHP2, encoded by the gene of PTPN11, has a critical role in signal transduction downstream of growth factor receptor signaling and was the first reported oncogenic tyrosine phosphatase (Chan and Feng, 2007; Huang et al., 2014; Rehman et al., 2018). Activating mutations in this gene have been associated with developmental pathologies such as

Noonan syndrome and are also frequently found in multiple cancers such as leukemia, lung and breast cancer, and neuroblastoma (Chan and Feng, 2007; Huang et al., 2014; Rehman et al., 2018; Martínez-Jiménez et al., 2020). SHP2 is ubiquitously expressed and regulates cell survival and proliferation primarily through the activation of the RAS–ERK and PI3K–AKT signaling pathways (Huang et al., 2014; Prahallad et al., 2015; Rehman et al., 2018). Additionally, it is a key mediator of the programmed cell death 1 (PD-1) and B- and T-lymphocyte attenuator immune checkpoint pathways (Prahallad et al., 2015; Zhao et al., 2019). Reduction in the SHP2 activity may suppress tumor cell growth and enhance the anti-tumor immune response (Prahallad et al., 2015; Zhao et al., 2019; Quintana et al., 2020). Thus, SHP2 is a potential target of cancer therapy, especially for many RTKs-driven tumors which depend on it for survival.

Epidermal growth factor receptor (EGFR) and multiple other RTKs are frequently over-expressed in CRC (García-Aranda and Redondo, 2019), which will usually result in the activation of SHP2 and its downstream signaling (Chen et al., 2016). Therefore, targeting SHP2 in CRC is a potential therapy (Prahallad et al., 2015; Chen et al., 2016; Rehman et al., 2018; García-Aranda and Redondo, 2019). However, KRAS or BRAF gain-of-function mutations are frequently observed in multiple cancer types, especially in CRC, pancreatic cancer, and non-small-cell lung cancers (NSCLC) (Network, 2012; Zehir et al., 2017), which may hijack the function of SHP2 as the key mediator of multiple RTKs to control the ERK and AKT signaling. Additionally, RAS-mutant tumors are insensitive to inhibition of upstream growth factor receptor signaling (Prahallad et al., 2015; Chen et al., 2016). Thus, SHP2 inhibition, which links RTKs to the RAS–RAF–MEK–ERK and RAS–PI3K–AKT–mTOR pathways, will be ineffective in KRAS-mutant or BRAF-mutant cancer cell lines. Previous data also indicate that SHP2 inhibition in KRAS-mutant NSCLC cell lines has little effect *in vitro* (Mainardi et al., 2018). However, inhibition of the RAS oncoproteins has been proven to be difficult, and attempts to target downstream effectors have been hampered by the activation of compensatory resistance mechanisms (Corcoran et al., 2012; Turke et al., 2012; Hirata et al., 2015; Kitai et al., 2016). Recently, SHP2 signaling response activation to the inhibitors of RAS downstream effectors has been reported in multiple cancer types, and combined targeting of RAS downstream effectors, especially for MEK inhibitors and SHP2, generated significant synthetic effects on tumor growth (Fedele et al., 2018; Wong et al., 2018; Ahmed et al., 2019; Lu et al., 2019). Thus, SHP2 is a promising target, especially as the combined therapy was used in RAS-mutant cancers.

RAS dominantly activates the ERK signaling and also controls the PI3K-AKT signaling by interacting with p110a (Zhang et al., 2002; Gupta et al., 2007; Castellano et al., 2013). However, the PI3K-AKT signaling may also be activated by SHP2 through moving p85, which is a suppressor for PI3K-AKT signaling independent of RAS mutation (Zhang et al., 2002). Recently, researchers have argued that the PI3K-AKT pathway requires RTK-induced activation, usually involving SHP2 as a critical mediator in KRAS-mutant cancers (Ebi et al., 2011; Navas et al., 2012; Hao et al., 2019). Thus, targeting the SHP2-PI3K-AKT pathway may still provide an attractive therapeutic strategy despite SHP2 downstream mutation. Moreover, SHP2 inhibition under growth factor-limiting conditions and in KRAS-mutant NSCLC xenografts provokes senescence responses (Mainardi et al., 2018). Therefore, the role and mechanism of SHP2 in CRC may be complex and require a profound study. The present study reported that most CRC cells are resistance to SHP2 inhibition, which is associated with a feedback reactivation of the AKT pathway. The underlying mechanism for AKT reactivation may be mediated by multiple RTKs and released *p*-FAK activation, followed by SHP2 inhibition. FAK co-inhibition prevented a more universal feedback reactivation after SHP2 inhibition, and the combined inhibition of SHP2 with AKT or FAK drove sustained AKT pathway suppression and improved antitumor efficacy both *in vitro* and *in vivo*. Therefore, the present study not only demonstrated the feedback reactivation of AKT pathway as a key mechanism for the resistance of CRC to SHP2 inhibition, but also provided the combination of SHP2 and each of the AKT and FAK pathway inhibition as potential strategies to enhance the efficacy of SHP2 inhibition.

MATERIALS AND METHODS

Bioinformatics

The *p*-SHP2 expression data in 7694 cancer specimens and 277 cancer cell lines, which were examined by reverse phase protein array (RPPA), were downloaded and retrieved manually from the website of the cancer proteome atlas (TCPA) portal (https://tcpaportal.org/). The half maximal inhibitory concentrations (IC_{50}) of 496 cancer cell lines including 41 CRC cell lines in response to SHP099 were also retrieved from a study (Hao et al., 2019) that studied the mutation status of KRAS and BRAF. All 496 cell lines past the information check of CCLE (https://portals.broadinstitute.org/ccle/about) and Cellosaurus (https://web.expasy.org/cellosaurus/). The cell lines with an IC_{50} value of SHP099 more than 30 μg/ ml were defined as the resistance phenotype (Hao et al., 2019). Then, the differences in *p*-SHP2 expression between CRC (n = 487) and other cancer types were ranked with the median and compared. The status of KRAS or BRAF mutation of all cancer cell lines were retrieved from CCLE and the *p*-SHP2 expression across the cancer cell lines was discriminated into high or low expression with a normalized RPPA expression of 0.1 as the cut-off value. Then, the distribution of the resistant cell lines concerning the subgroups of mutation status (KRAS or BRAF mutation), and p-SHP2 expression (high or low) was also investigated.

Patients

The present study was conducted in 365 patients with localized CRC who received curative surgery in Changhai Hospital, Second Military Medical University (Shanghai,

China) between January 2008 and October 2011. Less than 5% of patients with rectal cancer received preoperative radiotherapy in the cohort. The baseline information of patients, including age, gender, TNM stage (determined according to the American Joint Committee on Cancer Staging Manual, seventh edition), differentiation grades, carcinoembryonic antigen (CEA), and CA199 is presented in **Supplementary Table S1**. A written informed consent was obtained from each patient. The formalin-fixed paraffin-embedded specimens, including 365 cancerous and 75 noncancerous tissues, from the patients were collected and were used to construct tissue microarrays (TMAs) by a commercial company (Outdo Biotech, Shanghai, China). The TMA construction details were described in a previous study (Chang et al., 2014).

Cells and Reagents

Cell lines were obtained from the American Type Culture Collection (ATCC), which routinely performs cell line authentication by short tandem repeat analysis, and maintained in Dulbecco's modified eagle medium (CaCO2, CW2 and SW480) or RPMI-1640 (SW620, RKO, and Colo-205) supplemented with 10% heat-inactivated fetal calf serum (GIBCO), 100 U/ mL penicillin, and 100 mg/ ml streptomycin at 37°C in a humidified atmosphere containing 5% carbon dioxide (CO_2). The SHP2 inhibitor (SHP099, #HY-100388) and the AKT-1/2/3 inhibitor (MK-2206, #HY10358) were purchased from MedChemExpress. The FAK inhibitors of PF-573228 (#S2013) and VS-4718 (#S7653) were purchased from Selleckchem.

Cell Proliferation Assays

CRC cells were seeded in triplicate in 96-well plates at 4,000 cells per well and exposed to the inhibitors of SHP099 and MK-2206 both alone and in combination with indicated concentrations, and the dimethyl sulfoxide (DMSO) as control. The number of viable cells at 24, 48, and 72 h was assessed using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. The absorbance at 450 nm was measured to reflect the viable cell population. To determine the IC_{50} values, data were fitted using the dose response algorithm in Graphpad Prism as Y = Bottom + (Top − Bottom)/[1 + 10(X-LogEC50)], in which the top and bottom are plateaus in the units of the Y axis, and EC50 is the inhibitor concentration that gives a response half-way between the bottom and top.

Drug Combination Studies

The combination effect of SHP099 and MK-2206 on a panel of CRC cell growth was analyzed using CompuSyn 1.0 (Chou and Martin, 2005). The individual dose–effect of each drug was obtained by treating 5 CRC cell lines with SHP099 or MK-2206. The median effect dose (Dm) and linear correlation coefficient of the ME-plot (r) were analyzed. Optimal concentration ratios were obtained based on the Dm values, and six serial dilutions of the optimal ratio were used to measure the cytotoxic effect. Combination index (CI) of the combined use of different drugs was calculated using CompuSyn, which defined synergism (CI < 1), additive effect (CI = 1), and antagonism (CI > 1).

Colony Formation Assays

The cells were initially cultured in 6-well plates for colony formation assay (Corning, NY, United States) at a density of 2.0×10^3/well, and the regular medium supplemented with the inhibitors was refreshed every 2–3 days. After culturing for 2–3 weeks, the resulting colonies were fixed with ice-cold methanol and stained with a crystal violet solution for counting. The assay was performed in triplicate. The plates were scanned using a photo-scanner, and cell growth was quantified using ImageJ software.

Animal Studies

Experiments were performed on 4-week-old nu/nu athymic BALB/c male mice obtained from the Shanghai JiHui experimental animal breeding company, Shanghai, China, and all the mice were maintained in pressurized ventilated cages under an Institutional Animal Care and Use Committee-approved protocol and institutional guidelines for the proper and humane use of animals in research. Subcutaneous tumors were generated by transplanting $0.5–1.0 \times 10^7$ tumor cells (SW620 and Colo205) in phosphate buffered saline (PBS) into the right flank (200 μL/mouse) and randomized approximately 14 days post-implantation (size >100 mm^3). The mice were treated with SHP099, MK-2206, VS-4718, or their combination with the indicated doses. SHP099 was formulated in 30% hydroxypropyl-β-cyclodextrin, whereas MK-2206 was dissolved in 30% Capitisol and administered by oral gavage. For the VS-4718 treatment, drug was prepared in 0.5% carboxymethyl cellulose and 0.1% Tween 80 and the mice were treated at 35 mg/ kg BID by gavage. All inhibitors were administered orally every day. The reagents which used to dissolve the inhibitors were taken as the vehicle control in the study. Tumor dimensions were measured with vernier calipers at an interval of 3 days, and tumor volumes were calculated as follows: π/6 × larger diameter × (smaller diameter)^2. Animals were sacrificed by CO_2 euthanasia when tumors reached the maximum-allowed size or when signs of ulceration were evident. After image analysis, the isolated tumor specimens were further processed for western blotting and immunohistochemistry (IHC) examination as corresponding manual.

Western Blotting

The cells were washed with PBS once, disrupted on ice for 30 min by using radioimmunoprecipitation assay (RIPA) lysis and extraction buffer (Thermo, America). Pierce protease and phosphatase inhibitor mini tablets (Thermo, America) were added at one tablet per 10 ml solution and centrifuged for 15 min (14,000 ×g) at 4°C. Protein concentration was determined with bicinchoninic acid (BCA) reagent (Dingguo, Beijing). Equal amounts of protein (10–50 μg) in cell lysates were separated by 10%

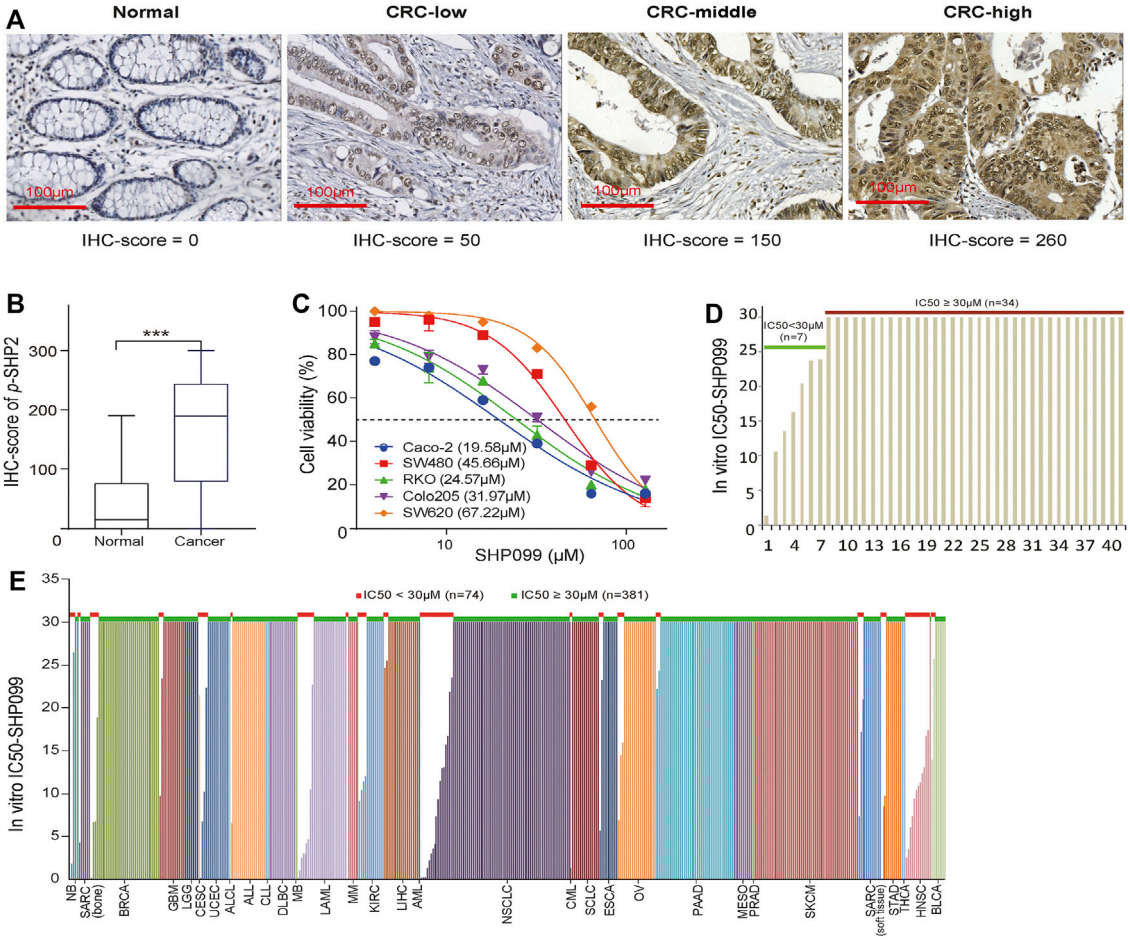

FIGURE 1 | The expression of p-SHP2 in CRC and the activity of its inhibitor (SHP099) in cancer cells. **(A)** Representative images of p-SHP2 expression in colorectal tissues based on IHC. **(B)** Increased p-SHP2 expression in CRC. (***< 0.001) **(C)** Activity of SHP099 in 5 CRC cell lines with indicated IC50s. **(D)** Analyses of IC_{50} values of SHP099 in 41 CRC cell lines. **(E)** Analyses of IC_{50} values of SHP099 in 455 cancer cell lines excluding CRC cell lines. CRC: colorectal cancer; IHC: immunohistochemistry; IC_{50}: the half maximal inhibitory concentration.

sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride membranes (Millipore), immunoblotted with specific primary and secondary antibodies, and detected through chemiluminescence by using the enhanced chemiluminescence (ECL) detection reagents from Millipore. Antibodies for western blotting against *p*-ERK1/2 (T202/Y204, 1:2000, #4370), ERK1/2 (1:1000, #4695), AKT (1:1000, #4691), *p*-AKT (S473, 1:1000, #4060), and *p*-FAK (Tyr397, 1:1000, #3283) were purchased from Cell Signaling Technologies (CST). GAPDH (1:5000, ab181602) and *p*-SHP2 (Y542, 1:1000, ab62322) were purchased from Abcam. The primary antibodies above are all from rabbit, so the secondary antibody of anti-rabbit IgG, HRP-linked Antibody (1:5000, #7074, CST) was used in the study.

RTK Arrays

Human phospho-RTK arrays (R & D Systems) were utilized according to the manufacturer's instructions. Cells were briefly washed with cold PBS, lysed in NP40 lysis buffer, and 100 mg of lysates was incubated overnight with blocked membranes. Membranes were subsequently washed and exposed to a chemiluminescent reagent and an X-ray film. Quantification of pixels was performed through densitometry by using Adobe CS2 and Fuji Film Multi Gauge software.

IHC Assay

IHC antibodies for *p*-SHP2 (1:200, sc-280, Santa Cruz) were used in this study. All TMAs were stained simultaneously. The p-SHP2 expression was semi-quantitated using the H-score method as our previously reported (Yuan et al., 2019). The score of the *p*-SHP2 expression was defined as the staining intensity (0, negative; 1, weakly positive; 2, moderately positive; 3, strongly positive) multiplied by the percent tumor-positive area (0–100%). IHC scores were independently assessed by two observers (Y.Y and Z.F) who were blinded to the information of the specimen donors. IHC scores from the two observers was average for further analysis, and controversial cases (defined as a

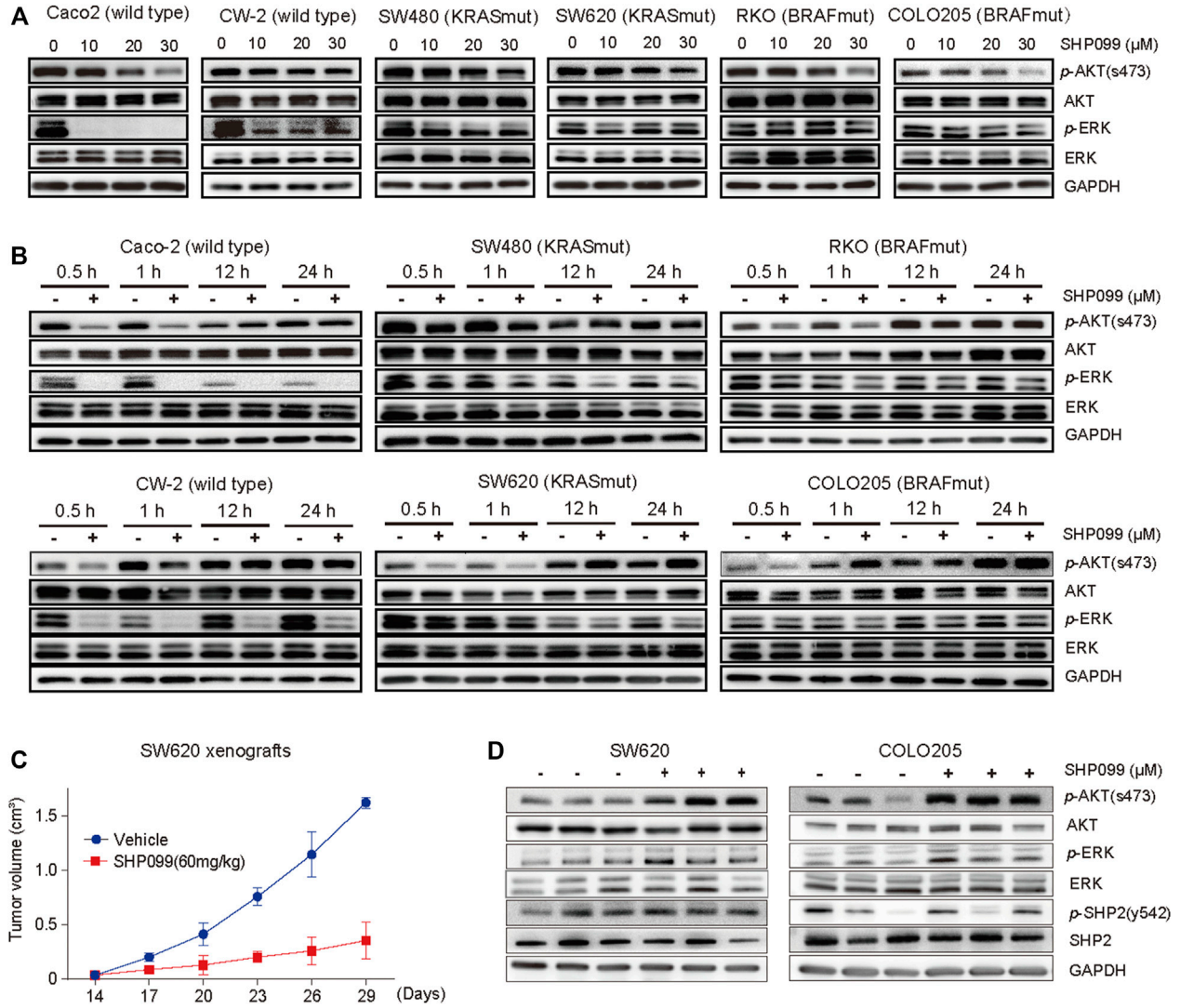

FIGURE 2 | Feedback activation of AKT signaling followed by SHP2 inhibition with SHP099 in CRC cells. **(A)** SHP099 reduces the expression of p-AKT in indicated CRC cells. **(B)** A transient inhibition of p-AKT followed by feedback activation of the signaling across all indicated CRC cells. The same cell lines treated with different concentrations of SHP099 such as Caco-2 (20 μM), CW-2 (20 μM), RKO (30 μM), Colo205 (30 μM), SW480 (30 μM), and SW620 (40 μM) and collected at indicated times and analyzed by immunoblotting. **(C)** SHP099 reduces the growth of xenograft tumors derived from SW620 cells. **(D)** Increased activity of p-AKT signaling after SHP099 treatment from isolated xenograft tumors derived from CRC cells.

difference in IHC scores more than 10% of the average score) were jointly re-evaluated until a consensus was reached.

Statistics

Expression levels of *p*-SHP2 in the CRC and adjacent normal tissues were compared using independent sample *t*-test for non-paired samples. The proportion of cancer cells with the resistance phenotypes between the subgroups concerning the mutation status of KRAS or BRAF or the levels of *p*-SHP2 was compared using chi-square test. All statistical tests were two-sided and were performed using SPSS version 22.0 for Windows (SPSS, Chicago, IL, United States). A p value of <0.05 was considered statistically significant.

RESULTS

universal Resistance to SHP2 Inhibitor SHP099 in CRC

The IHC examination of *p*-SHP2 in 365 CRC and 75 noncancerous specimens indicated that the protein is located mainly in the cytoplasm of epithelial cells (**Figure 1A**) and is significantly elevated in CRC (**Figure 1B**). According to the *p*-SHP2 expression from the RPPA examination across TCGA cancers, as illustrated in **Supplementary Figure S1**, the median of *p*-SHP2 levels in CRC was located in the front of the list (ranked 12) among 31 cancer types. The data indicated that *p*-SHP2 may be a

valuable target for CRC treatment. Next, we evaluated the effect of SHP2 inhibition with SHP099 on the growth of 5 CRC cell lines. IC_{50} values of more than 30 μM were observed in 3 of 5 tested CRC cells (**Figure 1C**), indicating a resistance to the blockage of SHP2 signaling. Moreover, bioinformatics analysis exhibited that only 17.1% (7/41) CRC cells (**Figure 1D**) and 16.3% (74/455) cell lines from other cancer types possessed IC_{50} values less than 30 μM (**Figure 1E**), confirming universal resistance to SHP099 among cancer cell lines. Although gain-of-function mutations of KRAS and BRAF have been identified as a contributor to SHP099 resistance (Mainardi et al., 2018), more than 77.4% (233/301) of all cancer cell lines with the wild types of both KRAS and BRAF were resistant to SHP099 treatment (**Supplementary Figure S2A**). We further considered the association between *p*-SHP2 status and the resistance to SHP099 in cells with both BRAF and KRAS wild types and observed only a marginal significance ($p = 0.052$) (**Supplementary Figure S2B**). Therefore, the potential mechanisms constraining the efficacy of SHP099 are still unclear and must be explored.

Rapid Feedback Reactivation of AKT Pathway Following SHP2 Inhibition

ERK and AKT signaling usually serve as the most crucial effectors for SHP2 inhibition. To investigate their changes in response to SHP2 inhibition, we evaluated the effects of SHP099 on a panel of CRC cell lines. As expected, we observed that the level of *p*-ERK in $CaCO_2$ and CW-2 cells, which express wild types of both KRAS and BRAF, was sharply reduced at 45 min in response to SHP099, whereas it was only moderately or slightly reduced at a higher dosage of SHP099 in cells with KRAS or BRAF mutations (**Figure 2A**). The level of *p*-AKT was significantly suppressed by SHP099 with dosage-dependent trends across almost all tested cells (**Figure 2A**), independent of the mutation status of KRAS or BRAF. However, following the suppression, a significant rebound was observed in the level of *p*-AKT in response to SHP099 at 12 h (**Figure 2B**), although the rebound of *p*-AKT in RKO cells occurred at approximately 24 h. Additionally, the *p*-ERK levels in response to SHP099 were still sharply reduced in the CaCO2 and CW-2 cells and slightly or moderately reduced in the other cells (SW620, RKO, Colo205, and SW480) even after an extension of the observed time to 24 h (**Figure 2B**). Moreover, the *p*-AKT reactivation in CRC xenograft models following SHP2 inhibition also have be investigated. Although the isolated xenograft tumors (from SW620 or Colo205) from SHP099-treated animals exhibited smaller tumor sizes than the control group (**Figure 2C**), they exhibited consistently higher *p*-AKT levels than the controls (**Figure 2D**). Moreover, the *p*-ERK level in the SHP099 treatment groups was slightly elevated than that in the control groups (**Figure 2D**). Therefore, the AKT pathway exhibits a clear early repressed and then rebounded response to SHP099, indicating a dynamic and complex interaction between SHP2 and AKT signaling.

Synergistic Suppression of CRC by SHP2 Inhibition and AKT Blockage

The inhibition of AKT signaling may sensitize the role of SHP099 because the AKT signaling activation is always associated with drug resistance (Datta et al., 2017; Song et al., 2017; Vitiello et al., 2019). By using siRNAs targeting AKT1-3, we observed that SHP099 significantly reduces the proliferation and colony formation of CRC cells (CaCO2 and SW480) as knocking down AKT (**Supplementary Figures S3A–3B**). Furthermore, we assessed the combined effect of SHP099 and MK-2206 (a specific AKT inhibitor) on CRC. The proliferation curves consistently exhibited that the combined treatment is the most effective inhibition of CRC growth among all groups across tested cell lines, particularly the inhibition effect on the third day that strongly indicated a synergistic interaction between SHP099 and MK-2206 (**Figure 3A**). The CI was employed to examine the presence of a synergistic effect by the combination of SHP099 and MK-2206. The CI values from the 5 cell lines were all less than 0.70, which suggested a stable synergism in all tested cell lines (**Figure 3B**). The strongest synergism ($CI \leq 0.3$) was observed in the RKO and CaCO2 cell models (**Figure 3B**). Moreover, the synergistic effect of SHP099 and MK-2206 was also supported by the results of colony formation (**Figure 3C**) and cell apoptosis assays (**Figure 3D**). Thus, the synergistic inhibitory effect of SHP2 and AKT on CRC growth exists universally.

Combined SHP2 and AKT Inhibition Drives Tumor Regressions *in vivo*

Having established the effect of combined SHP099 and MK-2206 on CRC cells, we set out to validate the findings with *in vivo* models. Firstly, we injected the Colo205 and SW620 cells into nude mice until the tumors reached the required volumes at approximately the sixth day. Then, daily oral administration of a single agent SHP099 or MK-2206 and their combination was employed according to the designed regimen. The tumor volume difference among the four groups was not significant until the 19^{th} day (**Figure 4A**). Although both SHP099 and MK2206 exhibited significant inhibition of tumor growth in animal models (**Figures 4A,B**), the combination exhibited the maximum inhibition of tumor volumes among all groups, which almost retained the original sizes throughout the experiment period. Moreover, the difference in body weight between groups was statistically nonsignificant (**Figure 4C**). Consistent with at least some non-autonomous effects, SHP099 decreased tumor vascularity, as monitored by CD31 immunostaining, and the proliferation marker Ki67 also exhibited the weakest intensity in the group of combined inhibitors (**Figure 4D**). Therefore, the xenograft models consistently exhibited that the combination of SHP099 and

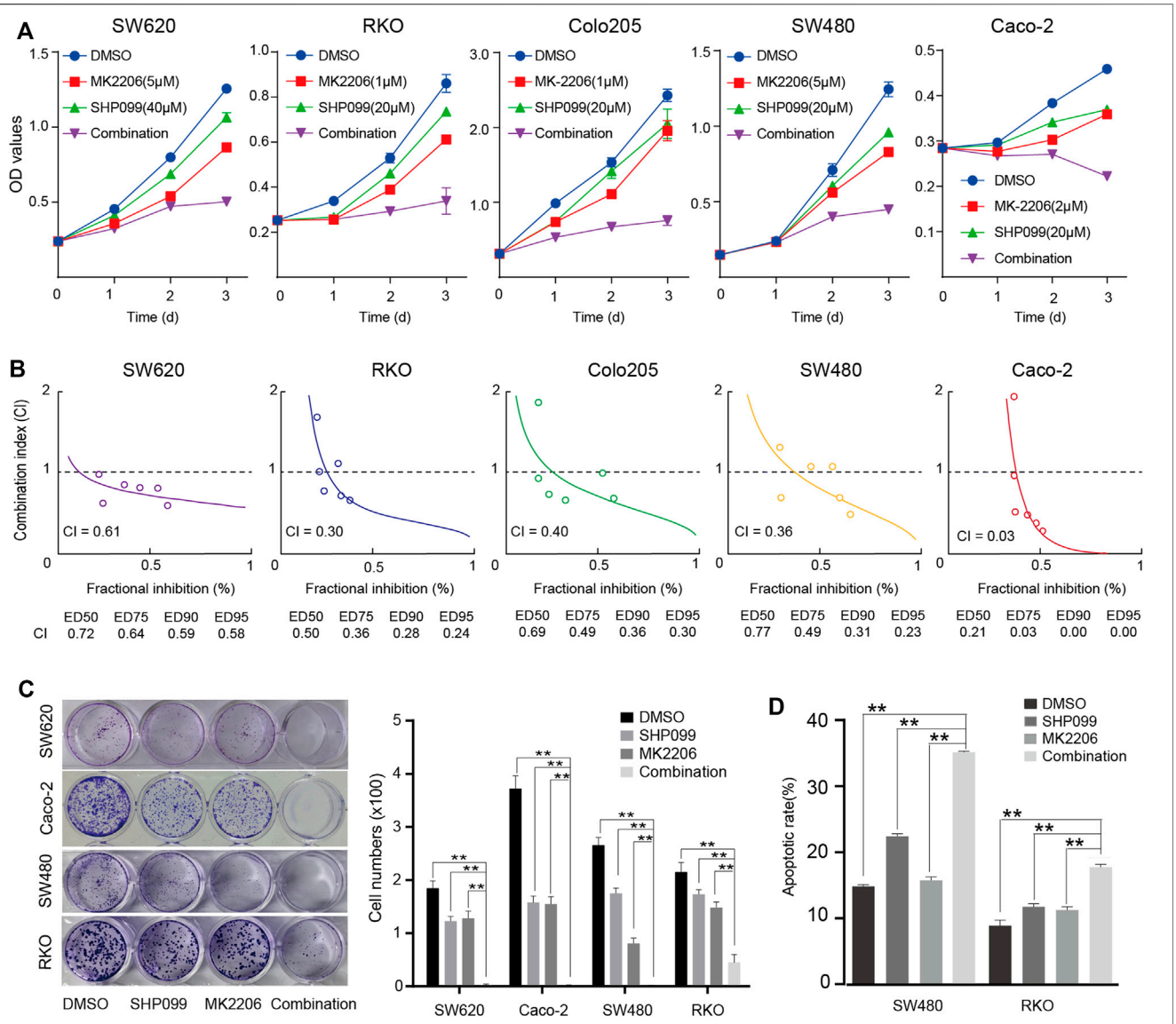

FIGURE 3 | Synergistic suppression effect of SHP099 and MK-2206 on CRC cell growth. **(A)** The effect of SHP099 and MK-2206 on the proliferation of the 5 indicated cell lines. **(B)** Combination index of SHP099 and MK-2206 of the 5 involved CRC cell lines. **(C)** The effect of SHP099 and MK-2206 on the colony formation of the four indicated cell lines. **(D)** The effect of SHP099 and MK-2206 on the apoptosis of the 2 indicated cell lines. (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

MK-2206 may overcome the adaptive feedback resistance and may represent a promising therapeutic strategy.

Induction of Phosphorylation of Multiple RTKs by SHP2 Inhibition

Multiple RTKs activated PI3K–AKT signaling in normal and tumor cells (García-Aranda and Redondo, 2019; Chandarlapaty et al., 2011). To investigate the role of RTKs in rebound of AKT signaling in response to SHP099, we employed an anti phosphotyrosine receptor antibody array to assess the levels of RTK activation at baseline and after 24 h of SHP099 treatment (**Figure 5A**). We observed that four phosphorylated RTKs (EGFR, IGF1R, Insulin R, and AXL) are the most prominent in at least 1 of the four tested cell lines. Additionally, following 24 h of SHP099 treatment, we observed that the phosphorylation of many RTKs (EGFR, ERBB3, FGFR1, FGFR3, EPHA2, EPHA4, EPHA7, EPHA10, HGFR, and ALK) is induced more than twofold in 24 h in at least 1 cell line (**Figure 5B**). However, the changed profile of the RTKs was extremely heterogenous across all indicated cell models, implying that multiple RTKs may play a role in the adaptive feedback of SHP099 treatment. The induction of tyrosine phosphorylation of EPHA2 following SHP099 treatment was consistently observed across four tested cell models, indicating that EPHA2 is a common target. However, the potential T594 site inhibited AKT activation (Miao et al., 2009). Therefore, the variable induction of multiple RTKs across different cell models following SHP099 treatment and different

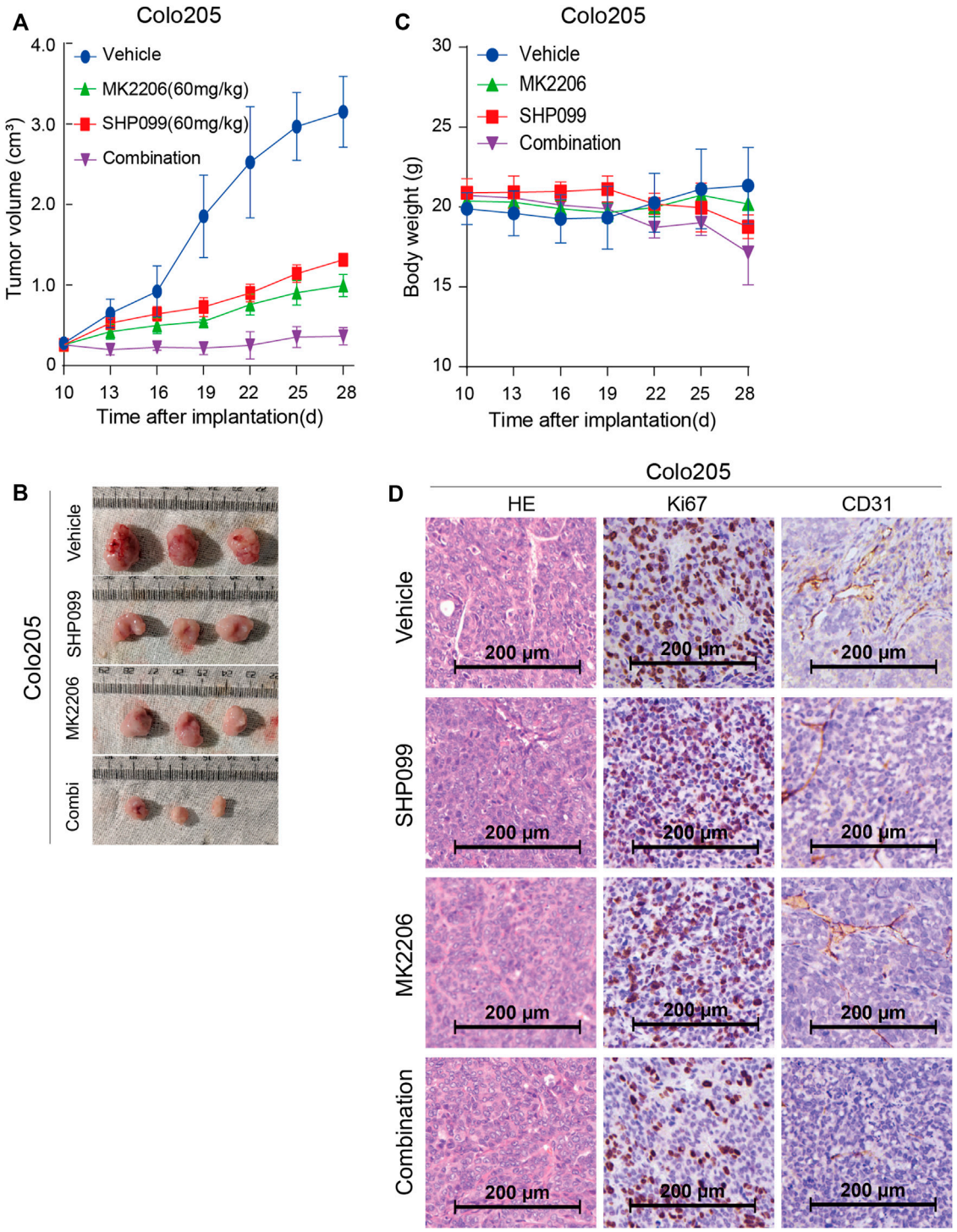

FIGURE 4 | Effect of SHP099 and MK-2206 on the growth of xenograft CRC tumors. **(A)** Dynamic effect of SHP099 and MK-2206 on the CRC tumor volumes. **(B)** Represented images from isolated CRC tumors receiving SHP099 and MK-2206 treatment. **(C)** The body weight of animals during the treatment of SHP099 and MK-2206. **(D)** The effect of SHP099 and MK-2206 on the expression pattern of Ki67, and CD31 in isolated xenograft tumors examined by IHC.

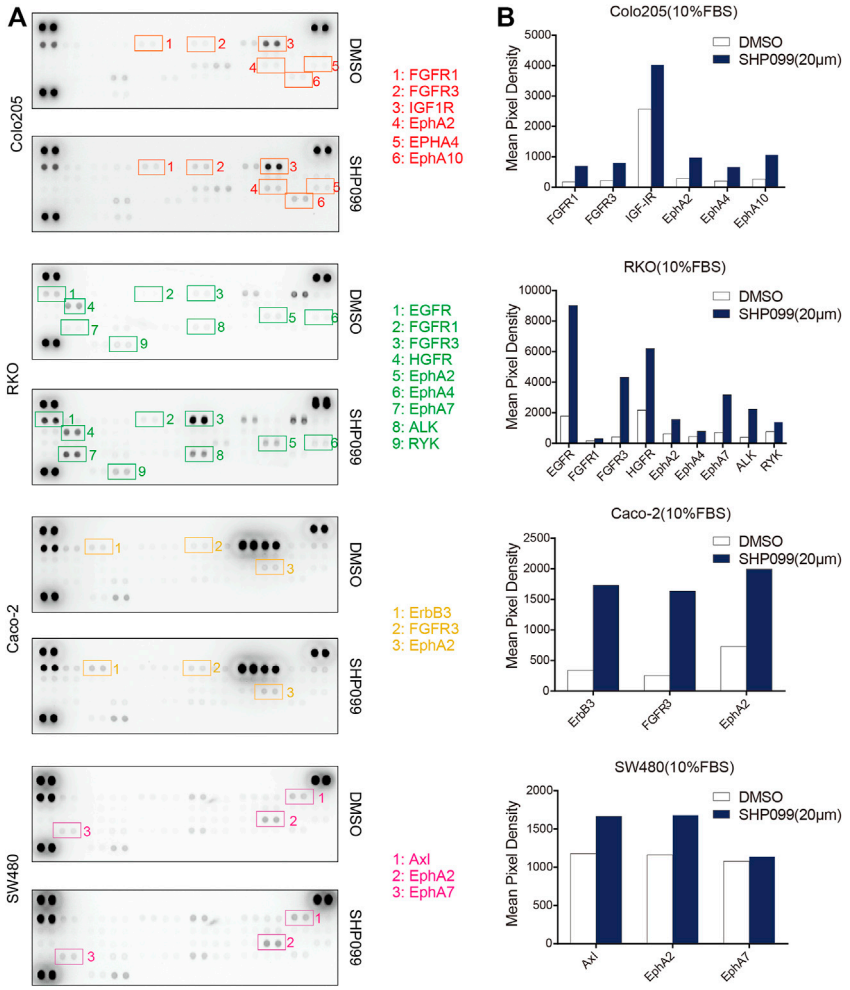

FIGURE 5 | SHP2 inhibition with SHP099 induces several phosphorylated RTKs. **(A)** Induced expression profiles of RTKs by SHP099 for 24 h in CRC cells with the examination by phospho-RTK arrays. Spots are in duplicate, and each pair corresponds to a specific p-RTK. **(B)** Comparison of typical induced p-RTK expression by SHP099 across several CRC cell lines.

levels of phospho-RTKs at baseline suggest that distinct and multiple RTKs may drive adaptive feedback across different CRC cell lines and that the strategies targeting a single RTK may not be universally effective.

FAK Mediation of the Feedback Reactivation of the AKT Pathway Following SHP2 Inhibition in a Subset of CRC Cells

Studies have reported the suppression of FAK signaling (Marin et al., 2008; Hartman et al., 2013; Lee et al., 2015) and activation of AKT–mTOR signaling (Ashton et al., 2010; Yoon et al., 2017) by SHP2. The present study evaluated the association between SHP2, FAK, and AKT signaling. The *p*-FAK induced by SHP099 treatment was significantly increased at 1 h for Colo205 and at 12 h for SW620 (**Figure 6A**), which is tightly correlated with the rebound of *p*-AKT, suggesting that FAK may be a key mediator for feedback reactivation of the AKT pathway following SHP2 inhibition. However, this phenomenon was not observed in other cell lines (**Supplementary Figure S4A**). The baselines of *p*-FAK were significantly higher in these cell lines than those in SW620 and Colo205 (**Supplementary Figure S4B**). To explore the role of induced FAK in the rebound of AKT pathway following SHP2 inhibition, we combined FAK inhibitor PF573228 and SHP099 to treat CRC cells. The combination of PF573228 and SHP099 not only eliminated the feedback reactivation of the AKT pathway but also generated stronger inhibition of *p*-AKT than SHP2 alone (**Figure 6B**). The result clearly demonstrated that released FAK activation following SHP2 inhibition may be a key mediator for the feedback reactivation of AKT pathway in SW620 and Colo205.

Sensitization of the Suppression of SHP2 Inhibition on CRC by FAK Blockage

To explore the function of induced or baseline *p*-FAK in the resistance to SHP2 inhibition, we investigated the effects of SHP2

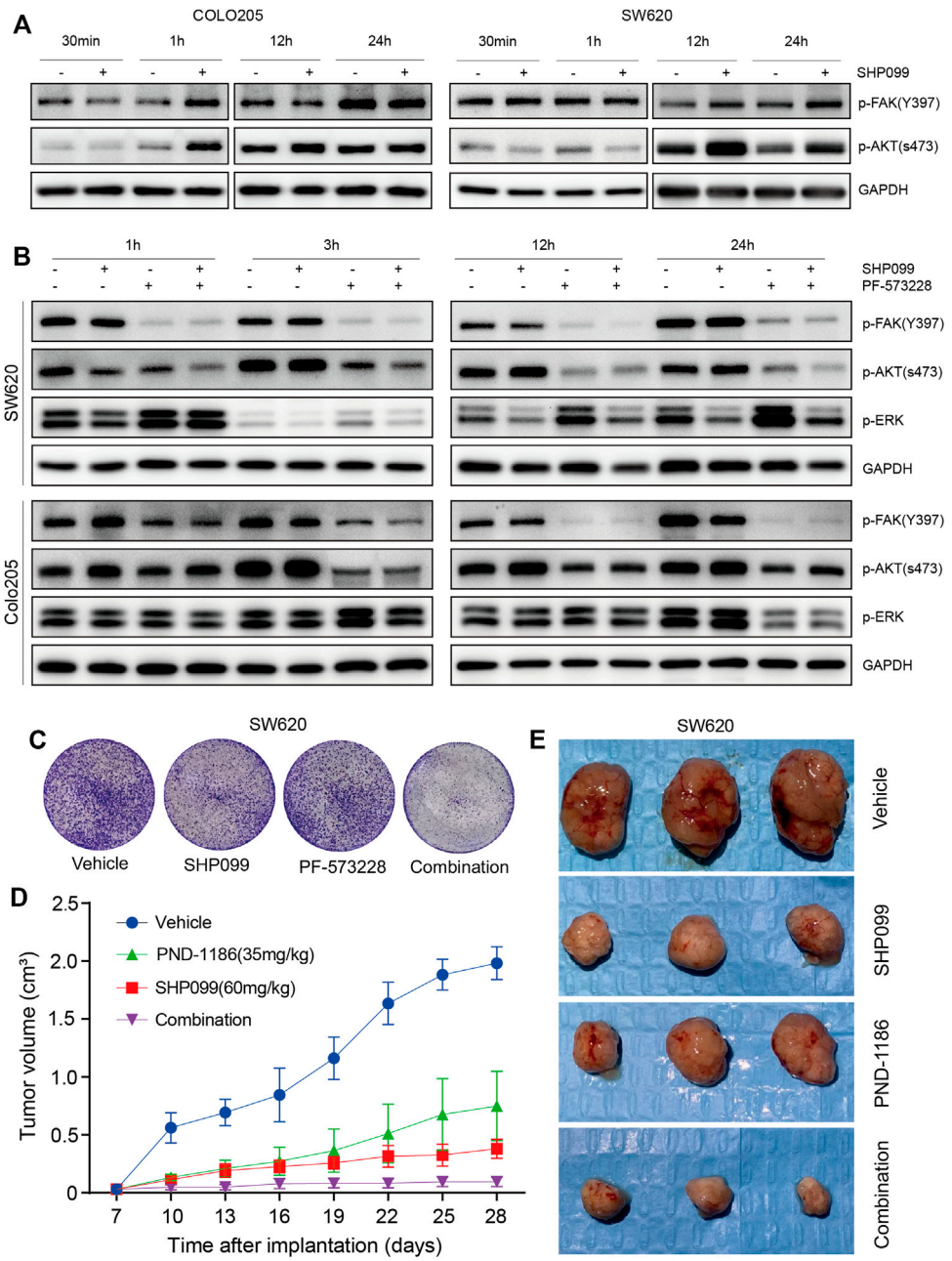

FIGURE 6 | p-FAK inhibition improves antitumor efficacy when combined with SHP099. **(A)** Dynamic p-FAK is associated with the feedback activation of AKT signaling. **(B)** Dynamic effect of SHP099 and PF-573228 (10 μM) on the AKT and ERK signaling in CRC cells. **(C)** PF-573228 sensitizes the colony inhibition of SHP099 in SW620 cells. **(D)** PND-1186 enhances the growth inhibition of SW620 xenografts by SHP099. **(E)** Images of isolated tumors from SW620 xenografts treated by SHP099 and/or PND-1186.

and FAK inhibitors, either alone or in combination, on CRC growth. The colony assays exhibited that the combined inhibition of SHP2 and FAK results in the strongest inhibition among all indicated groups, across not only SW620 (**Figure 6C**) but also the other four cell lines (**Supplementary Figure S4C**). Thus, the suppression of induced or baseline *p*-FAK can overcome the resistance to SHP099. With SW620-derived xenograft models, we further observed significant tumor suppression with a combination of SHP099 and FAK inhibitor PND-1186/VS4718 (**Figures 6D,E**). Thus, the findings indicate that the combination of SHP2 and a FAK inhibitor may be a board-spectrum and

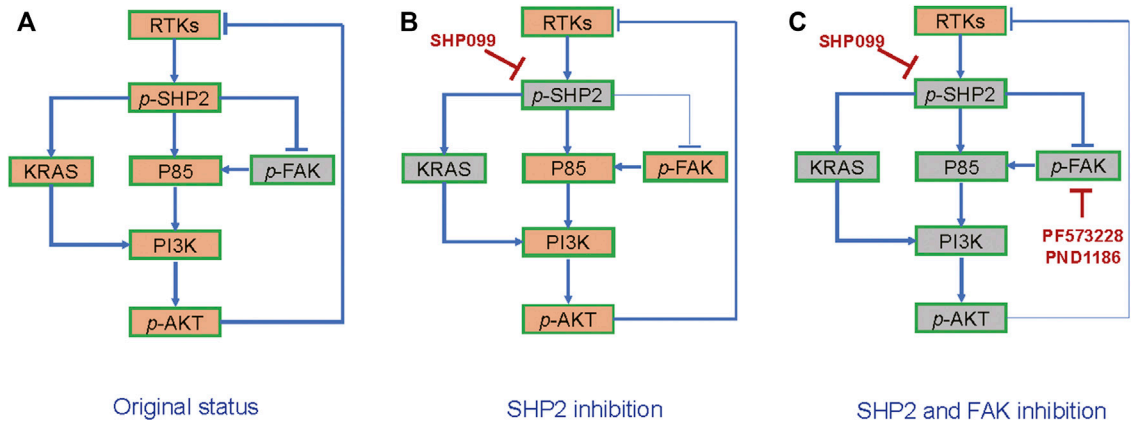

FIGURE 7 | The schematic diagram of combined suppression of SHP2/FAK-AKT signaling. **(A)** RTKs-SHP2 signal activates AKT but suppresses FAK. **(B)** FAK-AKT signal is released in response to SHP2 inhibition. **(C)** Combination of SHP2 and FAK blockages generates stronger AKT inhibition than the inhibitor alone.

promising treatment regimen for CRC, and the potential working mechanism of the combination therapy on AKT pathway is represented in **Figure 7**.

DISCUSSION

The activation of AKT and ERK pathways by the multiple RTKs is critical in the pathogenesis of cancer. As a key mediator, SHP2 inhibition represents a potential opportunity to block these pathways (Huang et al., 2014; Prahallad et al., 2015; Chen et al., 2016; Rehman et al., 2018). In the present study, we observed that *p*-SHP2 expression is significantly elevated in the CRC epithelial cells as compared with normal tissues, which supported the use of SHP2 as a target for CRC treatment. However, the use of an SHP2 inhibitor alone may be ineffective in most CRC cells, especially for cells with gain-of-function mutations of KRAS or BRAF (Chen et al., 2016; Mainardi et al., 2018). We observed that most of the cancer cells with the wild types of both KRAS and BRAF still exhibit resistance to SHP2 inhibition, which cannot be fully explained by the *p*-SHP2 levels, indicating an unknown mechanism for the resistance.

The ERK pathway has been investigated widely as a key effector of SHP2 inhibition in studies (Chan and Feng, 2007; Huang et al., 2014; Prahallad et al., 2015; Chen et al., 2016; Fedele et al., 2018; Mainardi et al., 2018; Rehman et al., 2018; Wong et al., 2018; Ahmed et al., 2019; García-Aranda and Redondo, 2019; Lu et al., 2019; Zhao et al., 2019; Martínez-Jiménez et al., 2020; Quintana et al., 2020) and may not be affected by SHP2 inhibitors in cancer cells with KRAS or BRAF mutations (Chen et al., 2016; Mainardi et al., 2018). Similarly, the inhibition of SHP2 with SHP099 generated the obvious reduction of *p*-ERK only in those cells with both wild types of KRAS and BRAF. Researchers have observed higher levels of PI3K/AKT pathway activation in CRC than those in other cancer types (even in BRAF mutated specimens) (Ebi et al., 2011), indicating a critical role of PI3K/AKT pathway in cancer. However, the variation in the AKT pathway, a key effector of SHP2, has still not been well investigated in response to SHP2 inhibition. For CRC cells, we clearly observed a rapid reduction in the AKT pathway activation after SHP099 treatment, which is independent of KRAS or BRAF mutation. This result is consistent with a recent notion that SHP2 has a major effect on AKT pathway in CRC, even in those cells with KRAS and BRAF mutations (Ebi et al., 2011). An alternative explanation for this data is the alternative regulation of SHP2-p85-p110α and SHP2-KRAS-p110α, and the former of which is not depended on KRAS mutation. Unexpectedly, in 12–24 h after the reduction of the pathway, a reactivation of the AKT pathway was observed consistently across all tested CRC cells. Furthermore, we also confirmed that the reactivation of the AKT pathway occurs in xenograft models of CRC. Although the reactivation of the AKT pathway by its inhibitors has been reported (Chandarlapaty et al., 2011), the reactivation of AKT pathway induced by its upstream inhibition such as SHP099 is scarcely reported until now (Lauriol et al., 2016).

Concerning that the activation of the AKT pathway is usually associated with drug resistance (Datta et al., 2017; Song et al., 2017; Vitiello et al., 2019), we propose that the blockage of the reactivation of the AKT pathway in response to SHP2 inhibition may overcome the resistance of CRC cells to SHP2 inhibitors (Song et al., 2017; Sun et al., 2019; Leung et al., 2020). In line with this hypothesis, we observed that the combined inhibition of SHP2 and AKT pathways with SHP099 and MK-2206 generated a synthetic suppression of the growth of all tested CRC cells. The efficacy of the combined treatment was further confirmed with colonic assays and in animal xenograft models. Additionally, the combination of SHP2 and AKT inhibitors is almost comparable with the combined inhibition of SHP2 and ERK pathways, which has been reported in recent studies (Fedele et al., 2018; Wong et al., 2018; Ahmed et al., 2019; Lu et al., 2019). Moreover, SHP099 exhibited synergistic potential when combined with PI3K inhibitors (such as Pictilishib) (Chen et al., 2016; Sun et al., 2019), which supported our hypothesis. Therefore, we report that targeting the AKT pathway also has the potential to sensitize the efficacy of SHP2 inhibitor.

RTK-driven feedback reactivation of AKT or ERK signaling has been identified as a key driver of drug resistance in cancers treated with AKT and mTOR inhibitors or BRAF and MEK inhibitors (Corcoran et al., 2012; Turke et al., 2012; Sun and Bernards, 2014; Datta et al., 2017; Fedele et al., 2018; Wong et al., 2018; Ahmed et al., 2019; Lu et al., 2019; Leung et al., 2020). Thus, the reactivation of AKT following SHP2 inhibition may also be associated with RTKs. We observed that the levels of multiple RTKs is elevated in response to SHP099. However, the profile of RTKs was extremely heterogeneous among CRC cells. Similarly, the heterogeneity of the profile of RTKs induced by another inhibitor was also reported (Hao et al., 2019). Thus, the results indicate that the blockage of the reactivation of AKT pathway is difficult by targeting a dominant RTK.

When SHP2 is inhibited by SHP099, other mediators such as FAK and RSK can also take over the signaling from RTKs to AKT and ERK. Due to the FAK dephosphorylation by SHP2 (de Oliveira et al., 2009; Hartman et al., 2013), the SHP2 inhibition may result in the released activation of FAK signaling. We observed obvious elevation in FAK signaling following SHP2 inhibition in a subset of CRC cells, which is tightly correlated with the reactivation of AKT signaling. Interestingly, other cells without the proposed association exhibited consistently high baseline expression of *p*-FAK. The nuclear FAK expression is also associated with a poor prognosis in CRC (Albasri et al., 2014; Davis et al., 2017). The results promoted us to investigate the effect of the combined inhibition of SHP2 and FAK on all CRC cells. Surprisingly, the combined treatment resulted in obvious growth inhibition across all the tested cells. Moreover, the combination of SHP2 and FAK inhibitors resulted in a stronger reduction of AKT signaling in CRC cells, indicating that the role of FAK inhibitor as a sensitizer on SHP2 inhibitor depends at least partly on the blockage of AKT pathway reactivation induced by SHP2 inhibition. Therefore, FAK is a promising combination partner for SHP2 inhibitors, capable of preventing adaptive feedback reactivation from multiple RKTs to maintain AKT pathway suppression and enhance efficacy *in vitro* and *in vivo*. Additionally, the inhibition of SHP2 and FAK signaling may also contribute to enhanced antitumor immune response (Prahallad et al., 2015; Serrels et al., 2015; Jiang et al., 2016; Serrels et al., 2017; Zhao et al., 2019; Quintana et al., 2020), which must be explored further.

Our study has also some limitation. First, it is insufficient to clarify the effect of the mutation patterns of KRAS, BRAF and PIK3CA on the FAK-AKT signal. Second, only 2 cell lines were used *in vivo* models to verify the combination regimens, and more models should be used further. Third, the effect of tumor microenvironment, especially for multiple immune cells, is not involved in the study. However, we have described the adaptive feedback of the AKT pathway through multiple RTKs or released FAK activation after SHP2 inhibition and confirmed that the reactivation can drive the resistance to SHP2 inhibition in both *in vivo* and *in vitro* models. The combination of SHP2 inhibitors with the inhibitors of either FAK or AKT pathway may represent a promising therapeutic approach against CRC.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Committee of Second Military Medical University, Shanghai, China. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Institutional Review Committee of Second Military Medical University, Shanghai, China. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

YL, YY, and FZ performed the experiments. YL, YY, WC analyzed the data and wrote the paper. AG and FC edited the manuscript. MS revised the article critically. YF and XX collected the baseline information of patients. HS provided guidance on experimental technology. WC, YP, and SZ are the corresponding authors and they designed the research. All authors read and approved the final manuscript.

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REVIEW
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Insight Into Nicotinamide Adenine Dinucleotide Homeostasis as a Targetable Metabolic Pathway in Colorectal Cancer

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Tumour cells modify their cellular metabolism with the aim to sustain uncontrolled proliferation. *Cancer* cells necessitate adequate amounts of NAD and NADPH to support several enzymes that are usually overexpressed and/or overactivated. Nicotinamide adenine dinucleotide (NAD) is an essential cofactor and substrate of several NAD-consuming enzymes, such as PARPs and sirtuins, while NADPH is important in the regulation of the redox status in cells. The present review explores the rationale for targeting the key enzymes that maintain the cellular NAD/NADPH pool in colorectal cancer and the enzymes that consume or use NADP(H).

Keywords: NAD, NADPH, NAMPT (nicotinamide phosphoribosyltransferase), isocitrate dehydrogenase (IDH), CD38, ALDH = aldehyde dehydrogenase, PARP, sirtuins

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INTRODUCTION

Colorectal cancer (CRC), a frequent cancer that occurs in both males and females (Kim HI. et al., 2018), is characterized by an high rate of mortality (Brody, 2015). Overall, 5-years survival rates are high, around 90%, but this percentage decreases to 66% in patients with regional lesions and metastasis (Koyanagi et al., 2008). From a therapeutic point of view, new drugs have extended the survival rate, but there is still a strong unmet medical need in advanced or metastatic CRC, in particular due to chemotherapy/target therapy resistance and unresponsiveness to new therapies (Brody, 2015; Loree and Kopetz, 2017). Finding new therapeutic strategies in CRC is therefore both challenging and necessary.

Among the cancer features that define aggressiveness, there is the reprogramming of cellular metabolism (Loree and Kopetz, 2017). Abnormal consumption of glucose and increased production of lactate, with a subsequent decrease of oxidative phosphorylation through aerobic glycolysis, is defined as the "Warburg effect", and allows cancer cells to win the evolutionary game of the "survival of the fittest" thereby allowing them to proliferate both in aerobic and anaerobic environments (Vander Heiden et al., 2009). Therefore, also gluconeogenesis, that is opposed to aerobic glycolysis, is able to hijack cell plasticity, promoting tumour growth (Grasmann et al., 2019). Nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADPH) are indispensable in regulating these metabolic reactions as well as for adenosine triphosphate (ATP) production. While the ubiquitous nature of these pathways renders their pharmacological targeting challenging, a number of groups have now focused their attention on this possibility. These strategies can be sub-divided in distinct chapters: 1) reducing the NAD(H) supply of cells by inhibiting the

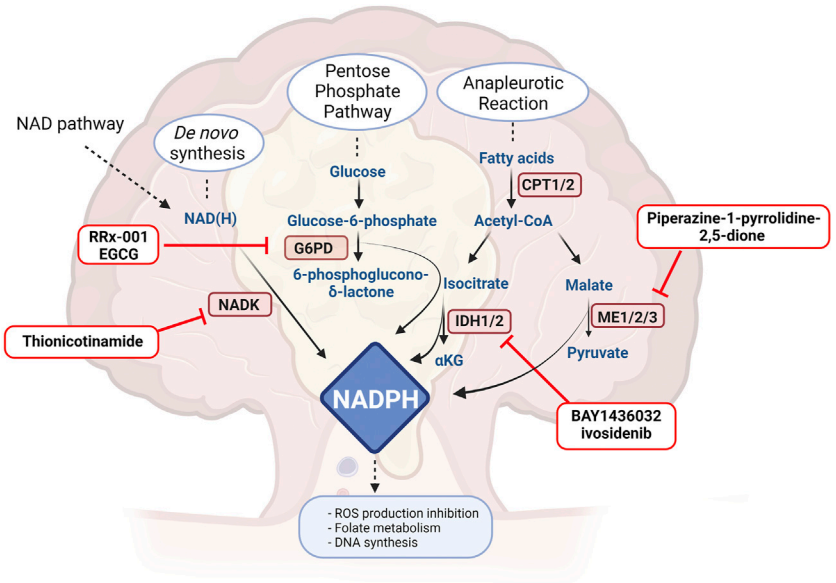

FIGURE 1 | Drugs or molecules that have been postulated as effective in CRC and that target NAD routes. NAMPT: nicotinamide phosphoribosyltransferase, NAPRT: nicotinic acid phosphoribosyltransferase, NMNAT: nicotinamide mononucleotide adenylyltrasnferase, NADSYN: NAD synthetase, PARP: Poly-ADP ribose polymerase, ALDHs: aldehyde dehydrogenase. Created with BioRender.com.

enzymes involved in its synthesis, thereby reducing the activity of all down-stream pathways dependent on this molecule; 2) selectively inhibiting NAD-consuming enzymes: 3) selectively inhibiting enzymes that use NAD(P)(H) as a co-factor.

Several reviews have addressed the advantage of targeting NAD/NADPH homeostasis and consuming enzymes in cancer (Audrito et al., 2019; Galli et al., 2020; Ju et al., 2020; Rather et al., 2021); thus, an extensive description is beyond the purpose of this manuscript, and we refer to other excellent reviews on the subject, including the ones that describe the entire NADome (Chiarugi et al., 2012). In the present review, we have searched on MEDLINE/PubMed the names more than of 400 NAD and NADPH-dependent enzymes followed by the term "colorectal cancer" and have chosen the most relevant, and less reviewed, targets in the context of CRC. In particular, we have included papers pointing out targets in a pharmacological perspective, avoiding results of proteins found exclusively in sequencing analysis.

NAD homeostasis results from the balance between NAD-consuming reactions and NAD-biosynthetic routes. Indeed, NAD-consuming enzymes (*e.g.*, poly-ADP-ribose polymerases (PARPs)) are upregulated and have a higher activity in cancer, and metabolic enzymes that require NAD(P) as a co-factor are also up-regulated, resulting in a higher demand of these molecules which is provided by NAD-synthetic paths. It is therefore not surprising that several manuscripts have investigated the effect of inhibition of these pathways in CRC, as summarized in **Figure 1**.

NICOTINAMIDE ADENINE DINUCLEOTIDE SYNTHESIS AS A TARGET

There are four main NAD biosynthetic pathways in eukariotic cells: *1*) the *de novo* biosynthetic *Pathway* from quinolinic acid, derived from dietary tryptophan, *2*) the *Preiss-Handler Pathway* (PH-pathway) from nicotinic acid (NA), *3*) the *Salvage Pathway* from nicotinamide (NAM), mediated by nicotinamide phosphoribosyltransferase (NAMPT) and *4*) the newly described route from nicotinamide riboside (Tempel et al., 2007) through nicotinamide riboside kinase (NMRK). These four pathways converge in the production of a mononucleotide, catalysed by different phosphoribosyltransferase enzymes depending on the starting precursor. In the last step, which is common to all routes, the formation of NAD is catalysed by nicotinamide mononucleotide adenylyltransferase (NMNAT). Nicotinamide is considered the main precursor of NAD due to its availability compared to nicotinic acid: it is present in higher concentrations in the bloodstream, it is easily introduced by diet, and it is the reaction product of NAD-dependent enzymes.

Due to the Warburg effect and to over-activation of NAD-consuming enzymes, proliferating cells necessitate larger NAD supplies (La Vecchia and Sebastián, 2020). Chowdhry et al. have highlighted that tumours are usually dependent on the PH-pathway (PH-dependent tumours) or from salvage pathway (salvage-dependent tumours). In general, this is usually linked to whether the original healthy tissue expressed higher levels of either nicotinic acid phosphoribosyltransferase (NAPRT) or NAMPT (Chowdhry et al., 2019). While these two enzymes

have received considerable attention in cancer, every enzyme that sustains NAD production might be a possible target for targeting CRC.

NAMPT and NAPRT as Drug Targets in Colorectal Cancer

Nicotinamide phosphoribosyltransferase is the key enzyme of the NAD Salvage Pathway and belongs to type II phosphoribosyltransferases. It exists as a dimer in two different forms: an intracellular form (iNAMPT) that is localized in the cytosol and in the nucleus, and an extracellular form (eNAMPT) that is secreted in extracellular fluids by various cell types. NAPRT is structurally similar and has also been reported to be both intracellular and extracellular.

iNAMPT is situated inside the cell as a homodimer with a molecular weight of about 110 kDa. It is involved in NAD biosynthesis and catalyses the production of nicotinamide mononucleotide (NMN) from NAM and 5-phosphoribosyl-1-pyrophosphate (PRPP). Since it controls the intracellular levels of NAD (*i.e.,* its inhibition leads to a significant drop in levels), iNAMPT modulates the activity of NAD-dependent enzymes, such as PARPs and sirtuins (SIRTs). NAPRT, instead, uses NA as a substrate.

These two enzymes therefore run parallel routes of synthesis. According to Chowdhry et al., colon cancer cells appear to be NAMPT-dependent, due to lower levels of NAPRT in the primary tissue (Chowdhry et al., 2019), even though other manuscripts reveal a dependence also from NAPRT (Hong et al., 2019) and a higher expression of this latter enzyme in healthy small intestine, colon cancer cell lines and biopsies (Hara et al., 2007; Duarte-Pereira et al., 2016; Cole et al., 2017).

While a number of drug discovery programs have initiated to develop NAPRT inhibitors, potent and selective ligands have so far been elusive, and it is therefore not known whether NAPRT inhibition may be a viable strategy to target CRC. On the contrary, the field of iNAMPT has capitalized on the report, back in 2003, of the first specific nanomolar inhibitor, FK866 or APO866, which has been followed by a number of other inhibitors which have either reached clinical stages (CHS828, GMX1777, KPT9274 and OT82) or have only been carried forward only at the preclinical level (Holen et al., 2008; Pishvaian et al., 2008; Goldinger et al., 2016; Galli et al., 2020).

Alongside the metabolic rationale, it is also known that iNAMPT overexpression is correlated to colorectal carcinoma rather than adenoma and it is therefore not surprising that its inhibition, using FK866, leads to cell death of a number of CRC cell lines, including HT29, HCT116 and Caco-2 (Lv et al., 2015). Similarly, the small molecule GMX1778, another inhibitor that has reached clinical trials, is able to induce antitumour responses in HCT-116 xenograft model (Watson et al., 2009). Similarly, another NAM-competitive inhibitor, named A-1293201, is also effective in the same model (Wilsbacher et al., 2017).

Tolstikov et al. have characterized iNAMPT inhibition in CRC, again using FK866 and mass-spectrometry-based metabolomics (Tolstikov et al., 2014). Using the human HCT116 cell line (Duke's staging type D) and have reported how FK866 is able to hinder several metabolic pathways and their related signalling pathways. In brief, FK866 is able to 1) attenuate glycolysis and the pentose phosphate pathway and thereby lead the accumulation of glycolytic substrates; 2) attenuate nucleotide and amino acid metabolism with a subsequent reduction of purine and pyrimidine metabolism; 3) induce reduction of pyruvate entry in the TCA cycle with a consequent reduction in aspartate and alanine synthesis; 4) induce an attenuation of purine biosynthesis, with an accumulation of xanthine; and 5) reduce fatty acid and lipid metabolism (Tolstikov et al., 2014). Alongside metabolism, iNAMPT has also been shown to sustain neoplastic progression acting on cancer stem cells and Wnt/β-catenin signalling (Lucena-Cacace et al., 2018; Ye et al., 2020). Indeed, iNAMPT is now considered an oncogene, overexpressed in colon cancer and correlated to poor prognosis, able to promote tumour initiation through cancer initiating cells (CICs), in CICs xenograft using HCT116 and LS180 cell lines (Lucena-Cacace et al., 2018). This overexpression determines chemoresistance mediated by an increase in CD133 + cancer stem cells, overexpressing PARPs and SIRT1, and the treatment with FK866 in combination with olaparib (PARP inhibitor) or sirtinol (SIRT inhibitor) is able to counteract tumour progression (Lucena-Cacace et al., 2018). Moreover, iNAMPT regulates Wnt/β-catenin pathway promoting CRC growth, so using FK866, β-catenin nuclear translocation and Wnt/β-catenin target gene cyclin D1 are hampered. Axin, a component of the β-catenin demolition complex, is subsequently elevated after FK866 treatment (Ye et al., 2020). On the other hand, the resistance of FK866 may sensitise cells to 5-fluorouracil, cisplatin and γ-rays in HCT116-resistant cells (Ogino et al., 2018, 2019). Both iNAMPT and NAPRT have been found overexpressed in a model of cancer-associated colitis (CAC), as azoxymethane (AOM)/dextran sodium sulphate (DSS), highlighting a possible effect on inflammation that undergoes cancer development.

In 1994, a cytokine, referable to the extracellular form of NAMPT (eNAMPT), was identified as an immunomodulatory agent able to promote pre-B cell colony formation synergizing with IL-7 and stem cell factor (SCF) (Samal et al., 1994). Indeed, it has now been ascertained that eNAMPT can also be secreted by a number of different cell types (including cancer cells) and acts as a cytokine that modulates the immune response (Audrito et al., 2015; Camp et al., 2015). It is still unclear whether enzymatic activity is maintained and if it is necessary for its cytokine-like action (Grolla et al., 2016). iNAMPT and eNAMPT have the same amino-acid sequence and the same quaternary structure, but specific post-translational modifications might be responsible for eNAMPT secretion. In particular, it has been suggested that the deacetylation of lysine 53 on iNAMPT, operated by SIRT1, predisposes the protein to secretion and enhances eNAMPT activity in adipocytes (Yoon et al., 2015). No testimony certifies a possible effect of targeting eNAMPT in CRC, even though it has been found elevated in CRC patients (Nakajima et al., 2010), and shown to promote multi-drug resistance (Yan et al., 2017) and increase reactive oxygen species (ROS) (Buldak et al., 2015). Our group and Garcia's have recently developed neutralizing monoclonal antibodies against eNAMPT (Colombo

et al., 2020; Garcia et al., 2021), and it is possible that in the future data in CRC will be gathered also in this respect although the relationship between eNAMPT and NAD homeostasis is unclear. More recently, also NAPRT has been found in the extracellular space (eNAPRT), elevated in septic patients. Managò et al. have highlighted also its presence in some tumour patients, compared to healthy subjects, but more information needs to be gathered (Managò et al., 2019).

Overall, therefore, iNAMPT inhibition would appear as a plausible target. While efficacy would be supported by the above data, there are nonetheless doubts regarding safety, with a number of on-target side effects (Olesen et al., 2010; Tarrant et al., 2015), probably due to the ubiquitous nature of this enzyme. The verdict is not final, though, as there are dual agents (PAK4 p21-activated kinase and NAMPT dual inhibitor) in the clinic and a series of NAMPT inhibitors has been reported as not having these side effects in preclinical tests (Abu Aboud et al., 2016; Travelli et al., 2019a; Galli et al., 2020). Moreover, iNAMPT has appeared to be a promising target also in the immune counterpart of tumours. NAMPT inhibitors appear to have a role in hijacking myeloid-derived suppressor cell (MDSC) mobilization, activating antitumor immune responses and sensibilizing tumours to immune checkpoint inhibitors (Travelli et al., 2019b) as a result of a direct control of the microenvironment. No one has hypothesized this strategy in CRC, but it appears to be promising in CRCs with high immune infiltration or dependent on bowel inflammation (IBD-CRCs). This suggests that targeting iNAMPT in CRC could have a direct effect on tumours and an indirect anticancer activity on the immune system.

Therapeutic role of NMNAT Targeting in Colorectal Cancer

The final products of iNAMPT and NAPRT, NMN and nicotinic acid mononucleotide, are then transformed in NAD and nicotinic acid adenine dinucleotide (NAAD) through NMNAT, which is present in cells in three different isoforms (NMNAT1,2,3). NMNAT1 is predominantly present in the nucleus, NMNAT2 in the Golgi apparatus (Berger et al., 2005), while NMNAT3 in mitochondria or cytoplasm (Cambronne and Kraus, 2020). NMNAT2 has been highlighted as a possible diagnostic target for CRC, as its levels correlate with p53 in more invasive tumours, even though it is not correlated with overall survival (Cui et al., 2016). Furthermore, NMNAT2 overexpression in CRC is linked to SIRT6 downregulation (Qi et al., 2018).

It has been found that overexpression of NMNAT2 sensitises Caco-2 and HT-29 to tiazofurin, inducing cell death, while low level-NMNAT2 cell lines appear to be refractory (Kusumanchi et al., 2013). This is most likely due to a link between the amount of NAD and the presence of inosine monophosphate dehydrogenase (IMPD), the target of the active metabolite of tiazofurin, which is an NAD analogue. While NMNAT2 upregulation favours tiazofurin action, which were abandoned in clinics due to adverse events (Popsavin et al., 2006), it is thought that inhibitors of this enzyme would be a significantly better therapeutic option. Yet, at present no potent NMNAT inhibitor has been developed (Petrelli et al., 2011; Buonvicino et al., 2018), with the exception of 2,3-dibromo-1,4-naphthoquinone (Haubrich et al., 2020), which nonetheless lacks selectivity against the different isoforms and possibly acts also on other targets. Yet, given the convergence of both iNAMPT and NAPRT on NMNAT2, it could be postulated that inhibitors of these isoform in CRC may be attractive.

NICOTINAMIDE ADENINE DINUCLEOTIDE-UTILIZING ENZYMES AS DRUG TARGETS

Therapeutic Role of Poly-ADP-Ribose Polymerase *Inhibitors in Colorectal Cancer*

Increased production of ROS in cancer cells determines DNA damage, with base lesions or single strand breaks (Saikolappan et al., 2019; Chang et al., 2020). While some of these might lead to pro-tumoural permissive mutations, an excess would invariably lead to apoptosis. This balance is maintained by PARPs, an 18-member nuclear enzyme family involved in DNA damage repair. PARPs orchestrate chromatin remodelling, transcription, replication, recombination and DNA repair (Morales et al., 2014) and are upregulated in CRC (Nosho et al., 2006; Dziaman et al., 2014). Of these 18 members, the most relevant ones in the context of cancer appear to be PARP1 and PARP2, recognized for their ability to activate base excision repair (BER) in response to single-stranded DNA breaks (SSBs). (Amé et al., 2004). Indeed, most tumours, to counterbalance ROS production, overexpress PARPs with the aim to increase genomic stability (Nomura et al., 2000). To support this, PARP-1 knock-out cells are more sensitive to exogenous DNA damage agents, such as alkylating drugs and irradiation (Menear et al., 2008). Briefly, in front of DNA damage response, there is the activation of several pathways that leads to poly ADP-ribosylation (PARylation) in the damage site by PARPs using NAD as a source of adenosine diphosphate ribose (ADPR).

Several PARP inhibitors have been developed and are already in the armamentarium of oncologists. Most of their indications at present pertain to patient populations (e.g., ovarian, breast, pancreatic, prostate cancer) in which there is a deficit of the BRCA1/2 genes, involved in homologous recombination and DNA repair, thereby providing an increased mutational burden that synergizes with PARP inhibition. Yet, in cancers in which BRCA1/2 is prevalent, PARP inhibitors have also shown an effect in the all-comer population. PARP inhibitors are increasing their importance in cancer therapy, and it is beyond the scope of the present manuscript to review them. It should be noticed, though, that although CRC is not yet an approved indication of any PARP inhibitor, several manuscripts have highlighted their potential, in particular in combination with DNA damaging agents such as irinotecan (Davidson et al., 2013; Genther Williams et al., 2015; Augustine et al., 2019), 5-fluorouracil and oxaliplatin (Jarrar et al., 2019), PI3K inhibitors in combination with anti-CTLA4 immunotherapy and X-ray radiation (Landry et al., 2020), p53 inhibitors (idasanutlin or pifithirin-β) (Smeby et al., 2020) or ATM inhibitors (Wang et al.,

TABLE 1 | PARP inhibitors in different cell models.

PARP inhibitor	Combinatory drug	Cell model	References
Veliparib (ABT-888)	Irinotecan (SN-38)	HCT-116	Davidson et al. (2013)
Olaparib	ATM inhibitor (KU55933)	SK-CO-1 and HCT 116	Wang et al. (2017b)
Rucaparib	Irinotecan	HCT-116	Augustine et al. (2019)
Talazoparib, niraparib	Idasanutlin (MDM2i) and pifithrin-β (p53i)	SK-CO-1, LS513, SW1222 and SNU61	Smeby et al. (2020)
Niraparib	HS-173 (PI3Ki) ± γH2AX or αCTLA-4	CT-26	Landry et al. (2020)
Niraparib	Irinotecan (SN-38)	Microsatellite stable/instable CRC cell lines	Genther Williams et al. (2015)
Olaparib	5-FU or oxalilplatin	CRC-patient-derived cell lines	Jarrar et al. (2019)

TABLE 2 | PARP inhibitors in clinical trials.

Study number	Phase	Drugs	Enrolment	Ref/Stage
NCT01051596	2	Veliparib + temozolomide	75 CRC patients incurable by surgery	Completed (Pishvaian et al., 2018)
NCT00912743	2	Olaparib	33 CRC patients incurable by surgery	Completed (Leichman et al., 2016)
NCT02484404	1/2	Cediranib+Olaparib+MEDI4736 (anti-PD-L1)	Phase 2 part of the study requests the participants to have tumor samples removed	Recruiting (clinicaltrial.gov)
NCT03875313	1/2	Talazoparib + telaglenastat (glutaminase inhibitor)	33 between different types of solid tumour	Terminated (clinicaltrial.gov)
NCT02305758	2	Velparib + FOLFIRI + bevacizumab	130 untreated metastatic colorectal cancer	Completed (Gorbunova et al., 2019)
NCT03337087	1/2	5-fluorouracil+leucovorin+liposomal irinotecan + rucaparib	CRC (up to 3 lines of prior therapy)	Recruiting (clinicaltrial.gov)
NCT03761914	2	Olaparib+durvalumab	Advanced Mismatch Repair Proficient Colorectal *Cancer* (MMRp-CRC)	Active (clinicaltrial.gov)
NCT04171700	2	Rucaparib	Mutations in Homologous Recombination Repair (HRR) genes	Recruiting (clinicaltrial.gov)
NCT01618136	1/2	E7449 (PARP/tankyrase inhibitor) + temozolomide or carboplatin and paclitaxel	41 patients with solid tumours	Completed (Plummer et al., 2020)
NCT00535353	1	Olaparib + irinotecan hydrochloride	26 patients with locally advanced or metastatic colorectal cancer	Completed (Chen et al., 2016)
NCT04456699	3	Olaparib+bevacizumab	Metastatic colorectal cancer (CRC) who have not progressed following first-line induction of FOLFOX with bevacizumab	Recruiting (clinicaltrial.gov)
NCT04166435	2	Olaparib + temozolomide	patients with MGMT promoter hypermethylated advanced colorectal cancer	Recruiting (clinicaltrial.gov)

2017a). Mauri et al. have highlighted the "biomarkers of PARPness" which includes BRCA1/2 mutations in solid tumours, the sensitivity to platinum agents, but also ATM and CHK1 loss (Wang et al., 2017b; Mauri et al., 2020) (**Table 1**).

Different PARP inhibitors have already entered clinical trials in CRC (**Table 2**; clinicaltrials.gov). Several of them are still recruiting patients but others have already published encouraging results, despite the advanced settings. Pishvaian at al. have highlighted the efficient combination of veliparib and temozolomide in metastatic CRC population, refractory to other therapies, with a median OS of 6.6 months. More recently, Plummer et al. have demonstrated the efficacy of E7449 (PARP/tankyrase inhibitor) in patients with solid tumours, including CRC, showing a promising antitumoral activity with 50–800 mg oral dose. Furthermore, Chen et al. have evaluated that the combination of olaparib and irinotecan have appeared to be beneficial with intermittent administrations and dose reduction, avoiding side effects of both the molecules. On the other hand, Goburnova's and Leichman's groups have guided the only two terminated clinical trials in which, veliparib plus FOLFIRI and olaparib respectively, do not appear to be beneficial comparing to FOLFIRI alone or as a single agent. The conclusions of these studies are supported by the controlled nature of the trial, but both enrolled a very small number of patients in phase II trials. It is likely, therefore, that larger trials are needed to understand the potential of PARP inhibitors in CRC. For this to be successful, though, the correct patient population (in particular referring to the DNA repair machinery) and combination strategy should be optimized first.

Therapeutic Role of Sirtuin Inhibitors in Colorectal Cancer

Another class of NAD-consuming enzymes that has a role in genomic stability, metabolism and senescence are SIRT1-7

TABLE 3 | SIRT properties in CRC.

Isoform	Localization	Properties in CRC	Inhibitor
SIRT1	Nucleus/Cytoplasm (O'Callaghan and Vassilopoulos, 2017)	Promotes EMT and metastasis formation (Cheng et al., 2016)	4bb, evodiamine, MHY2256b (Ghosh et al., 2017; Zhou et al., 2019; Kim et al., 2020)
SIRT2	Cytoplasm/Nucleus (O'Callaghan and Vassilopoulos, 2017)	Promotes tumour angiogenesis (Hu et al., 2018)	MHY2256, AK-1, AF8, AF10, AF12 (Cheon et al., 2015; Farooqi et al., 2019; Kim et al., 2020)
SIRT3	Mitochondria (O'Callaghan and Vassilopoulos, 2017)	Modulates mitochondria fission, mobility and proliferation (Wang et al., 2018a, 3)	-
SIRT4	Mitochondria (O'Callaghan and Vassilopoulos, 2017)	Increased sensitivity to -FU (Huang et al., 2016) and tumour-suppression (Miyo et al., 2015)	-
SIRT5	Mitochondria/Cytoplasm (O'Callaghan and Vassilopoulos, 2017)	-	-
SIRT6	Nucleus (O'Callaghan and Vassilopoulos, 2017)	Prognostic favourable (Zhang et al., 2019)	-
SIRT7	Nucleus (O'Callaghan and Vassilopoulos, 2017)	Radiosensitivity in CRC (Tang et al., 2017)	-

(Vassilopoulos et al., 2011). Sirtuins are class III histone deacetylase which are highly conserved and use NAD as an acetyl acceptor leading to O-acetyl-ADPR and free nicotinamide. While first described as histone deacetylates, these enzymes have multiple targets and is therefore not surprising that the different isoforms shown different subcellular localization: SIRT1, SIRT6 and SIRT7 are mostly localised in the nucleus; SIRT3, SIRT4 and SIRT5 are in mitochondria; while SIRT2 is restricted to the cytoplasm (O'Callaghan and Vassilopoulos, 2017). They exert different activities, summarized in **Table 3**. All the different isoforms have been studied as putative prognostic biomarkers in CRC, and some of them, as SIRT1 and 2 have been considered papabile targets (Huang et al., 2016; Zu et al., 2016).

Several sirtuin inhibitors have been developed through the years, but none has so far entered clinical trials for cancer. The most studied inhibitors developed are against SIRT1 and SIRT2. Ghosh et al. designed a SIRT1 inhibitor named 4bb which is able to induce HCT116 apoptosis via p53-acetylation and Bax and caspase overexpression (Ghosh et al., 2017). Moreover, also MHY2256, a SIRT1/2 inhibitor has been shown to reduce viability of both p53 wild-type or mutant colorectal cancer lines (HCT116, HT29 and DLD-1) (Kim et al., 2020). Given that SIRT2 is thought to promote vascular endothelial growth factor A (VEGF-A) signalling and endothelial-like tube formation in tumour angiogenesis (Hu et al., 2018), specific inhibitors have also been developed. For example, AK-1, a cell-permeable benzylsulfonamide, is able to induce Snail down-regulation and the consequent block in G1-phase of HCT116 cell line (Cheon et al., 2015), while several lysine-based thioureas named AF8, AF10 and AF12 are able to counteract viability in HCT116 cell line and in the xenograft murine model (Farooqi et al., 2019).

No other inhibitors have so far been tested in CRC, but it is possible that also other sirtuins might be promising therapeutic targets.

Therapeutic role of CD38 Inhibitors/ Antibodies in Colorectal Cancer

CD38 is an ADP-ribosyl glycohydrolase mainly expressed in hematopoietic and non-hematopoietic cells, orchestrating activation and differentiation. CD38 is a transmembrane protein, localised on the cell membrane endowed of both enzymatic and receptor activity. As an ectoenzyme, it promotes the catabolism of extracellular NAD and NADP into ADPR or, in smaller amounts and depending on pH conditions, into the Ca2+-mediating second messengers cyclic adenosine diphosphate ribose (cADPR) and NAADP (van de Donk et al., 2018). The reaction produces cADPR, ADP ribose, and NAADP that stimulate Ca2+ mobilization (Malavasi et al., 2021). Whether in cancer biology this enzymatic activity is important has never been ascertained. On the other hand, as a receptor, CD38 is responsible of T cell activation, in particular in lamina propria cells, promoting colitis (Lischke et al., 2013) and it has been considered a positive prognostic marker for CRC (Perenkov et al., 2012).

Karakasheva et al. have highlighted that CD38 might be an interesting target for metastatic CRC. Using peripheral blood mononuclear cells (PBMCs) from CRC patients, compared to healthy donors, they observed an increased frequency of $CD38^+$ monocytic-myeloid-derived suppressor cells (M-MDSCs) and $CD38^+$ ppolymorphonuclear-myeloid-derived suppressor cells (PMN-MDSCs) compared to healthy monocytes, with the ability to induce immunosuppressive properties (Karakasheva et al., 2018). Moreover, $CD38^+$M-MDSCs and $CD38^+$PMN-MDSCs of patients who have previously undergo treatment are increased compared to naïve patients. Other Authors have highlighted the promising use of anti-CD38 antibodies in CRC patients refractory to other therapies, nonetheless no clinical trials or approvals have been proposed for CRC, even though anti-CD38 antibodies have represented a break-through in multiple myeloma (Nooka et al., 2019) and are at present approved in this indication.

Therapeutic Role of Aldehyde Dehydrogenases *in Colorectal Cancer*

Among the different NAD-consuming enzyme, the aldehyde dehydrogenase (ALDHs) superfamily, composed by 19 different members, is known to be essential in the irreversible oxidation of a wide range of endo- and xenobiotic aldehydes to

the respective carboxylic acid (Vassalli, 2019). Briefly, upon the activation of the catalytic cysteine (C302, numbering based on the ALDH2 sequence) by the glutamic acid (G268, numbering based on the ALDH2 sequence), the thiolate group interacts with the carbonyl carbon of the target aldehyde. After the deacylation, the hydride is transferred from the tetrahedral intermediate to the NAD pyridine ring. Finally, the thioester intermediate is hydrolysed, the reduced cofactor is dissociated and the enzyme can bind a new NAD molecule (Marchitti et al., 2008). The catalytic activity of ALDHs is essential to counterbalance the intrinsic toxicity of aldehydes, an extremely reactive chemical species extremely whose accumulation leads to DNA alkylation (LoPachin and Gavin, 2014). ALDHs are also involved in drug metabolism, and, for example, the active metabolite of cyclophosphamide, is a substrate of these enzymes (Tomita et al., 2016).

The relevance of this enzyme in the context of gastroenteric tumour is also given by the fact that CRCs are at times linked to high consumption of alcohol (Ferrari et al., 2012; Na and Lee, 2017). This could in part be attributable to acetaldehyde accumulation, produced by the alcohol oxidation (Zhang and Fu, 2021). Indeed, an unhealthy lifestyle with high alcohol consumption can be considered as one of the most common and important causes related to the potential development of CRCs and other significant cancers as head and neck, oesophageal and gastric (Zhang and Fu, 2021). Besides, a higher risk of CRCs has been demonstrated in Asian populations affected by a genetic polymorphism on the ALDH isoform 2, the key enzyme in acetaldehyde metabolism. The *ALDH2*2* allele is extremely common in Est Asians, with the glutamine 487 substituted by a lysine (E487K) which intrinsic activity is reduced from 60 to 100% (Chang et al., 2017; Rossi et al., 2018; Wang et al., 2018b). Based on these suggestions, a small molecule activator of the enzyme, known as alda-1, has been developed and characterized. This molecule increases the catalytic activity of wild-type ALDH2 just two-fold but increases 11-fold the activity of homozygous ALDH2*2 (Chen et al., 2014). The high-resolution crystal structure of the ALDH2*2-Alda1 complex describes the peculiar mechanism of interaction in which the small ligands can reconstitute the correct folding at the level of the αG structure of the Rossman-fold essential for the interaction with the NAD^+ adenosine ring and for the maintenance of the correct oligomerization, allowing the recovery of the full enzymatic activity (Perez-Miller et al., 2010).

Alongside activators in selected genetic contexts, inhibitors have also been proposed as possible therapeutics in CRC, due to an increased expression and activity of ALDHs (Khoury et al., 2012; Li et al., 2018; van der Waals et al., 2018; Yang et al., 2018; Attia et al., 2020). Interestingly, this is also true in the cancer stem cells (CSCs) (Clark and Palle, 2016), that are known as the most challenging to be targeted, refractory to common chemotherapeutics. It is also likely that this increased expression leads to chemotherapy and radiotherapy resistance of cancer cells and CSCs (Januchowski et al., 2013; Kim et al., 2017; Dinavahi et al., 2019; Muralikrishnan et al., 2020; Rebollido-Rios et al., 2020). ALDHs inhibitors have been proposed as monotherapies as well as in combination or as a tool for the early diagnosis (Koppaka et al., 2012; Anorma et al., 2018; Kim D. et al., 2018; Ferraris et al., 2020; Gelardi E. et al., 2021).

Among the 19 different isoforms, ALDH1A1 and 1A3, two key enzymes that participate in retinaldehyde oxidation (Moretti et al., 2016), have been scrutinized closely in several solid tumour, included the CRCs. Kovoska et al. have shown that abrogating *ALDH1A3* by siRNA-mediated gene silencing increases raltitrexed and 5-fluorouracil sensitivity of HCT-116 (Kozovska et al., 2018). Similarly, our group has recently described new ALDH1A3 inhibitors with a conserved imidazo [1,2- α]pyridine ring scaffold, considered as synthetic analogues of daidzin, a known reversible inhibitor of the ALDH isoenzymes (Quattrini et al., 2020b; Quattrini et al., 2020a). In particular one of the most promising compounds, called NR6, showed a strong potency and selectivity both against recombinant enzymes and cells overexpressing ALDH1A3 (Gelardi E. L. M. et al., 2021). The *in vitro* treatment with NR6 led to a strong cytotoxic effect only in cancer cell lines (HCT-116) overexpressing the target protein without inducing any significant toxicity in non-cancerous fibroblasts (CCD-18Co). The role of ALDH1A1 in CRCs is still unclear, outside the capacity to reduce the oxidative stress and the activity of alkylating agent, but it is considered as promising marker for the characterization of tumour malignancy and to locate the cancer stem cells (Ciccone et al., 2020). A small series of ALDH1A1 and ALDH1A pan-inhibitors are available, called CM10, A37 and NCT 501 hydrochloride (Morgan and Hurley, 2015; Yang et al., 2015; Chefetz et al., 2019) that were deeply investigated against ovarian cancer (Nwani et al., 2019), but that could be also useful to characterize the potential validity of ALDH1A1 as target in CRCs.

Another isoform that has gained interest in the past few years is mitochondrial ALDH1B1, the second most important enzyme in the alcohol metabolism, highly expressed in the crypts of intestinal tissues and a possible actor for tumour progression (Langan et al., 2012; Singh et al., 2015). Also in this case, immunohistochemistry experiments have shown a higher expression levels in cancer stem cells (Chen et al., 2011).

Taken together, these data suggest the relevance of this enzymatic superfamily as an intriguing and innovative target for cancer therapy, not only from the common perspective of the small inhibitor development but also as a marker for the early diagnosis.

NADPH SYNTHESIS AS A TARGET

As mentioned above, ROS production may determine DNA damage and apoptosis. *Cancer* cells have the ability to scavenge ROS formation by activating antioxidant mechanisms, but usually these require high NADPH levels (Ju et al., 2020). There are several mechanisms orchestrated by NADPH balance, including antioxidant reactions that lead to reduced glutathione, necessary for hydrogen peroxide reduction and fatty acids, amino acids and nucleotides synthesis able to promote tumour growth (Ju et al., 2020; Rather et al., 2021). Moreover, NADPH works as an essential electron donor and

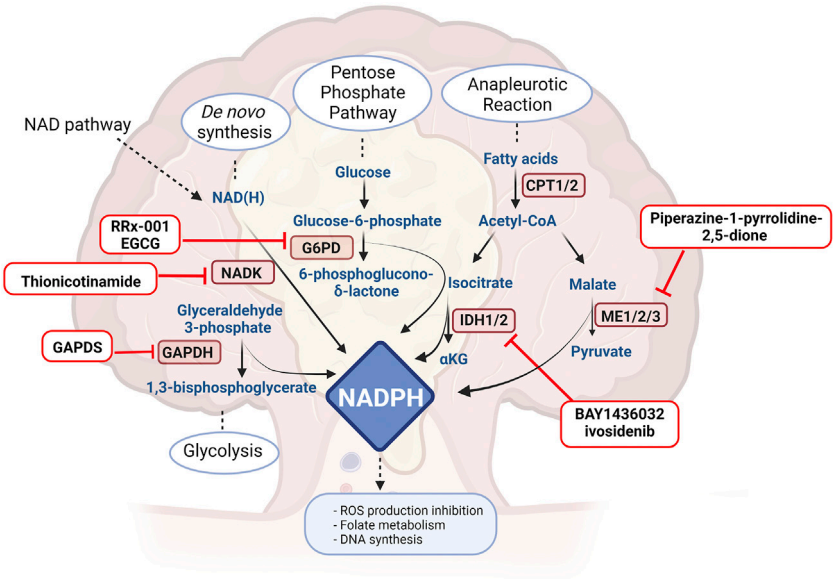

FIGURE 2 | Drugs or molecules that have been postulated as effective in CRC and that target NADPH routes. G6PH: glucose 6-phosphate dehydrogenase, NADK: NAD kinase, CPT: carnitine palmitoyl transferase, ME: malic enzyme, IDH: isocitrate dehydrogenase, GAPDH: glyceraldehyde 3-phosphate dehydrogenase. Created with BioRender.com.

cofactor maintaining reduction potential in anabolic reactions. In cancer cells, NADPH levels are controlled by several pathways, with the aim not only to contrast ROS production, but also in order to promote several metabolic reactions able to induce tumour proliferation, including NAD kinase (NADK), malic enzymes (ME) and NADP-dependent isocitrate dehydrogenases (IDH1 and IDH2) (Ju et al., 2020) but also glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Also in this scenario, a number of agents have been developed and proposed to contrast NADPH homeostasis in cancer (**Figure 2**).

Therapeutic Role of NADK Inhibitors in Colorectal Cancer

De novo synthesis of NADPH is orchestrated by NADK, localised in mitochondria and in the cytosol, which phosphorylates NAD to NADP (Katsyuba et al., 2020; Pramono et al., 2020). Cytosolic NADK is overexpressed in several tumours, including colorectal cancer (Ju et al., 2020; Pramono et al., 2020). More importantly, cancer cells bear NADK mutations endowed of increase enzymatic activity, inducing higher production of NADPH and reduction of ROS, sustaining cancer cell viability (Tedeschi et al., 2016). It is well known that the silencing of NADK impairs cancer growth in human colorectal cancer (Tedeschi et al., 2015; Yau et al., 2017), but only one inhibitor has so far been described. Thionicotinamide, a structural analogue of NAD, is able to counteract the growth of C85 (human colorectal cell line) both *in vitro* and *in vivo*, impairing the NADPH pool and augmenting ROS production (Tedeschi et al., 2015). The possibility to target only the mutated form of NADK is intriguing, but at present no specific inhibitor has been described.

Therapeutic Role of Malic Enzymes in Colorectal Cancer

Malic Enzymes contribute to both anabolic and catabolic reactions. They are tetrameric proteins with a double dimer structure, acting as oxidative decarboxylases. Malic enzymes are responsible for different actions which includes the decarboxylation of malate into pyruvate using NADP and producing NADPH, into Krebs cycle. Malic enzymes display different localisations: malic enzyme 1 (ME1) is in cytosol while malic enzyme 2 (ME2) and malic enzyme 3 (ME3) are located in mitochondria. ME1 participates in glycolysis and Krebs cycle but also in fatty acid and cholesterol synthesis using NADPH. ME1 produces NADPH stocked in the cytosol, which will be used by fatty acid synthase. (Fernandes et al., 2018).

Increased expression of MEs or increased activity has been associated to several types of cancer (Loeber et al., 1994; Ju et al., 2020) and all three isoforms have been associated with a worse prognosis in several type of cancer. In CRC, the literature has focused its attention on ME1, that, as other NADPH-dependent enzymes, also maintains the redox homeostasis in cells (Ju et al., 2020). Fernandez et al. have shown the importance of ME1 in the development of the tumoral mass in APCmin/+ mice. In detail, the number and the area of adenomas are increased in mice overexpressing ME1, which directly activates and sustains the Wnt/β-catenin pathway. Moreover, silencing ME1 in several CRC cell lines determines cell death and senescence (Murai et al., 2017). Furthermore, using piperazine-1-pyrrolidine-2,5-dione, a non-specific inhibitor (Zhang et al., 2006), they observed a decreased viability of HCT116 and HT29 treated or not in combination with a Wnt-canonical pathway inhibitor, confirming the potential of MEs as drug targets (Fernandes et al., 2018; Ju et al., 2020).

Therapeutic Role of G6PD in Colorectal Cancer

The pentose phosphate pathway (PPP) is among the principal cellular NADPH suppliers. The PPP is divided into an oxidative branch in which glucose-6-phosphate dehydrogenase (G6PD) converts glucose-6-phosphate into pentose phosphate metabolites, synthetizing NADPH, and in a non-oxidative branch that recycles pentose phosphates to glycolytic intermediates or produces ribose-5-phosphate (Boren et al., 2002). G6PD has been found over-expressed and over-activated in several cancers, determining poor prognosis and chemoresistance (Zhang et al., 2017; Yu et al., 2019; Ju et al., 2020), while its deficiency appears to decrease predisposition to cancer (Pes et al., 2019).

Several G6PD inhibitors have been developed. In CRC, RRx-001 has been broadly studied (Reid et al., 2014; Oronsky et al., 2016). A comparative clinical trial in phase 2 (NCT02096354) is active comparing RRx-001 in metastatic colorectal cancer in combination with irinotecan. Moreover, epicatechin gallate (EGCG), a putative G6PD inhibitor, has also been investigated in CRC, and has been shown to reduce the *de novo* synthesis of fatty acids and the pentose phosphate pathway in HT29 cells (Sánchez-Tena et al., 2013). EGCG has entered an early phase 1 clinical trial (NCT02891538) as chemo-preventive drug in patients who have undergone surgery and who do not require treatment after resection because of a benign prognosis.

Therapeutic Role of Isocitrate Dehydrogenase in Colorectal Cancer

Isocitrate dehydrogenase, an enzyme involved in TCA, synthetizes isocitrate from α-ketoglutarate, converting NADP to NADPH (Clark et al., 2016). It is present in three different isoforms: IDH1, situated in the cytosol and in peroxisomes, IDH2 and IDH3, which are located in mitochondria (Pramono et al., 2020). Both IDH1 and IDH2 are overexpressed in several types of tumours, decreasing ROS production and increasing concomitantly NADPH. Targeting IDH1 seems to be appealing in both wild-type and mutant forms. Indeed, IDH1 and IDH2 mutations occur in several tumours (Whitehall et al., 2014). In particular, the R132H IDH1 mutation lacks the classical enzymatic activity and is endowed of a particular synthetic pathway in which α-ketoglutarate is converted to 2-hydroxyglutarate. 2-hydroxyglutarate appears to induce cell proliferation via the mTOR-signalling pathway (Neitzel et al., 2020) and increases lipogenesis during hypoxia (Reitman et al., 2014). The mutated $IDH1^{R132H}$ substantially decreases NADPH production (Bleeker et al., 2010). Tougeron et al. have not manged to observe IDH1/2 in CRC patients (Tougeron et al., 2016), while Whitehall et al. have underlined that these mutation are present in correspondence to the CpG island methylator phenotype and in the presence of BRAF mutations (Whitehall et al., 2014). The idea of targeting both the wild-type form or the mutated form appears to be beneficial in CRC (Neitzel et al., 2020). IDH1 mutations appear to be important also in IBD-CRCs where sporadic cancer gene mutations occur less often. In this case, targeted IDH therapy appears to be helpful (Alpert et al., 2019). Moreover, 2-hydroxyglutarate has been found elevated also in presence of wild-type IDH, in HCT116 and RKO cell lines, responsible of epithelial-mesenchymal transition and development of metastases (Fallah-Rad, 2016). On the other hand, wild-type IDH1 silencing determines NADPH reduction and the consequent sensibilization to chemotherapy (Ju et al., 2020), while HCT116 cell line, endowed of the mutated form, are more sensitive to cisplatin treatment, and this is reverted by AGI-5198, an inhibitor of IDH1 mutated form (Khurshed et al., 2018). In contrast, the use of the IDH1 inhibitor BAY1436032 in mutant HCT116 (on R132H) leads to cell death (Pusch et al., 2017). Another important evidence is that the inhibitors of IDH mutated enzymes, as ML309 in combination with vitamin C, are responsible for the decrease of 2-hydroxyglutarate. This reduction determines an increase of DNA hydroxymethylation (Gerecke et al., 2020). What is also important is the post-translational modifications on IDH1. Wang et al. have highlighted that IDH1 is hyperacetylated at lysine 224, promoting tumoral expansion and metastases, sign of poor prognosis. SIRT2 is responsible of the deacetylation of lys224 in IDH1, increasing IDH1 enzymatic activity and reducing liver metastases (Wang et al., 2020). In conclusion, targeting mutated IDH in CRC seems to be appealing for IBD-CRCs, while in sporadic CRC wild-type IDH seems to be more plausible. Several mutant IDH1 inhibitors have entered clinical trials for hematologic malignancies (Rather et al., 2021), while only ivosidenib has been proposed as wild-type inhibitor in myeloid neoplasms (NCT03564821). The only clinical trial proposing IDH1 inhibitor for CRC is NCT04584008, aiming to use DNA sequencing in order to enrich patient populations with selected genotypes.

Therapeutic Role of GADPH in Colorectal Cancer

Glyceraldehyde 3-phosphate dehydrogenase is an enzyme that catalyses the sixth step of glycolysis and, surprisingly, is also a moonlighting protein, *i.e.*, a protein that possesses other cellular functions alongside its prototypical one. Given the relevance in glycolytic metabolism it has obviously been linked to the energy metabolism of cancer cells. A genome-wide microarray analysis has revealed that GAPDH is highly expressed in rapidly proliferating colon cancer cells. Moreover, the glycolytic inhibition with Na iodoacetate promotes *in vitro* the reduction of growth of different colorectal cancer cells (Bazzocco et al., 2015) and the regression of xenograft tumours inducing necrosis (Sánchez-Aragó and Cuezva, 2011).

Given that GAPDH nitrosylation has been demonstrated to trigger nuclear translocation and initiate p53-mediated cell death (Hara et al., 2005), a recent article has provided the mechanism by which microcystins-LR (MC-LR), a toxin produced by cyanobacteria, induces colon cancer cells apoptosis. MC-LR cytotoxicity is associated with nitric oxide (NO) increases that induce GAPDH nitrosylation with the consequent nuclear translocation and colorectal cancer cell p53-mediated

apoptosis (Li et al., 2020). Other evidence suggests that nuclear translocation of GAPDH increases under stressful stimuli. Grolla and collaborators have indeed demonstrated that GAPDH can be the shuttle for NAMPT from the cytosol in the nucleus under stress condition as oxidative stress, NO-induced stress and DNA damage to sustain the NMN/NAD pool (Grolla et al., 2016). Yet, the inhibitor of GAPDH shuttling to the nucleus, omigapil (Erb et al., 2009), has been evaluated in neurodegenerative disorders but has had little success in the cancer field.

Among several catalytic inhibitors, the triazine-based small molecule GAPDS has been reported to exert a specific anticancer activity in human carcinoma cells by preventing GAPDH tetramerization. GAPDS reduces cell viability in both normoxic and hypoxic conditions with an enhanced expression of apoptotic markers. Moreover, the effects on tetramerization result in a reduction in cytosolic GAPDH and tubulin expression which have a direct impact on the ability of cancer cells to invade and migrate (Jung et al., 2014). The involvement of GAPDH on cell motility has also been confirmed (Liu et al., 2017).

Studies on patient samples confirm the involvement of GAPDH in colorectal cancer. GAPDH mRNA levels were shown to have a 1.6-fold expression increase between normal and colorectal cancerous tissues and 2.3-fold expression comparing colorectal liver metastases and liver tissues (Rubie et al., 2005). A study on vitamin C on human colorectal cancer cells suggests that ROS are implicated in the mechanism by which vitamin C inhibits GAPDH. Vitamin C intracellular accumulation leads to the production of endogenous ROS, resulting in the reduction of GAPDH activity via post-translational modifications and NAD depletion (Yun et al., 2015).

Despite one study shows a reduction of GAPDH expression in presence of metastasis (Tarrado-Castellarnau, 2017) some contrasting evidence also suggests a relationship between motility of cancer cells and this protein. Indeed, an increased expression of GAPDH in human colorectal cancer cells was associated with epithelial to mesenchymal transition (EMT) accompanied by the upregulation of mesenchymal markers (Liu et al., 2017). EMT is an important process involved in tumour invasion and metastasis formation and requires several regulators. The zinc-finger protein Snail binds to E-cadherin, suppresses the expression of the adhesion molecule and promotes the phenotypic transition (Cano et al., 2000). The silencing of GAPDH was shown to revert this phenomenon downregulating Snail expression associated with a reduction of vimentin and an increased expression of E-cadherin. Chromatin immunoprecipitation reveals that GAPDH physically interacts with the transcriptional factor Sp1 that binds to Snail promoting EMT (Liu et al., 2017). A recent study demonstrates the toxicity of a lipopeptidyl benzophene, asperphenin B, on human colorectal cancer cells associated with the downregulation of GAPDH expression. Moreover, GAPDH upregulation was found in metastasized cells and the anti-metastatic activity of the compound *in vitro* was correlated with the modulation of EMT signalling pathways (Byun et al., 2021).

GAPDH expression levels, considering all the stages of tumour progression, were not correlated with survival in patients with colorectal cancer (Tarrado-Castellarau, 2017) despite the evidence are not univocal.

Barbazán et al., have reported a multimarker expression panel of circulating tumour cells (CTC) to forecast prognosis in patients with metastatic colorectal cancer both at baseline and during treatment to monitor therapy response. GAPDH expression levels were included in the six-gene panel markers and Kaplan-Meier plots reveal an inverse correlation between high CTC group, progression-free survival and overall survival, elucidating an increased risk of tumour progression (Barbazán et al., 2014).

Therefore, GAPDH expression in colorectal cancer can contribute to highlight mechanisms involved in tumour progression and univocal data on patients will prompt the development of GAPDH targeted therapy in the clinical set.

CONCLUSION

The aim of this review was to understand how NAD and NADPH routes may be important in CRC progression, highlighting the enzymes that might represent pharmacological targets. NAD biosynthesis, the use of NAD as an ADPR donor and NADPH homeostasis are crucial for cellular metabolism and signalling. Not surprisingly, therefore, these pathways are upregulated in cancer, and this is true also for CRC. Yet, few of the players of the NADome have been investigated thoroughly in this setting, despite an important unmet therapeutic need. NAMPT and PARP at present appear to be the most promising targets. iNAMPT inhibitors have possibly been abandoned too early, for on target side effects, without giving enough emphasis on the identification of the patient population that might benefit more from these treatments, thereby generating a favourable benefit/risk. This may possibly be given by understanding how to determine the NAMPT-dependence of tumours. Moreover, the evidence on eNAMPT deriving from different settings support a potential beneficial effect of eNAMPT neutralization also in CRC.

PARP inhibitors have entered several clinical trials, primarily in combination. For some of the concluded trials, the combination of a PARP inhibitor with other molecules (e.g., irinotecan) appears to be therapeutically superior compared to classical alkylating agents, while the outcome of the other trials is awaited.

Last, the other NAD and NADPH routes targets reviewed by us appear to be either CRC markers or promoters of CRC progression. Yet, specific molecules to unravel their potential in CRC treatment have yet to be disclosed. SIRT, G6PD and ALDH inhibitors have never entered clinical trial, but their ability to contrast CRC development, as highlighted by our study, prompts the investigation of the molecules that will arise in this setting. Importantly, the innovative IDH inhibitor ivosidenib, that has a wild-type inhibitor active in myeloid neoplasms, may also be an alternative to be tested in IBD-CRCs, where IDH is usually not mutated.

AUTHOR CONTRIBUTIONS

GC and EG collected information and wrote the manuscript. FB and MM collected and analysed the information. GC, CT, and AG supervised the conception and writing of the manuscript.

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REVIEW
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The Crosstalk Between Signaling Pathways and Cancer Metabolism in Colorectal Cancer

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Colorectal cancer (CRC) is one of the most frequently diagnosed cancers worldwide. Metabolic reprogramming represents an important cancer hallmark in CRC. Reprogramming core metabolic pathways in cancer cells, such as glycolysis, glutaminolysis, oxidative phosphorylation, and lipid metabolism, is essential to increase energy production and biosynthesis of precursors required to support tumor initiation and progression. Accumulating evidence demonstrates that activation of oncogenes and loss of tumor suppressor genes regulate metabolic reprogramming through the downstream signaling pathways. Protein kinases, such as AKT and c-MYC, are the integral components that facilitate the crosstalk between signaling pathways and metabolic pathways in CRC. This review provides an insight into the crosstalk between signaling pathways and metabolic reprogramming in CRC. Targeting CRC metabolism could open a new avenue for developing CRC therapy by discovering metabolic inhibitors and repurposing protein kinase inhibitors/monoclonal antibodies.

Keywords: colorectal cancer, metabolism, metabolic reprogramming, protein kinase, signaling pathways

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1 INTRODUCTION

Despite the advancement in early detection and medical treatments in recent decades, cancer incidence and mortality continue to increase gradually, as estimated in Global Cancer Statistic (GLOBOCAN) database under The International Agency for Research of Cancer (IARC) and the World Health Organization (WHO). Based on the latest GLOBOCAN 2021 statistics, colorectal cancer (CRC) remains the third most common cancer and the second leading cause of cancer mortality globally (Sung et al., 2021). CRC accounts for 10 percent of 19.3 million new cancer cases and 9.4 percent of about 10 million cancer deaths worldwide (Sung et al., 2021). The high incidence rate of CRC is correlated with genetics, environmental factors, and lifestyle development (Dunlop et al., 2013; Johnson et al., 2013; Rawla et al., 2019). Previous studies estimated that about 65–70 percent of CRC is thoroughly sporadic with no known family history (Samadder et al., 2015). About 25 percent of CRC cases are known to have a family history related to inflammatory bowel syndrome (Yurgelun et al., 2017). Only 5 percent of CRC cases are hereditary CRC syndrome, namely FAP (familial adenomatous polyposis) and Lynch syndrome (Samadder et al., 2015). CRC arises from the glandular epithelial cells of the large intestine when specific cells undergo a series of genetic and epigenetic mutations to become hyper-proliferative and cancerous over time (Ewing et al., 2014; Dekker et al., 2019). These cancerous cells form a benign adenoma at an early stage, in which some progress into carcinoma and metastasize to other organs at the later stage of malignancy (Dekker et al., 2019).

Like any other cancer, CRC cells undergo rewiring of cellular metabolism via the dysregulation of oncogenes and tumor suppressors during carcinogenesis (Brown et al., 2018). Metabolic

reprogramming is an essential cancer hallmark in many cancers, including CRC (Cantor and Sabatini, 2012). By altering core metabolism pathways, such as glutaminolysis, glycolysis, lipid synthesis, and mitochondrial oxidation, metabolic reprogramming allows CRC cells to sustain rapid cell proliferation with high demand for energy and biosynthetic precursors to drive tumor development and cancer metastasis (Brown et al., 2018). Regulation of metabolic reprogramming involves a complex network of different signaling pathways in cancer cells, which various protein kinases can regulate (Garcia-Ortega et al., 2017; Lu and Hunter, 2018). Protein kinases (PTKs) are enzymes that selectively modify the biological activity of biomolecules (lipids, proteins, and carbohydrates) via phosphorylation with ATP as the primary source of phosphate (Cheng et al., 2011). Dysregulation of protein kinases has been reported in multiple cancers, such as breast (Templeton et al., 2014), stomach (Shiroki et al., 2017), pancreas (James et al., 2020), and CRC (Asante et al., 2019), to affect metabolic reprogramming of cancer cells through the manipulation of signaling pathways. This review will discuss the importance of protein kinases as crucial regulators of signaling pathways in the metabolic reprogramming of CRC cells. A deeper understanding of the protein kinases and signaling pathways involved in the metabolic reprogramming in CRC will provide insight into discovering new therapeutic targets.

2 The Warburg Effect in Colorectal Cancer

In 1924, Otto Warburg discovered that, even in the presence of oxygen, cancer cells preferentially alter the ATP production towards aerobic glycolysis followed by lactic acid fermentation rather than oxidative phosphorylation (OXPHOS) (Devic, 2016). Compared to normal cells, most pyruvate in cancer cells is distributed into lactate fermentation in the cytosol instead of entering the mitochondrial tricarboxylic acid (TCA) cycle and OXPHOS (Vaupel et al., 2019). The unique metabolic phenotype of cancer cells is known as the Warburg effect (Vaupel et al., 2019). In comparison, OXPHOS produces more ATP per molecule of glucose, but aerobic glycolysis produces ATP more rapidly, favoring the actively proliferating cancer cells (Vaupel et al., 2019). Aerobic glycolysis also generates more glycolytic intermediates and pyruvate to synthesize macromolecules and support ATP production (Blanco and Blanco, 2017b). Subsequently, pyruvate is converted into lactate to enter the Krebs cycle and rapidly produce ATP (Blanco and Blanco, 2017b). Other glycolytic intermediates such as glucose-6-phosphate, fructose-6-phosphate, and glyceraldehyde-3-phosphate are converted into macromolecules (nucleotides, phospholipids, and fatty acids) to sustain cell growth and maintenance (Blanco and Blanco, 2017c).

Certain protein kinases are involved in glucose uptake and aerobic glycolysis as rate-limiting enzymes, such as hexokinase (HK), phosphofructokinase (PFK), and pyruvate kinase (PK) (Blanco and Blanco, 2017b). Accumulated evidence shows that dysregulation of these glycolytic regulatory enzymes is vital to modulate the Warburg effect in CRC via different signaling pathways (Brown et al., 2018). Hexokinase (HK) serves as the rate-limiting enzyme to catalyze the first irreversible step of glucose uptake by converting glucose to glucose-6-phosphate (G6P) upon phosphorylation with ATP (Blanco and Blanco, 2017b). Interestingly, G6P then suppresses the activity of HKII through a feedback inhibition mechanism (Blanco and Blanco, 2017b). G6P is the precursor for major pathways of glucose metabolism, including glycolysis, pentose phosphate pathway, oxidative phosphorylation (OXPHOS), and hexosamine biosynthesis pathways (Blanco and Blanco, 2017b). Therefore, HK is regarded as an essential regulator of glucose metabolism. The mammalian HK family has five isoforms: HKI, HKII, HKIII, HKIV, and HKDC1 (HexoKinase Domain-Containing protein 1) (Wilson, 2003; Irwin and Tan, 2008). Among all, HKII is frequently upregulated in multiple cancers, including breast (Sato-Tadano et al., 2013), glioblastoma (Wolf et al., 2011), prostate (Lee et al., 2019), and CRC (Ho and Coomber, 2016). Previous studies report that HKII plays dual cancer-promoting effects by inducing glycolysis and inhibit apoptosis. HKII binds to voltage-dependent anion channel (VDAC) in the outer membrane of mitochondria to suppress apoptosis by closing the permeability transition pores and preventing cytochrome c release (Yuan et al., 2008; Tait and Green, 2013). Notably, growth factor-induced oncogenic signaling pathways, such as EGFR and PI3K/Akt/mTOR signaling, regulate the mitochondrial translocation of HKII (Makinoshima et al., 2014; Liu et al., 2015; Ciscato et al., 2020). Mitochondrial binding of HKII also reduces the feedback inhibition from G6P to increase its stability and promote glycolytic flux, producing more ATP (Wolf et al., 2011). HKII overexpression is correlated with poor prognosis in CRC and has been proposed as a therapeutic target due to its importance in glycolysis and cellular survival (Hamabe et al., 2014; Ho and Coomber, 2016; Krasnov et al., 2016).

2.1 EGFR/Akt in the Warburg Effect

Xanthohumol is a natural compound extracted from hops (*Humulus lupulus*), and it exerts antitumor effects in CRC cells (Jiang et al., 2018). Liu et al. discovered that xanthohumol inhibited glycolysis in CRC cells via EGFR/Akt/HKII axis (Liu et al., 2019). Xanthohumol directly suppressed the phosphorylation of EGFR and EGFR downstream kinases Akt to downregulate the activity of HKII, resulting in a lower rate of glycolysis and activation of mitochondrial-induced apoptosis in CRC cells (Liu et al., 2019). Overexpression of HKII reversed the inhibitory effect of xanthohumol and increased the glycolytic rate in CRC cells (Liu et al., 2019). Epidermal growth factor receptor (EGFR), also known as ErbB1/Her1, is a member of the membrane-bound receptor tyrosine kinase family, which can be activated by binding of its specific ligands, namely epidermal growth factor (EGF) and transforming growth factor α (TGFα) (Wang, 2017). EGFR is overexpressed in many cancers to function as an oncogene (Nishimura et al., 2015; Cairns et al., 2018). Additionally, EGFR can be mutated in cancers to become constitutively active without ligand binding (Cairns et al., 2018). When activated, EGFR phosphorylates the

downstream effectors and signaling pathways to initiate a wide range of oncogenic activities such as cell survival and proliferation (Templeton et al., 2014; Cairns et al., 2018; Fernandes et al., 2018).

Meanwhile, Akt is a serine/threonine kinase frequently upregulated in tumor cells, activated by a wide range of growth factors and receptor stimuli, such as EGFR, PI3K, and PTEN (Nitulescu et al., 2018). Akt mainly functions as a central anabolic and survival effector to regulate cellular metabolism and survival through the phosphorylation of target molecules at different cellular compartments (Song et al., 2019; Hoxhaj and Manning, 2020). The EGFR/Akt signaling cascade has been identified as a crucial oncogenic regulator in various cancers, including non-small cell lung cancer(NSCLC) (Chandrasekaran et al., 2017) and prostate cancer (Gan et al., 2010). Additionally, the direct interaction between HKII and the EGFR/Akt pathway could be essential to modulate cancer progression. Another study by Zou et al. reported that the ubiquitin-like protein FAT10 promoted bladder cancer progression by upregulating HKII via the EGFR/Akt pathway (Zou et al., 2021). FAT10 stabilizes EGFR expression by reducing its degradation and ubiquitination (Zou et al., 2021). Similarly, the EGFR/Akt/HKII regulatory circuit modulates the CRC metabolism. Thus, this axis could become a therapeutically significant target for CRC.

2.2 C-MYC Signaling in the Warburg Effect

The c-MYC is a member of the MYC family that includes MYCN and MYC (Rahl and Young, 2014). MYC encodes the Myc transcription factor, which dimerizes with Max to bind DNA and regulate about 15 percent of total gene expression in human cells (Dang et al., 2006). The c-MYC expression is tightly regulated by growth factor-dependent signals in normal cells (Dong et al., 2020). However, c-MYC is a proto-oncogene overexpressed in many cancers, including CRC (Gabay et al., 2014). The c-MYC dysregulation in cancers is mainly induced through gene amplification, chromosome translocation, super-enhancer activation, and loss of upstream repressors to stabilize the c-MYC protein expression (Dong et al., 2020; Wolf and Eilers, 2020). c-MYC functions as a critical regulator of malignant transformation by promoting multiple processes, including cell proliferation, cell growth, and genomic instability (Gabay et al., 2014). More importantly, c-MYC contributes to metabolic reprogramming via several effective mechanisms, such as glycolysis, glutaminolysis, mitochondrial biogenesis, and lipid synthesis (Wahlstrom and Henriksson, 2015). Accumulated evidence suggests that c-MYC-driven metabolic reprogramming in cancer cells is mainly characterized by increased uptake of precursors, enhanced rate of glycolysis and glutaminolysis, as well as increased synthesis of fatty acids and nucleotides (Cunningham et al., 2014; Broecker-Preuss et al., 2017; Cai et al., 2019; Casciano et al., 2020). Tang et al. reported that lncRNA GLCC1 regulates CRC progression and glucose metabolism by stabilizing c-MYC to promote the transcription of glycolytic genes in CRC cells (Tang et al., 2019). GLCC1 was significantly upregulated in glucose-depleted CRC cells and bound explicitly with heat shock protein 90 (HSP90) chaperon upon glucose depletion (Tang et al., 2019). GLCC1 modulates the interaction between HSP90 and c-MYC complex to stabilize c-MYC from degradation (Tang et al., 2019). Indirectly, GLCC1 coordinates the localization and binding pattern of c-MYC genome-wide, promoting the expression of LDHA to upregulate glycolytic metabolism for CRC proliferation (Tang et al., 2019). Lactate dehydrogenase A (LDHA) is a cytosolic enzyme encoded by the LDHA gene on the short p arm of chromosome 11 (11p15.4), which plays a critical role in anaerobic and aerobic glycolysis (the Warburg effect) (Kolappan et al., 2015). LDHA facilitates the interconversion of pyruvate to lactate coupled with the recycling of NAD^+ from NADH during the last step of glycolysis (Blanco and Blanco, 2017b). LDHA is frequently upregulated in CRC and identified as a direct target gene of the c-MYC oncogenic transcription to reprogram cancer metabolism (Wang et al., 2015; Satoh et al., 2017).

Similarly, N-MYC downstream-regulated gene 2 (NDRG2), a well-known tumor suppressor, inhibits glycolysis in CRC cells by modulating c-MYC expression (Xu et al., 2015). NDRG2 is widely expressed in normal tissue and downregulated in various tumors, including CRC (Kloten et al., 2016; Zhang et al., 2017; Morishita et al., 2021). Overexpression of NDRG2 suppresses cellular growth, proliferation, and invasion in cancers (Li et al., 2013; Hong et al., 2016; Yang CL. et al., 2018; Kang et al., 2020). In CRC metabolism, NDRG2 inhibits c-MYC expression by suppressing β-catenin, the critical effector of the Wnt signaling pathway (Xu et al., 2015). Under normal conditions, β-catenin can be degraded via upstream regulators, such as adenomatous polyposis coli (APC), GSK-3β, and casein kinase 1α (CK1α) (Duchartre et al., 2016). When Wnt signaling is often dysregulated in cancers, possibly due to APC mutation, cytoplasmic β-catenin translocates to the nucleus to activate c-MYC transcription (Duchartre et al., 2016). NDRG2 directly inhibits β-catenin to repress the c-MYC expression at the transcriptional level (Xu et al., 2015). c-MYC is the oncogenic transcriptional factor responsible for modulating the Warburg effect in CRC cells through the regulation of glucose transporters (GLUTs) and other glycolytic enzymes, namely HKII, PKM2, and LDHA (Wahlstrom and Henriksson, 2015). NDRG2 suppresses glycolysis in CRC cells by downregulating the expression of GLUT1, HKII, PKM2, and LDHA through c-MYC inhibition (Xu et al., 2015).

Another carcinogenic modulator, CD36, is a membrane glycoprotein that is associated with elevated fatty acids absorption to modulate cancer progression and metastasis in various cancers such as ovarian (Ladanyi et al., 2018), cervical (Yang P. et al., 2018), liver (Nath et al., 2015), and stomach (Pan et al., 2019). However, CD36 has been reported to target β-catenin/c-MYC-mediated glycolysis to repress CRC tumorigenesis (Fang et al., 2019). Early evidence showed that Wnt signaling could play a role in modulating the Warburg effect in CRC cells via the nuclear accumulation of β-catenin (Pate et al., 2014). CD36 was significantly downregulated in CRC tissue, and its expression level was negatively associated with cancer progression (Fang et al., 2019). Ectopic expression of CD36 directly promoted proteasome-dependent ubiquitination of Glypican 4 (GPC4), which is a member of the heparan sulfate

proteoglycans (HSPGs) family (Zhao et al., 2016; Fang et al., 2019). Previous studies suggest that lipid raft localization of GPC4 is required to activate the Wnt/β-catenin pathway (Fico et al., 2012; Sakane et al., 2012; Cao et al., 2018). GPC4 degradation reduced the nuclear translocation of β-catenin, leading to the downregulation of c-MYC and downstream glycolytic genes (GLUT1, LDHA, HK2, and PKM2) in CRC cells (Fang et al., 2019). Collectively, CD36 promotes the ubiquitination of GPC4 to de-activate β-catenin/c-MYC signaling cascades and downstream glycolytic target genes, repressing the glycolysis and tumorigenesis in CRC cells (Fang et al., 2019).

HK II is another downstream effector of c-MYC, which could be targeted to modulate glycolysis in CRC cells. Dioscin is a kind of steroid saponins isolated from *Dioscoreae rhizome* and *Paridis rhizome*, which has potent activities against various cancers, including CRC (Si et al., 2016; Li et al., 2018; Mao et al., 2019). A recent study by Wu et al. showed that dioscin inhibits glycolysis and induces apoptosis in CRC cells by targeting c-MYC and HKII (Wu et al., 2020). Upon treatment with dioscin, the interaction between FBW7 and c-MYC in CRC cells was enhanced, leading to the ubiquitination of c-MYC (Wu et al., 2020). Consequently, dioscin promoted c-MYC degradation, and the downstream HKII was suppressed, resulting in glycolysis inhibition (Wu et al., 2020). Dioscin also impaired the interaction between HKII and VDAC-1 on the outer mitochondrial membrane, in which Bax could bind to the VDAC-1 more efficiently (Wu et al., 2020). This phenomenon increased membrane permeability and the release of cytochrome C, which ultimately led to cellular apoptosis (Wu et al., 2020). Thus, HKII is essential for dioscin-mediated glycolysis inhibition and apoptosis in CRC cells.

2.3 STAT3 Signaling in the Warburg Effect

Apart from EGFR/Akt and c-MYC signaling pathways, other pathways target HKII to modulate the glucose metabolism in CRC cells. The polo-like kinases (PLKs) belong to a family of highly conserved serine/threonine kinases, in which all five members (PLK1–5) possess a conserved N-terminal kinase domain and one or more polo-box domains (PBDs) at the C-terminus (Barr et al., 2004). Initially, PLKs were found to be critical regulators of cell cycle checkpoint, mitosis, and DNA damage response (Xie et al., 2005). Recent studies suggest that PLKs can modulate tumor growth, apoptosis, and metabolism (Ou et al., 2016; Gutteridge et al., 2017). Ou et al. revealed that PLK3 inhibits glucose metabolism in CRC by targeting HSP90/STAT3/HKII signaling (Ou et al., 2019). PLK3 was significantly downregulated in CRC tissues and correlated with poor prognosis (Ou et al., 2016; Ou et al., 2019). PLK3 was directly bound to HSP90 to trigger proteasome-mediated degradation of HSP90, which reduced the phosphorylation of signal transducer and activator of transcription 3 (STAT3) at S727 residue (Ou et al., 2019). STAT3 directly binds to the promoter region of the HKII gene and upregulates the protein expression HKII. The STAT3 dephosphorylation downregulated the transcriptional activation of HKII, resulting in a lower glycolytic rate in CRC cells (Ou et al., 2019). The STAT family consists of seven proteins (STAT 1, 2, 3, 4, 5a, 5b, and 6), which have dual roles as signaling molecules and transcription factors (Villarino et al., 2015). STATs transduce signals from activated cytokine and growth factor receptors into the nucleus to initiate the transcription of target genes (Villarino et al., 2015). Specific cytokines and growth factors activate each STAT protein to regulate downstream target genes. For instance, cytokine receptors activate receptor-associated tyrosine kinases, such as the Janus kinase (JAK) family kinases, to phosphorylate the tyrosine 705 residue (Y705) in STAT3 protein (Parganas et al., 1998). Growth factor receptors associated with their intrinsic receptor tyrosine kinase (RTK) phosphorylate STAT3 at the same tyrosine 705 residue (Y705).

STAT3 is activated in response to a defined set of cytokines, namely IL-6, interferon-gamma (IFNγ), and erythropoietin, in addition to growth factors including epidermal growth factor (EGF) and fibroblast growth factor (FGF) (O'Sullivan et al., 2016). Tyrosine phosphorylation activates STAT3 to undergo homo/hetero-dimerization with another STAT protein(O'Sullivan et al., 2016). STAT3 dimers bind to specific DNA response elements in the promoter regions of target genes to regulate the gene transcription (O'Sullivan et al., 2016). Activated STAT3 dimer induces the expression of multiple genes associated with anti-apoptosis, proliferation, angiogenic, and metastatic properties in cancer cells (Timofeeva et al., 2013; Liu et al., 2015; Zhu et al., 2019). Moreover, serine/threonine kinases phosphorylate STAT3 on S727 in the cytoplasm or nucleus (Zhang et al., 1995). The serine phosphorylation of STAT3 is required for maximal transcriptional activity but not DNA binding (Wen et al., 1995). Multiple kinases are responsible for S727 phosphorylation, more prominently the mitogen-activated protein kinase (MAPK) family members (Shaheen and Broxmeyer, 2018). The MAPK family consists of the extracellular signal-regulated kinase (ERK) as well as JNK/stress-activated protein kinase and p38/HOG1 (p38 MAPK) (Shaheen and Broxmeyer, 2018). STAT3 signaling is well studied as a significant intrinsic pathway for cancer inflammation due to its frequent activation in cancer cells that promotes inflammatory genes and suppresses anti-tumor immunity (Yu et al., 2009). Nevertheless, recent studies suggest that STAT3 signaling contributes to metabolic reprogramming in cancers. Lin et al. reported that palmitic acid inhibits glycolysis in hepatocellular carcinoma by silencing the STAT3 pathway (Lin et al., 2017). STAT3 signaling is also essential in PKM2-mediated glucose metabolism in breast cancer cells via the let-7a-5p/Stat3/hnRNP-A1 regulatory feedback loop (Yao A. et al., 2019).

It was observed that STAT3 signaling plays a crucial role in the PLK3-inhibited glucose metabolism of CRC cells by targeting HKII expression (Ou et al., 2019). The interplay between STAT3 signaling and c-MYC has altered glycolysis in CRC cells in response to inflammation (Qu et al., 2017). IL-6 is a pro-inflammatory cytokine frequently detected in the tumor microenvironment to induce inflammation and activate STAT3 signaling in cancer cells (Yu et al., 2009; Kim et al., 2016; Arora et al., 2018). *In vitro* studies revealed that the addition of IL-6 into CRC cell lines activated the phosphorylation of STAT3 and increased the expression of c-MYC and glycolytic enzymes, such as GLUT1 and LDH, resulting in higher glucose

uptake and lactate production (Qu et al., 2017). These results suggest that inflammation could induce the reprogramming of glucose metabolism in CRC cells via the STAT3/c-MYC pathway. JAK2 is an upstream regulator of STAT3, which phosphorylates STAT3 at the Y705 residue. A recent study by Li et al. identified that JAK2/STAT3 signaling was targeted by atractylenolide-I to induce apoptosis and suppress glycolysis in CRC cells (Li Y. et al., 2020). Atractylenolide-I (AT-I) is a natural derivative of *Rhizoma Atractylodis* macrocephalus that has been shown to demonstrate anti-tumor activities in a wide range of cancers. Mechanistically, AT-I could directly bind to JAK2 to inhibit the JAK2 activity and suppress the downstream phosphorylation of STAT3 (Li Y. et al., 2020). Subsequently, the inactivation of STAT3 contributed to the downregulation of HKII, resulting in a lower rate of glycolysis and lactate production in CRC cells (Li Y. et al., 2020). Hence, AT-I inhibits glycolysis via JAK2/STAT3 signaling to suppress HKII expression in CRC cells (Li Y. et al., 2020).

2.4 The PKM2 Paradox in the Warburg Effect

Pyruvate kinase (PK) is a rate-limiting enzyme in the final, irreversible step of the glycolysis, which is responsible for catalyzing the transphosphorylation between phosphoenolpyruvate and ADP to generate pyruvate and ATP (Blanco and Blanco, 2017b). There are four mammalian PK isoforms: PKL, PKR, PKM1, and PKM2, each with distinct kinetic properties and tissue distribution (Clower et al., 2010). PKL is mainly expressed in the liver and kidneys, while PKR is exclusively expressed in red blood cells (Israelsen et al., 2013). PKM1 is primarily expressed in differentiated tissues with high energetic demands, such as myocardium, skeletal muscle, and brain tissue (Chiavarina et al., 2011). PKM2 is distributed in tissues, such as the brain and liver, and is highly expressed in rapidly proliferating tissues, including cancers (Shiroki et al., 2017). The PK isoforms are encoded by two genes (PKLR and PKM), respectively, through the alternative splicing of pyruvate kinase mRNA (PKL and PKR; PKM1 and PKM2) (Chen et al., 2010). The human PKM gene with a length of 12 exons is alternatively spliced to generate transcripts based on the mutually exclusive selection between 9th and 10th exons: exon 9 is specific to PKM1, exon 10 is specific to PKM2 (Israelsen and Vander Heiden, 2015). Multiple splicing factors regulate the PKM1/PKM2 ratio in cancerous tissue, in which PKM2 is more favorable in most cancer types to modulate the Warburg effect (Israelsen et al., 2013). For instance, polypyrimidine tract binding protein (PTB), heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1), and A2 (HNRNPA2B1) repress exon 9 and promote exon 10 to upregulate the PKM2 expression (Clower et al., 2010). Another splicing factor, namely the serine/arginine-rich splicing factor 3 (SRSF3), directly binds the PKM transcript to promote the inclusion of exon 10 for enhancing the PKM2 expression (Chen et al., 2010). Previous evidence also suggests that c-MYC activates the expression of HNRNPs to maintain a high PKM2/PKM1 ratio in cancer cells (David et al., 2010).

PKM2 exists in two oligomeric states: an active tetramer and a less active dimer/monomer, due to tetramerization upon binding with fructose-1,6-bisphosphate (FBP) (Sciacovelli et al., 2014). The moderately active, dimer form of PKM2 mainly participates in the Warburg effect in cancers by producing glycolytic intermediates to support tumor growth and proliferation. The PKM2 dimers also induce transcriptional co-activation and function as protein kinase targeting histones and transcription factors (Lu, 2012). PKM2 dimers translocate into the cell nucleus upon signaling from the extracellular signal-regulated kinase (ERK1/2) to initiate the expression of other glycolytic genes (GLUT1, LDHA, PDK) (Yang et al., 2012). The nuclear translocation of PKM2 is crucial for the autoregulation of PKM2 expression by upregulating its upstream activators, such as HIF1α and β-catenin (Luo et al., 2011; Yang et al., 2012; Prigione et al., 2014). More importantly, nuclear PKM2 interacts with HIF1α and β-catenin to regulate the expression of glycolytic enzymes and initiate the Warburg effect in cancer cells (Luo et al., 2011; Yang et al., 2011). In contrast, the tetramer form of PKM2 is fully active to maximize the efficiency of glycolysis and generate pyruvate for the utilization of oxidative phosphorylation but is excluded from nuclear translocation (Prakasam and Bamezai, 2018).

Recently, Sam68, an RNA-binding protein (RBP), has been identified to regulate glycolysis in CRC cells by controlling the alternative splicing of the PKM gene (Zhao J. et al., 2020). Sam68 is well recognized as a critical oncogenic factor associated with cancer progression and poor prognosis in CRC (Liao et al., 2013; Fu et al., 2016; Wang et al., 2018). Ectopic expression of Sam68 upregulates the glycolysis and proliferation in CRC cells, associated with decreased PKM1/PKM2 ratio (Zhao J. et al., 2020). Sam68 binds to the EI9 region of the PKM gene to promote the inclusion of exon 9 and enhance the formation of PKM2 mRNA (Zhao J. et al., 2020). Overexpression of Sam68 significantly reduces the PKM1/PKM2 ratio in CRC cells, resulting in the metabolic shift from oxidative phosphorylation to glycolysis (Zhao J. et al., 2020). Sam68 increases the PKM2 mRNA transport into the cytoplasm to enhance the PKM2 protein synthesis, promoting the pyruvate kinase activity and lactate production in CRC cells (Zhao J. et al., 2020). In addition, polypyrimidine tract binding protein 1 (PTB1) is a positive regulator of the Warburg effect in cancer cells by regulating the PKM2 expression (Clower et al., 2010). PTB1 is an exonic splicing silencer of the PKM mRNA that promotes the PKM2 expression by including exon 10 in alternative splicing (He et al., 2014). c-MYC can regulate PTB1 to promote cancer progression and the Warburg effect (David et al., 2010). Taniguchi et al. have presented several works on the regulatory role of miR-124 on PTB1 and PKM1/PKM2 ratio in modulating the Warburg effect in CRC (Taniguchi et al., 2015a; Taniguchi et al., 2015b). MiR-124 was downregulated in CRC clinical samples, while *in vitro* analysis revealed that miR-124 induced apoptosis and suppressed the Warburg effect in CRC cells (Taniguchi et al., 2015b). PTB1 promotes the production of PKM2, which inhibits miR-124 in a feedback loop (Taniguchi et al., 2015b). MiR-124 induces the switching of PKM isoforms from PKM2 to PKM1 by downregulating PTB1

and its upstream regulators, namely c-MYC, E2F1, and STAT3 (Taniguchi et al., 2015b).

In addition, miR-124 can also regulate the Warburg effect in CRC cells via the DDX6/c-MYC/PTB1 positive-feedback mechanism (Taniguchi et al., 2015a). DDX6 is an oncogenic RNA helicase frequently overexpressed in multiple cancers, including CRC (Akao et al., 2006; Cordin et al., 2006; Akao, 2009; Tajirika et al., 2018). DDX6 expression is associated with the IRES-dependent c-MYC translation to regulate cancer cell growth and differentiation (Hashimoto et al., 2001; Akao et al., 2006; Taniguchi et al., 2018). DDX6 is significantly overexpressed in CRC tissues, in which the authors suggest that low expression of miR-124 contributes to the high DDX6 expression (Taniguchi et al., 2015a). MiR-124 directly targets DDX6 in CRC cells, in which miR-124 knockdown releases DDX6 to promote the c-MYC expression, and c-MYC upregulates PTB1 directly, contributing to the Warburg effect (Taniguchi et al., 2015a). PTB1 knockdown upregulates the miR-124 expression, subsequently suppressing the expression of DDX6 and c-MYC and inhibiting the Warburg effect in CRC cells (Taniguchi et al., 2015a). All this evidence supports that PTB1 is an essential modulator of CRC metabolism that can be regulated by miRNAs and oncogenic upstream activators, like c-MYC, to modulate the expression of PKM1 and PKM2 in the Warburg effect. Evidence shows that modulation of the Warburg effect involving the metabolic role of PKM2 could lead to the acquisition of other cancer phenotypes, such as chemoresistance. CD44 is a non-kinase transmembrane glycoprotein frequently overexpressed in cancers and cancer stem cells (Chen et al., 2018). CD44 functions as an adhesion molecule in many aspects of tumorigenesis, including migration, proliferation, and metastasis, as well as to function as a surface marker for cancer stem cells (Du et al., 2008; Liu et al., 2011; Louderbough and Schroeder, 2011; Cho et al., 2012; Wang et al., 2012; Senbanjo and Chellaiah, 2017). Overexpression of CD44 directly phosphorylates PKM2 at threonine (T105) residue to suppress its glycolytic activity and promote the Warburg effect in CRC cells (Tamada et al., 2012). CD44 knockdown also induces the metabolic shift from aerobic glycolysis to mitochondrial respiration with increased reactive oxygen species (ROS) production, which significantly re-sensitizes CRC cells towards cisplatin (Tamada et al., 2012). Hence, PKM2 is crucial for the CD44-mediated Warburg effect with enhanced cisplatin resistance in CRC cells.

Multiple signaling pathways regulate the metabolic role of PKM2 in CRC cells. The Wnt/β-catenin signaling pathway is aberrantly activated in CRC due to the adenomatous polyposis coli (APC) gene mutation (loss of function) in nearly 90 percent of CRC patients (Coppede et al., 2014). APC protein is required to form the β-catenin destruction complex for β-catenin degradation and inhibition of the Wnt/β-catenin signaling pathway (MacDonald et al., 2009). The loss of function in the APC gene is commonly associated with the early transformation of normal colon epithelium into adenoma (Powell et al., 1992). Mutant APC protein cannot form the destruction complex, leading to stabilization and accumulation of β-catenin expression in the cytosol (Requena and Garcia-Buitrago,

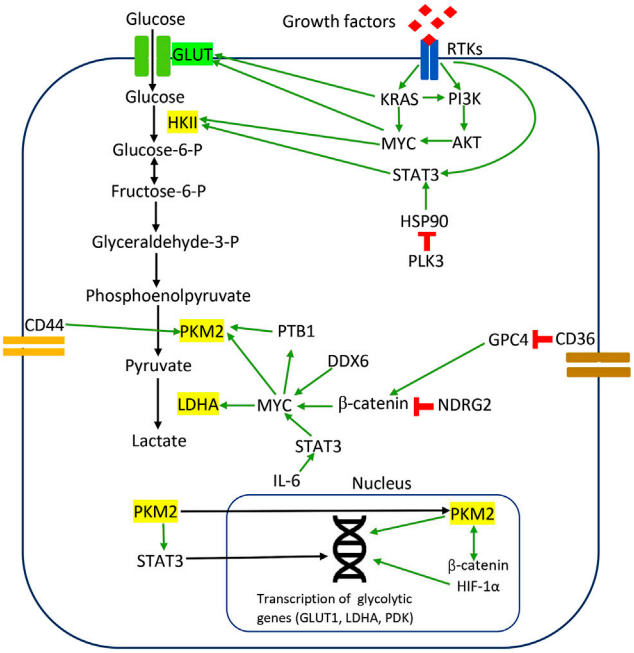

FIGURE 1 | Regulation of the Warburg effect via oncogenic signaling in CRC. The red sign indicates inhibition, while the green arrow indicates promotion.

2020). The subsequent nuclear translocation of β-catenin activates inappropriate target genes, namely c-MYC and cyclin D, associated with tumor proliferation, migration, invasion, and metastasis (Nguyen and Duong, 2018). More recently, the APC mutation in CRC cells has been reported to induce the Warburg effect via the Wnt/β-catenin signaling pathway to target PKM2 (Cha et al., 2021). The loss of function in APC stabilizes the β-catenin and increases the nuclear translocation of β-catenin (Cha et al., 2021). Subsequently, this enhances the β-catenin/Tcf4 binding on PKM2 promoter regions and promotes PKM2 transcription (Cha et al., 2021). The PKM2 upregulation further increases the expression of other glycolytic enzymes, namely LDHA, GLUT1, PFK1-M, and PFKBP1, to enhance the Warburg effect in CRC cells (Cha et al., 2021). PKM2 also accelerates the activation of Wnt/β-catenin signaling via a positive feedback loop in CRC cells (Cha et al., 2021). Collectively, the Wnt/β-catenin signaling activated by APC mutation requires PKM2 as the critical mediator to modulate the Warburg effect in CRC.

In CRC, PKM2 induces direct phosphorylation of transcription activators and signaling molecules to mediate metabolic reprogramming and proliferation. Previous studies observed that PKM2 phosphorylated STAT3 at Tyr705 to activate STAT3-controlled genes, namely MEK5, for cell proliferation (Gao et al., 2012). Yang et al. reported that PKM2 facilitates CRC cell migration via the STAT3 signaling cascade (Yang P. et al., 2014). PKM2 overexpression in DLD1 cells upregulated STAT3 gene transcription and activated downstream snail-2 and β1-

integrin-FAK signaling to induce tumor migration (Yang P. et al., 2014). PKM2 overexpression facilitated STAT3 nuclear translocation to upregulate the expression and function of PKM2 in migration and adhesion-associated signaling, suggesting the feedback mechanism between PKM2 and STAT3 (Yang P. et al., 2014). These results demonstrate that the protein kinase activities of dimeric PKM2 but not its metabolic functions are essential for CRC cell migration and cell adhesion. **Figure 1** illustrates the regulation of the Warburg effect via oncogenic signaling in CRC.

3 PKM2 in Glutaminolysis

Glutamine is the most abundant non-essential amino acid in most cancer cells, which is highly required for cellular proliferation (Sciacovelli et al., 2014). Glutamine is catalyzed by glutaminase (GLS) to generate glutamate and converted into other intermediates, namely α-ketoglutarate, pyruvate, lactate, and malate, which can be utilized in different metabolic pathways (Blanco and Blanco, 2017a). Hence, glutamine metabolism or glutaminolysis is equally essential in the metabolic reprogramming of CRC cells by supporting ATP production and biosynthesis of proteins, lipids, and nucleic acids. The less glycolytic, dimer form of PKM2 is crucial to coordinate the metabolism change between glycolysis and glutaminolysis in CRC cells (Li L. et al., 2020). PKM2 dimer facilitates glutaminolysis upon EGFR stimulation by promoting IRES-dependent c-MYC translation (Li L. et al., 2020). Previously, c-MYC regulates PKM2 expression by controlling PKM2 pre-mRNA splicing, and subsequently, PKM2 regulates c-MYC gene transcription in a direct feedback mechanism (David et al., 2010; Luo et al., 2011; Yang et al., 2012). The recent work by Li et al. demonstrates that PKM2 interacts with the c-MYC/IRES complex to regulate c-MYC translation via another IRES-dependent mechanism (Li L. et al., 2020). There are two pathways in c-MYC protein synthesis at the translational level: canonical cap-dependent translation and internal ribosome entry site (IRES)-dependent translation (Godet et al., 2019). The IRES-dependent c-MYC translation is an oncogenic pathway that allows the c-MYC protein synthesis under critical conditions, such as apoptosis and hypoxia, when the canonical cap-dependent translation is largely attenuated (Stoneley et al., 2000; Spriggs et al., 2009). As PKM2 dimer promotes IRES-dependent c-MYC translation in CRC cells, the activated c-MYC protein subsequently upregulates GLS-I to increase glutamine consumption (Li L. et al., 2020). Glutaminase I (GLS-I) is the mitochondrial enzyme that catalyzes the first, irreversible step of glutaminolysis by converting glutamine to glutamate (Jin et al., 2016). GLS-I plays a vital role in glutaminolysis and is frequently dysregulated in cancers (Pan et al., 2015; Daemen et al., 2018; Xiang et al., 2019; Ren et al., 2020). The less active form of PKM2 dimer modulates glutaminolysis in CRC cells by targeting IRES-dependent c-MYC translation to coordinate cell response to hypoxia environment. The oligomeric switching between tetramer and dimer forms of PKM2 corresponds to the metabolic change between glycolysis and glutaminolysis in CRC cells upon the stimulation of growth factors and environmental stress.

Another study proposed that PKM2 depletion could promote the β-catenin signaling and its downstream c-MYC to enhance glutamine metabolism in CRC cells (Wu et al., 2014). CRC cells enhance glutamine metabolism to compensate for glycolysis impairment upon PKM depletion (Wu et al., 2014). Previously, it was shown that c-MYC regulates mitochondrial glutaminase expression and glutamine metabolism in cancer cells (Gao et al., 2009). Interestingly, PKM2-knockdown in CRC cells significantly upregulates c-MYC protein expression and β-catenin expression at mRNA and protein levels, suggesting that PKM2 regulates glutaminolysis mainly via β-catenin/c-MYC signaling (Wu et al., 2014). Nuclear translocation of dimeric PKM2 negatively regulates the β-catenin mRNA at the transcriptional level through the action of miR-200a that directly targets the 3' UTR of β -catenin mRNA (Wu et al., 2014). Collectively, PKM2 functions as the protein kinase that negatively affects the β-catenin/c-MYC signaling pathway through miR-200a to modulate glutaminolysis in CRC cells.

4 KRAS Mutations and PI3K Signaling in Glutamine Metabolism

KRAS mutation is common in many cancers, including CRC (Ewing et al., 2014). KRAS mutation causes the protein to become constitutively active AND promote the signaling through growth and survival pathways, namely the phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) cascades (Kerk et al., 2021). Mutant KRAS has been used as a clinical biomarker to predict resistance to anti-epidermal growth factor receptor (EGFR) therapy in treating metastatic CRC (Kudryavtseva et al., 2016). Previous studies suggest that KRAS mutation could be essential to modulate glucose uptake, glutaminolysis, and mitochondrial ROS production in CRC metabolism. Yun et al. reported that CRC cell lines with mutations in either KRAS or BRAF upregulate GLUT-1 with increased glucose uptake and lactate production (Yun et al., 2009). Another proteomic study showed that mutant KRAS and BRAF in CRC cell lines could impact multiple aspects of metabolism, including glycolysis, phosphoserine biosynthesis, glutamine metabolism, and the non-oxidative pentose phosphate pathway, by modulating the expression of corresponding proteins/enzymes (Hutton et al., 2016). KRAS and BRAF mutations contribute to metabolic reprogramming in CRC to support rapid proliferation and sustain biosynthetic needs. Additionally, it was demonstrated that glutamine-based mitochondrial metabolism is essential for KRAS mutant CRC cells to support cell growth and proliferation (Weinberg et al., 2010). KRAS mutant CRC cells acquire a high glycolytic flux to provide glycolytic intermediates for the pentose phosphate pathway to produce nucleotides and phospholipids for rapid proliferation (Weinberg et al., 2010). Glutamine metabolism provides the alternative carbon source for the TCA cycle in mitochondrial respiration to generate ATP for cellular proliferation and tumorigenesis (Weinberg et al., 2010).

Knockdown of the glutamine transporter in KRAS mutant CRC cells reduces the proliferation rate and inhibits other oncogenic activities, including migration, invasion, and

metastasis (Wong et al., 2016). A recent study by Wong et al. also reveals that glutamine metabolism in mutant KRAS CRC cells contributes to the activation of Wnt signaling, cancer stemness, and drug resistance by reducing DNA methylation through SLC25A22, a mitochondrial glutamine transporter (Wong et al., 2020). SLC25A22 expression is associated with poor prognosis in advanced-stage CRC with mutant KRAS (Wong et al., 2020). SLC25A22 is upregulated in mutant KRAS CRC cells to induce succinate accumulation in the cell nucleus and subsequently modulate epigenetic regulators' expression to enhance WNT/β-catenin signaling and LGR5 expression (Wong et al., 2020). Indirectly, SLC25A22 promotes cancer stemness and drug resistance in CRC cells.

Phosphoinositide 3-kinases (PI3Ks) comprise a large family of lipid kinases that function as intracellular signal transducers (Noorolyai et al., 2019). PI3K signaling can be activated through the upstream RAS isoforms, tyrosine kinase receptors, and mutations in PI3K signaling components (43). The activation of the PI3K pathway is mainly associated with cell cycle progression, proliferation, differentiation, and metabolism in cancer cells (Fernandes et al., 2018). PIK3CA gene encodes the p110α catalytic subunit of PI3K, and its mutation represents one of the most common genetic aberrations in human cancers (Zardavas et al., 2014). PI3KCA mutations reprogram glutamine metabolism in CRC cells by upregulating glutamate pyruvate transaminase 2 (GPT2) (Hao et al., 2016). Consequently, CRC cells with PI3KCA mutations are more sensitive to glutamine deprivation and substantially increase glutamine metabolism to replenish the TCA cycle and generate ATP (Hao et al., 2016). Mutant p110α upregulates GPT2 gene expression through novel PDK1-RSK2-ATF4 signaling that is AKT-independent (Hao et al., 2016). Mutant p110α activates RSK2 kinase through pyruvate dehydrogenase kinase 1 (PDK1) (Hao et al., 2016). PDK1 is a downstream effector of PI3K via the signal transduction from phosphatidylinositol-3,4,5-triphosphate (PIP3), whereas RSK2 is a serine/threonine kinase that phosphorylates ATF4 at the serine 245 residue (S245) (Frodin et al., 2002; Hao et al., 2014). Activated RSK2 phosphorylates ATF4 to recruit the deubiquitinase USP8, preventing ATF4 from ubiquitin-mediated degradation (Hao et al., 2016). ATF4 (activating transcription factor 4) has been reported to modulate glutamine metabolism in different cancer studies (Qing et al., 2012; Csibi et al., 2013). Eventually, ATF4 activates GPT2 gene transcription directly to promote PI3K-mediated glutamine metabolism (Hao et al., 2016).

5 SIGNALING PATHWAYS IN OXIDATIVE PHOSPHORYLATION

KRAS mutation is also associated with the regulation of mitochondrial respiration in CRC cells. Mitochondrial respiration comprises two major components: the tricarboxylic acid cycle (TCA cycle) and oxidative phosphorylation (OXPHOS) (Blanco and Blanco, 2017c). TCA cycle occurs in the inner mitochondrial space to generate NADH and FADH2 via acetyl-CoA metabolism (Blanco and Blanco, 2017b). The NADH and $FADH_2$ generated by the TCA cycle will be oxidized in OXPHOS, which takes place on the inner membrane of mitochondria, to generate ATP and reactive oxygen species (ROS) as a byproduct (Blanco and Blanco, 2017b). In CRC, KRAS mutations have decreased ROS production and enhanced mitochondrial OXPHOS efficiency by activating mitochondrial phospholipid synthesis via the upregulation of transcriptional factors HIF-1α and HIF-2α (Chun et al., 2010). The hypoxia-inducible factors-1α and -2α (HIF-1α and HIF-2α) are frequently upregulated in cancers and associated with tumor angiogenesis, cell growth and survival, and metastasis (Li T. et al., 2020). It was shown that HIF-1α and HIF-2α proteins translocate to the nucleus and dimerize with HIF-1β to transactivate target genes (Koh et al., 2011). Previously, HIF-1α and HIF-2α regulated the exchange of COX4 (cytochrome c oxidase 4) subunits under hypoxic conditions to increase mitochondrial respiration efficiency and reduce ROS production (Fukuda et al., 2007).

6 SIGNALING PATHWAYS IN LIPID METABOLISM

Dysregulation of lipid metabolism is equally essential for CRC tumor growth and survival. Fatty acids are essential components of all biological membranes and the critical carbon source for energy metabolism in the TCA cycle and OXPHOS (Blanco and Blanco, 2017d). Fatty acids can act as signaling molecules associated with multiple aspects of tumorigenesis, such as migration, invasion, and drug resistance (Nath et al., 2015; Blanco and Blanco, 2017c; Ladanyi et al., 2018; Pan et al., 2019). In CRC, multiple studies have shown that the *de novo* synthesis of fatty acids is enhanced with the upregulation of critical enzymes, including ATP citrate lyase (ACLY), acetyl CoA carboxylase (ACC), and fatty acid synthase (FASN) (Zaytseva et al., 2012; Hofmanova et al., 2021). Upregulation of FASN has been reported in mutant KRAS cell lines, which supports cellular respiration through mitochondrial fatty acid β-oxidation (FAO) (Zaytseva et al., 2015). FAO is the primary pathway for degrading long-chain fatty acids to produce acetyl-CoA, which participates in the TCA cycle to replenish ATP, NADPH, NADH, and $FADH_2$ (Houten et al., 2016). A recent study by Wang et al. demonstrates that epigallocatechin-3-gallate (EGCG), a water-soluble polyphenol and the main active ingredient of green tea, inhibits fatty acid *de novo* synthesis and lipid droplets formation in CRC cells via AMPK activation (Wang et al., 2021). AMP-activated protein kinase (AMPK) is a serine/threonine protein kinase, which serves as a metabolic sensor and core regulator of energy metabolism in cancers (Steinberg and Carling, 2019). Previously, AMPK activation could decrease *de novo* lipogenesis (Smith and Steinberg, 2017). In addition, studies have shown that AMPK activation inhibits the Warburg effect and *de novo* lipogenesis while increasing OXPHOS to suppress cancer cell growth (Chen et al., 2012; Smith and Steinberg, 2017; Holczer et al., 2018). EGCG significantly increases the phosphorylation of AMPKα (Thr172) to inhibit

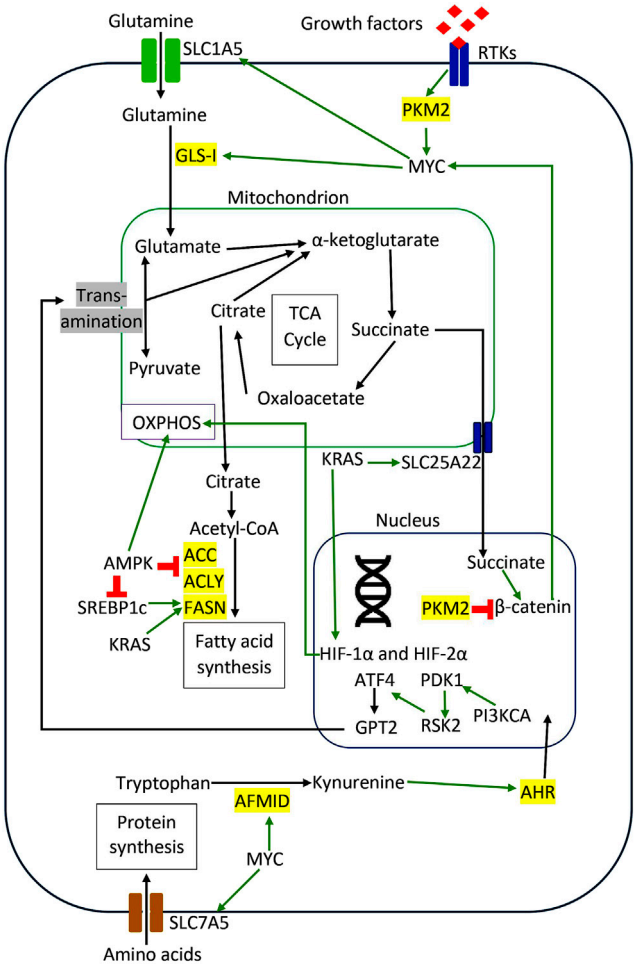

FIGURE 2 | Regulation of amino acids and lipid metabolism via oncogenic signaling in CRC cells. The red sign indicates inhibition, while the green arrow indicates promotion.

the expression of FASN, ACLY, ACC, and the transcriptional factor sterol regulating element-binding protein 1c (SREBP1c), resulting in *de novo* synthesis inhibition and reduced cell viability in CRC cells (Wang et al., 2021).

7 SIGNALING PATHWAYS IN TRYPTOPHAN METABOLISM

There are many other types of amino acid metabolism in cancer cells. Tryptophan (Trp) is an essential amino acid for protein synthesis, and it is the least abundant amino acid in most proteins (Adams et al., 2012). Venkateswaran et al. have presented a comprehensive work on the tryptophan metabolism in CRC cells. They discovered that CRC cells increase the tryptophan uptake and metabolism compared to normal colonic cells and tissues (Venkateswaran et al., 2019). Similarly, CRC cells were more sensitive to tryptophan depletion than their normal counterpart (Venkateswaran et al., 2019). Tryptophan can be metabolized in three different pathways (Blanco and Blanco, 2017a). Firstly it is incorporated into newly synthesized proteins (Blanco and Blanco, 2017a). Secondly, it enters the serotonin pathway to produce serotonin and melatonin (Blanco and Blanco, 2017a). Lastly, tryptophan enters the kynurenine pathway to generate kynurenine (Kyn), a biologically active metabolite (Van der Leek et al., 2017). The proto-oncogene c-MYC activates the kynurenine pathway in CRC cells by promoting the transcription of the tryptophan importers SLC1A5 and SLC7A5 and the tryptophan metabolizing enzyme arylformamidase AFMID (Venkateswaran et al., 2019). In the kynurenine pathway, tryptophan is metabolized by one of the three enzymes: indoleamine 2,3-dioxygenase1 (IDO1), indoleamine 2,3-dioxygenase2 (IDO2), and tryptophan 2,3-dioxygenase 2 (TDO2) to generate N-formyl kynurenine, which is converted into kynurenine by AFMID (Van der Leek et al., 2017). Previously, IDO1 expression correlates with impaired immune response, hepatic metastases, and poor clinical outcomes in CRC (Brandacher et al., 2006). IDO1 and kynurenine pathway metabolites activate PI3K-AKT signaling to promote nuclear translocation of β-catenin, enhancing cancer cell proliferation and inhibit apoptosis in CRC cells. (Thaker et al., 2013; Bishnupuri et al., 2019). SLC7A5, SLC1A5, and AFMID were upregulated in CRC clinical samples with the increased level of kynurenine (Venkateswaran et al., 2019). Kynurenine functions as an oncometabolite to activate the nuclear translocation of the transcription factor AHR (aryl hydrocarbon receptor), which regulates target genes associated with proliferation in CRC cells (Venkateswaran and Conacci-Sorrell, 2020). **Figure 2** illustrates the regulation of amino acids and lipid metabolism via oncogenic signaling in CRC cells. **Table 1** summarizes the main contributions of previous studies to the field of CRC metabolism, as discussed earlier.

8 METABOLIC REPROGRAMMING IN COLORECTAL CANCER CANCER STEM CELLS

Cancer stem cells (CSCs) represent a subpopulation of tumor cells with self-renewal and multi-lineage differentiation to regulate tumor growth and heterogeneity (Walcher et al., 2020). CSCs have been identified as the critical regulator of cancer progression in various solid tumors, including CRC, driving cancer initiation and cancer relapse (Ayob and Ramasamy, 2018). CSCs undergo metabolic adaptation to support their stemness properties and promote tumor development (Batlle and Clevers, 2017). In CRC, the metabolic reprogramming in CSCs is closely associated with the expression of signaling molecules and stem cell markers. CRC CSCs are known to express specific stem cell markers, such as CD133, CD44, leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5), and epithelial cell adhesion molecule (EpCAM) (Munro et al., 2018). CD133 is a well-characterized marker in CRC CSCs, while CD133(+) cells can initiate tumor formation in the animal model (Wang et al., 2012; Li, 2013). Based on the

TABLE 1 | Summarizes the main contributions of previous studies to the field of CRC metabolism.

Molecule/Signaling pathway involved	Target enzyme/Effector	Function	Reference
The Warburg Effect			
EGFR/Akt	HKII	Inhibits glycolysis upon suppression by xanthohumol	Liu et al. (2019)
GLCC1/c-MYC	LDHA	Promotes CRC progression and glucose metabolism	Tang et al. (2019)
NDRG2/β-catenin/c-MYC	GLUT1, HKII, PKM2, and LDHA	Inhibits glycolysis through c-MYC suppression	Xu et al. (2015)
CD36/GPC4/β-catenin/ c-MYC	Multiple glycolytic genes	Inhibits glycolysis and tumorigenesis in CRC cells	Fang et al. (2019)
CD44	PKM2	Promotes Warburg effect	Tamada et al. (2012)
Nuclear PKM2/HIF1α/ β-catenin	Multiple glycolytic genes	Promotes glycolysis and autoregulation of PKM2	Luo et al. (2011), Yang et al. (2012), Prigione et al. (2014)
IL-6/STAT3/c-MYC	GLUT1, LDH	Increases glucose uptake and lactate production	Qu et al. (2017)
JAK2/STAT3	HKII	Inhibits glycolysis and lactate production	Li et al. (2020c)
c-MYC	HKII	Inhibits glycolysis and induce apoptosis in CRC cells upon dioscin stimulation	Wu et al. (2020)
PLK3/HSP90/STAT3	HKII	Inhibits glucose metabolism	Ou et al. (2019)
Sam68	PKM1/PKM2	Upregulates glycolysis and proliferation in CRC cells	Zhao et al. (2020a)
miR-124/c-MYC/E2F1/ STAT3/PTB1	PKM1/PKM2	Promotes PKM2 production and glycolysis	Taniguchi et al. (2015b)
miR-124/DDX6/c-MYC/ PTB1	PKM2	Promotes Warburg effect	Taniguchi et al. (2015a)
Wnt/β-catenin/PKM2	LDHA, GLUT1, PFK1-M, and PFKBP1	Promotes Warburg effect	Cha et al. (2021)
Glutaminolysis			
EGFR/PKM2/c-MYC/IRES	GLS-I	Promotes glutaminolysis	Li et al. (2020a)
KRAS mutation	Multiple glutaminolysis enzymes	Regulates glutaminolysis, mitochondrial respiration, and ROS production	Weinberg et al. (2010), Hutton et al. (2016)
KRAS mutation	Mitochondrial glutamine transporter SLC25A22	Regulates glutamine metabolism, Wnt signaling, cancer stemness, and drug resistance	Wong et al. (2016)
Nuclear PKM2/miR-200/ β-catenin/C-MYC	Mitochondrial glutaminase	Promotes glutaminolysis	Wu et al. (2014).
PI3K/PDK1/RSK2/ATF4	GPT2	Increases glutamine metabolism to replenish the TCA cycle and generate ATP	Hao et al. (2016)
Oxidative Phosphorylation (OXPHOS)			
KRAS mutations	HIF-1α and HIF-2α	Increases mitochondrial respiration and reduces ROS production	Chun et al. (2010)
Lipid Metabolism			
AMPK	FASN, ACLY, ACC, SREBP1c	Inhibits fatty acid synthesis and reduces cell viability	Wang et al. (2021).
KRAS mutations	FASN	Upregulates fatty acid β-oxidation	Zaytseva et al. (2015)
Tryptophan Metabolism			
C-MYC	SLC7A5, SLC1A5, AFMID	Upregulates tryptophan metabolism via the kynurenine pathway	Venkateswaran et al. (2019)

analysis of microarray data from the GEO database, which included sorted CD133(+) and CD133(−) subfractions of CRC CSCs, Chen et al. revealed that genes involved in glycolysis, TCA cycle, and one-carbon metabolism were upregulated in CD133(+) cells. However, at the same time, genes involved in fatty acid biosynthesis were downregulated (Chen et al., 2014). Their findings suggest that CD133(+) CRC CSCs upregulates glycolysis for energy production while suppressing fatty acid biosynthesis.

CD133(+) CRC CSCs also exhibit an altered lipid metabolism as compared to non-CSC counterparts. By using transmission electron microscopy imaging and flow cytometry analysis, Tirinato et al. discovered that CD133(+) CSCs contain more lipid droplets in the cytoplasm as compared to CD133(−) cells (Tirinato et al., 2015). The authors also observed a similar result by stratifying cells based on Wnt/β-catenin activity instead of CD133 expression level, suggesting a possible correlation between CD133 and Wnt signaling to modulate the lipid metabolism in CRC stem cells (Tirinato et al., 2015). Functionally, CRC stem cells with high lipid droplet content demonstrated a higher *in vitro* sphere-forming ability and a more significant tumor formation upon subcutaneous injection in the animal model. The

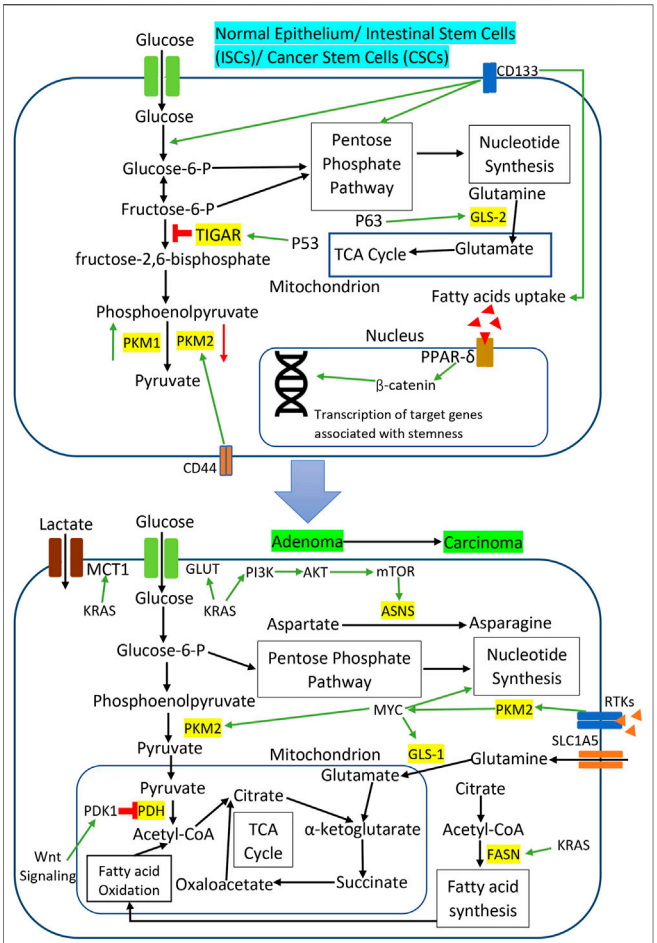

FIGURE 3 | Metabolic reprogramming through oncogenic signaling during CRC initiation and progression as well as metabolic reprogramming in CRC stem cells. The red sign indicates inhibition while the green arrow indicates promotion.

reprogramming of lipid metabolism could be essential in promoting the tumorigenesis of CRC stem cells.

CD44 is another stem cell marker in CRC CSCs, which also acts as the cell surface glycoprotein to participate in cellular interactions and cellular migration (Morath et al., 2016; Senbanjo and Chellaiah, 2017). CD44 transcription is activated by β-catenin/Wnt signaling, while its overexpression is frequently associated with the early transformation of colorectal adenoma to carcinoma (Orian-Rousseau, 2015). Knockdown of CD44 in CRC cell line HCT116 decreased the glucose uptake and consumption by downregulating PKM2 activity and lactate production (Tamada et al., 2012). Simultaneously, CD44 ablation in HCT116 cells also reduced glucose utilization by the pentose phosphate pathway (Tamada et al., 2012). CD44 could play an essential role in the metabolic reprogramming of CRC stem cells by inducing the switching of glucose metabolism from OXPHOS to glycolysis and pentose phosphate pathways. Moreover, CD44(+) CRC CSCs express a higher glutamine level as compared with the CD44(−) counterparts, but the actual interaction between CD44 and glutamine metabolism is yet to be investigated (Huang et al., 2016). The increased glutamine in CD44(+) CSCs can support CRC energy production to promote cell survival, growth, and proliferation.

The transcription factor p63 is a member of the p53 transcription factor family, which has been expressed in CRC CSCs, while p63 mutation is associated with poor clinical prognosis among CRC patients (Pignon et al., 2013; Bahnassy et al., 2014). Through its N-terminal *trans*-activator domain, p63 activates mitochondrial glutaminase 2 (GLS2) in CRC cells to catalyze the conversion of glutamine into glutamate and α-ketoglutarate for subsequent ATP production via the TCA cycle (Giacobbe et al., 2013). Glutamate is an essential precursor of glutathione, which is the primary ROS intracellular scavenger. The authors observed that GLS2 was significantly upregulated in CRC tissue samples with decreased cellular levels of ROS, suggesting that p63/GLS2 axis could be essential to protect CRC cells against oxidative stresses (Giacobbe et al., 2013). Furthermore, p63 overexpression in CRC is also associated with the upregulation of glycolysis in CSC cultures, implicating that p63 could be a crucial glycolytic regulator in CRC stem cells (D'Aguanno et al., 2014).

9 CROSSTALK BETWEEN ONCOGENIC SIGNALING AND METABOLIC PATHWAYS IN COLORECTAL CANCER PROGRESSION

CRC pathogenesis is a multi-step transformation that involves multiple genetic alterations to promote cancer initiation and progression. The activation of oncogenic signaling is essential to drive the stepwise progression of CRC via metabolic reprogramming to promote cancer cell survival, proliferation, epithelial-mesenchymal transition (EMT), angiogenesis, invasion, and migration. Metabolic reprogramming plays a vital role in different stages of CRC progression, from the early transformation of normal epithelial cells into the entire adenoma-carcinoma sequence. $Lgr5^{+}$ intestinal stem cells (ISCs) are the progenitors of secretory cells and enterocytes that produce mature intestinal epithelial cells, so ISCs are also the cancer stem cells for CRC, as shown by lineage tracing studies (Barker et al., 2009; Merlos-Suarez et al., 2011; Kemper et al., 2012). TIGAR (TP53-inducible glycolysis and apoptosis regulator) is transcriptionally activated by p53 tumor suppressor protein to decrease the intracellular levels of fructose-2,6-bisphosphate (Fru-2,6-BP) and downregulate the glycolytic pathway (Bensaad et al., 2006). Subsequently, TIGAR diverts the glucose metabolism towards the pentose phosphate pathway (PPP) to produce NADPH for antioxidant function and ribose-5-phosphate for nucleotide synthesis (Li and Jogl, 2009). Eric et al. reported that TIGAR was required for injury-induced regeneration in ISCs, by promoting PPP to generate NADPH and ribose-5-phosphate rapidly (Cheung et al., 2013). TIGAR was upregulated in human CRC cell lines and tumors regardless of p53 status, while the authors also observed that TIGAR promoted adenoma formation in a mouse model of intestinal cancer (Cheung et al., 2013). All this evidence suggests that TIGAR is essential to support cell proliferation by reprogramming glucose metabolism during CRC initiation.

TABLE 2 | List of small molecule inhibitors and monoclonal antibodies for CRC therapy in clinical trials or approved by the FDA.

Drug	Target	Clinical stage	Reference
Small Molecule Inhibitors			
Afatinib	EGFR	Phase II	De Pauw et al. (2016)
Alpelisib	PI3K	Phase II	Tabernero et al. (2016)
Cobimetinib	MAPK	Phase II	Klute et al. (2020)
Dabrafenib	BRAF	Phase II	Corcoran et al. (2015)
Dasatinib	Src	Phase II	Scott et al. (2017)
Encorafenib	BRAF	FDA approval in 2020	Kopetz et al. (2019)
Enzastaurin	AKT	Phase II	Wolff et al. (2012)
Erlotinib	EGFR	Phase III	Hagman et al. (2016)
Everolimus	mTOR	Phase II	Ng et al. (2013)
Gedatolisib	PI3K/mTOR	Phase II	Wainberg et al. (2017)
Gefitinib	EGFR	Phase II	Troiani et al. (2016)
Mk-2206	AKT	Phase II	Do et al. (2015)
Napabucasin	STAT3	Phase III	Jonker et al. (2018)
Niclosamide	STAT3	Phase I	Burock et al. (2018)
Pelitinib	EGFR	Phase II	To et al. (2020)
Regorafenib	VEGFR, KIT, PDGFR, RET, TIE2,EPH2A	FDA approval in 2012	Papadopoulos and Lennartsson, (2018)
Sonolisib	PI3K	Phase II	Bowles et al. (2016)
Trametinib	BRAF	Phase II	Corcoran et al. (2015)
Temsirolimus	mTOR	Phase II	Spindler et al. (2013)
Vemurafenib	BRAF	Phase II	Klute et al. (2020)
Monoclonal Antibodies			
Bevacizumab	VEGF-A	FDA approval in 2004	Rosen et al. (2017)
Cetuximab	EGFR	FDA approval in 2004	Bokemeyer et al. (2012)
Panitumumab	EGFR	FDA approval in 2006	Hocking and Price, (2014)
Pertuzumab	HER2	Phase II	Meric-Bernstam et al. (2019)
Ramucirumab	VEGFR2	FDA approval in 2015	Verdaguer et al. (2016)
Rilotumumab	HGF	Phase II	Van Cutsem et al. (2014)
Trastuzumab	HER2	Phase II	Meric-Bernstam et al. (2019)

Additionally, reprogramming of lipid metabolism is also associated with CRC initiation and maintenance of ISCs. Prolonged high-fat diet (HFD) in mice activates PPAR-δ (peroxisome proliferator-activated receptor-delta) in ISCs to upregulate a subset of β-catenin target genes associated with cancer cell stemness (Beyaz et al., 2016). Consequently, ISCs proliferate rapidly and increase adenoma formation in a mouse model of APC loss-induced intestinal tumorigenesis (Beyaz et al., 2016). Kim et al. reported that loss of PKM2 in Lgr5(+) ISCs promoted inflammation-associated CRC in the mouse model, while a similar result was also reported in the APC-driven colon cancer mouse model (Lau et al., 2017; Kim et al., 2019). These studies suggest that PKM2 expression is not required for CRC initiation, although PKM2 overexpression was reported in advanced stages of CRC (Lau et al., 2017). The downregulation of PKM2 also significantly led to the activation of PKM1, which may suggest a compensatory expression of PKM1 by deletion of PKM2 to support the metabolic requirement and proliferation of CRC cells (Kim et al., 2019). Meanwhile, Satoh et al. describe that c-MYC is responsible for inducing a global metabolic reprogramming in CRC, which starts from the adenoma stage and remains similar through all cancer stages (Satoh et al., 2017). c-MYC induces the upregulation of glycolysis and nucleotide metabolism to support the rapid proliferation of CRC cells during tumorigenesis (Satoh et al., 2017). Additionally, the protein kinase function of PKM2 activates c-MYC upon the signaling from RTKs (Li L. et al., 2020). The activated c-MYC also increases the glutamine uptake and metabolism in CRC cells by promoting the glutamine transporter SLC1A5 and glutaminase GLS-I (Li L. et al., 2020).

Accumulating evidence suggests that Wnt signaling and KRAS mutations induce metabolic reprogramming in the adenoma and carcinoma stage of CRC to support the energetic and biosynthetic requirements of rapidly proliferating CRC cells. Activation of Wnt signaling in CRC cell lines promotes glucose metabolism and lactate production by upregulating the transcription of pyruvate dehydrogenase kinase 1 (PDK1) and lactate transporter, MCT-1 (Pate et al., 2014; Sprowl-Tanio et al., 2016). Wnt signaling-induced PDK1 prevents pyruvate flux to mitochondrial respiration and indirectly promotes cancer proliferation and angiogenesis in xenograft CRC tumors in the mouse model (Pate et al., 2014). KRAS mutations in CRC also promote tumor growth and cancer progression by rewiring glucose, amino acids, and lipid metabolism. KRAS mutations increase the glucose uptake in CRC cells via the upregulation of GLUT1 to generate more glycolytic intermediates for the utilization in different metabolic pathways in tumor expansion (Kawada et al., 2012; Iwamoto et al., 2014). Toda et al. observed that KRAS mutation altered the amino acid metabolism in CRC cells by reducing aspartate level and increasing asparagine level to

TABLE 3 | List of potential metabolic inhibitors that target CRC cells.

Compound	Target molecule/pathway	Function	Reference
2-DG	Glycolytic enzymes	Inhibition of glycolysis with reduction of tumor invasion and metastasis	Park et al. (2017), Xiang et al. (2019)
DON	Multiple glutamine-utilizing enzymes	Inhibition of glutaminolysis and induction of cellular ROS	Lemberg et al. (2018); Schcolnik-Cabrera et al. (2020)
AZD3965	MCT1	Inhibition of lactate transport, glycolysis, and lipid biosynthesis	Beloueche-Babari et al. (2020)
CB839	GLS1	Inhibition of glutaminolysis	Zhao et al. (2020b), Cohen et al. (2020)
Cerulenin plus oxaliplatin	FASN	Inhibition of proliferation and metastasis with induction of apoptosis	Murata et al. (2010); Shiragami et al. (2013)
EGCG	FASN	Downregulation of STAT3 and reduced proliferation	Wang et al. (2021)
GSK165	ACLY	Activation of AKT signaling	Zhou et al. (2013)
IDF-11774	HIF1α	Inhibition of glycolysis and angiogenesis	Misale et al. (2012)
L-Aspartate plus Rapac-MYCin	ASNS and mTOR	Inhibition of asparagine biosynthesis	Toda et al. (2016)
Luteolin	FASN	Modulation of Wnt/β-catenin signaling	Horinaka et al. (2005), Pandurangan and Esa (2014), Yao et al. (2019b)
Metformin	Mitochondrial electron transport complex I	AMPK activation and PKM2 inhibition	Zhang et al. (2011); Carr et al. (2016)
Mito-Metformin	Mitochondrial electron transport complex I	ATP depletion	Hosono et al. (2010)
Oxamate	LDHA	Inhibition of glycolysis	Yang et al. (2014b), Salgado-Garcia et al. (2021)
RO5126766	RAF/MEK	Downregulation of GLUT1	Wu et al. (2015)
TVB-3166	FASN	Inhibition of *de novo* palmitate synthesis	Ventura et al. (2015)
TVB-3664	FASN and CD36	Suppression of lipid metabolism and transport, Modulation of AKT and ERK1/2 signaling	Zaytseva et al. (2018)
Vitamin C	EGFR/MAPK	Decreased phosphorylation of PKM2 and reduced GLUT1 expression	Aguilera et al. (2016)
WZB117	GLUT1	Downregulation of GLUT1	Misale et al. (2012)
WZB117 plus Ficlatuzumab	GLUT1 and HGF	Inhibition of glucose uptake and blocking HGF activity	Song et al. (2017)

maintain cell viability and tumor growth under the glutamine-depleted condition (Toda et al., 2016). Mutant KRAS upregulated asparagine synthetase (ASNS) expression through PI3K/AKT/mTOR signaling to increase the asparagine biosynthesis and promote CRC cell growth (Toda et al., 2016). Meanwhile, fatty acid synthase (FASN) is overexpressed in KRAS mutant CRC cell lines to support cellular respiration via lipid oxidation and subsequently provide a survival advantage under metabolic stress (Zaytseva et al., 2015). **Figure 3** illustrates specific metabolic pathways rewired through oncogenic signaling during CRC initiation and progression, as well as metabolic reprogramming in CRC stem cells.

10 TARGETING COLORECTAL CANCER METABOLISM

As discussed above, metabolic reprogramming is critical in the CRC tumorigenesis to support cancer initiation, proliferation, invasion, and metastasis. The first line of treatment for early-stage CRC patients (stage I and II) is surgical resection, while chemotherapy and radiotherapy are mostly recommended for late-stage II and above (Dekker et al., 2019). Chemotherapy for CRC patients usually includes the combination of chemotherapy agents, such as FOLFOX (5-FU, leucovorin, and oxaliplatin) or FOLFIRI (5-FU, leucovorin, and irinotecan), to maximize the treatment efficacy (Keum and Giovannucci, 2019). However, chemotherapy often comes with serious side effects, such as nausea, vomiting, loss of hair, and body weakness, which affect the life quality of CRC patients (Onyoh et al., 2019). Drug resistance becomes another difficulty in chemotherapy for CRC, as it quickly gives rise to cancer relapse and metastasis that significantly reduce the survival rate and prognosis among CRC patients (Rawla et al., 2019). Over the years, researchers have been looking into alternatives for chemotherapy in CRC management, possibly with more minor side effects and similar efficacy. Thus, targeting CRC metabolism could become another potential option for CRC therapy.

The advancement in the molecular biology of cancer has allowed researchers to identify the dysregulation of protein kinases as the effectors of signaling pathways in cancer development and progression. These relevant findings have led to pharmacological inhibitors that directly target protein kinases and signaling pathways in cancers to kill the cancer cells more effectively (Butti et al., 2018; Jiang and Ji, 2019). As protein kinases are the essential regulators of metabolic reprogramming, protein kinases inhibitors could be repurposed to target the CRC metabolism. In general, most of the inhibitors available can be grouped into two categories: small molecule intervention and antibody blocking (Lemmon and Schlessinger, 2010). Small-molecule inhibitors can target the ATP-binding site of protein kinases specifically to inhibit phosphorylation, while monoclonal antibodies are developed to bind to the extracellular domain of protein kinases and suppress kinase-ligand interaction

(Gaumann et al., 2016; Gan et al., 2017). Many of these inhibitors are being investigated in phase II clinical studies in CRC, while the US FDA has approved several drugs for targeted therapy in clinical application, as listed in **Table 2**. These small molecule inhibitors and monoclonal antibodies can be used as monotherapy or combined with conventional therapies to increase the therapeutic efficacy and cancer specificity (To et al., 2020; Hwang et al., 2021). For instance, cetuximab and panitumumab are monoclonal antibodies that target EGFR and can be used as monotherapy or combined with chemotherapy to treat patients with RAS wild-type metastatic CRC (Hayashi et al., 2017; Garcia-Foncillas et al., 2019).

Some natural compounds and synthetic agents have been discovered to target metabolic enzymes and signaling molecules directly. Consequently, these compounds can function as metabolic inhibitors which could potentially target CRC metabolism, as listed in **Table 3**. Some studies demonstrate that naturally derived metabolic inhibitors can be administered without side effects and are much safer than conventional chemotherapeutics (Pandurangan and Esa, 2014; Wang et al., 2021). Obesity and a high-fat diet are commonly associated with a higher risk of CRC, in which upregulation of lipid metabolic enzymes such as FASN and ACC often leads to CRC progression and metastasis through activation of oncogenic pathways including Wnt, PI3K/AKT, AMPK/mTOR. Thus, research on compound targeting enzymes involved in lipid metabolism could be promising in the future management of CRC cases. For example, luteolin (3,4,5,7-tetrahydroxyflavone) which is found in vegetables, fruits, and medicinal herbs, functions as a fatty acid synthase (FASN) inhibitor to modulate lipid metabolism in CRC cells via Wnt/β-catenin signaling (Pandurangan and Esa, 2014). Luteolin can be orally administrated in a dosage of up to 500 mg twice a day without side effects (Luo et al., 2017). Notably, the combination of metabolic inhibitors and chemotherapy drugs has been shown to enhance the therapeutic efficacy and reduce the toxicity simultaneously, which is promising for the future development of CRC therapy. For instance, a combination of glutaminase (GLS) inhibitor CB839 and 5-FU chemotherapy drug increases the anti-cancer effect on the xenograft growth of PIK3CA-mutant CRC cells without significant dose-limiting toxicity (Zhao Y. et al., 2020).

11 CONCLUSION

The reprogramming of cellular metabolism is vital in tumor development. Metabolic reprogramming constitutes an important cancer hallmark in CRC, which gives rise to the alteration in aerobic glycolysis, glutaminolysis, OXPHOS, and lipid metabolism. Numerous studies have demonstrated that oncogenic mutations and loss of tumor suppressor genes contribute to CRC cell metabolic reprogramming by modulating the downstream signaling pathways. Protein kinases, such as AKT and c-MYC, regulate the expression of metabolic enzymes at both transcriptional and translational levels. The crosstalk between oncogenic pathways and the metabolic pathways represents another new avenue to develop therapeutics for CRC. Small molecule inhibitors and monoclonal antibodies currently available could be repurposed to target CRC metabolism by regulating protein kinases and signaling pathways involved. The discovery of metabolic inhibitors could become the alternative to the current treatment options, such as chemotherapy and radiotherapy, that are associated with severe side effects. The development of therapeutic based on CRC metabolism is challenging when most of the research data is generated from cell line models. Given the complexity of the tumor microenvironment and the heterogeneity of actual tumors, many questions regarding dosage safety and the therapeutic efficacy in clinical trials are waiting to be answered.

AUTHOR CONTRIBUTIONS

Conceptualization, KWH and RN; writing—original draft preparation, KWH; writing—review and editing, SA, IO, and RN. All authors have read and agreed to the published version of the manuscript.

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Cyclin-Dependent Kinase Inhibitors and Their Therapeutic Potential in Colorectal Cancer Treatment

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Cyclin-dependent kinases (CDKs) are key players in cell cycle regulation. So far, more than ten CDKs have been described. Their direct interaction with cyclins allow progression through G1 phase, transitions to S and G2 phase and finally through mitosis (M). While CDK activation is important in cell renewal, its aberrant expression can lead to the development of malignant tumor cells. Dysregulations in CDK pathways are often encountered in various types of cancer, including all gastrointestinal (GI) tract tumors. This prompted the development of CDK inhibitors as novel therapies for cancer. Currently, CDK inhibitors such as CDK4/6 inhibitors are used in pre-clinical studies for cancer treatment. In this review, we will focus on the therapeutic role of various CDK inhibitors in colorectal cancer, with a special focus on the CDK4/6 inhibitors.

Keywords: cyclin-dependent kinases (CDKs), CDK inhibitors, CDK4/6 cell cycle inhibitors, colorectal cancer, CRC therapy, cell cycle

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CYCLIN-DEPENDENT KINASES AND THEIR ROLE IN CELL CYCLE PROGRESSION

Cell cycle is defined as the process through which the cell replicates all its genomic material and divides into two identical cells (Alberts et al., 2002). It consists of four phases: gap 1 (G1), where the cell grows in size and transcribes the RNA and protein necessary during cell division; synthesis or S phase, where all chromosomes are being replicated; gap 2 (G2), where cell growth and protein synthesis continue; and mitosis or M phase, where the cell restructures its membrane and organizes the newly synthesized chromosomes and then divides into two daughter cells. Before entering cell cycle, highly proliferative cells such as stem cells and lymphocytes are in a reversible cell cycle arrest, known as quiescence or gap 0 (G0). However, other cells such as neurons or adipocytes are irreversibly arrested in G0 phase, a phenomenon often described as cellular senescence. Senescence is also predominant in highly damaged cells, acting as a protective mechanism during the DNA damage response (DDR) (Terzi et al., 2016).

Each cell cycle phase, as well as transitions from one phase to the other, are tightly regulated by interactions between cyclins and cyclin-dependent kinases (CDKs) (Johnson and Walker, 1999). In general, cyclins directly bind CDKs and induce the formation of cyclin—CDK complexes. This promotes CDK activity and therefore ensures activation of specific transcriptional programs that allow cell cycle progression. More than ten CDKs are known to be involved in various events during cell cycle. From these, CDK1, 2, 3, 4, and 6 directly mediate cell cycle progression.

Transition from quiescence or G0 phase in G1 phase is modulated by growth factor signals or mitogenic stimulation. These result in the upregulation of Cyclin D, which binds to and activates CDK4 and CDK6 to promote cell commitment to enter G1 phase (Jinno et al., 1999; Lea et al., 2003). High CDK4/6 expression and activation ensures cell progression through G1 phase (Mende et al., 2015; Topacio et al., 2019).

On the molecular level, CDK4 and 6 phosphorylate Retinoblastoma (Rb) and promote the accumulation of E2F, a direct regulator of genes necessary during DNA synthesis. Furthermore, CDK4 and CDK6 activation initiates cell growth through activation of mammalian target of rapamycin complex 1 (mTORC1) (Romero-Pozuelo et al., 2020). Besides, CDK4 and 6 are involved in the control of DNA replication mechanisms (Braden et al., 2008). Along with CDK4/6, CDK2 and CDK3 are also activated during G1 phase. Rb phosphorylation, and therefore the accumulation of E2F during G1 phase, directly mediate the upregulation of Cyclin E in late G1 phase, which binds and activates CDK2. Formation of CDK2/Cyclin E complex maintains Rb phosphorylated in order to promote G1/S phase transition (Massague 2004; Horiuchi et al., 2012). However, CDK3 upregulation during late G1 phase seems to be independent of Cyclin D, E or A binding (Braun et al., 1998). Interestingly, the upregulation of CDK2 has been also shown to be important during the G1/S checkpoint in response to DNA damage. For example, knocking-down CDK2 in the HCT116 tumor cell line significantly reduced p53 phosphorylation in response to hydroxyurea (HU) and suppressed G1/S cell cycle arrest (Bacevic et al., 2017). Some recent studies also described a role of CDK2 directly after mitosis, as an intermediate level will remain in the cells that continue proliferating, while those that lack CDK2 can enter quiescence or so called gap 0 (G0) (Spencer et al., 2013; Gookin et al., 2017). On the other hand, high levels of Cyclin C/CDK3 have been reported to directly mediate quiescence (Ren and Rollins 2004).

The beginning of S phase is marked by increasing levels of Cyclin A, which binds CDK2. The complex formed by Cyclin A/CDK2 drives the cells through S phase and promotes DNA replication. During late S/G2 phase, increased levels of Cyclin A induce CDK1 activation, which drives entry into mitosis (Gavet and Pines, 2010; Kalous et al., 2020). Later, the formation of CDK1/Cyclin B complex triggers progression through M phase. Along with its important role in successful cell mitosis (Vassilev et al., 2006), CDK1 can also influence the remodeling of cell adhesion complexes during G1, S and G2 cell cycle phases (Jones et al., 2018) and promotes protein synthesis during proliferation (Haneke et al., 2020). Interestingly, CDK1 is reported to be the only necessary cyclin-dependent kinase during cell cycle, being able to bind to all cyclins and drive all events during cell division (Santamaria et al., 2007).

Several other CDKs are known to be involved in cell cycle progression as well. CDK7, for example, is an important cell cycle regulator. Its binding to Cyclin H and mating-type 1 protein (Mat1) induces the formation of CDK-activating kinase (CAK) complex. CAK activity is crucial to promote CDK2 and CDK1 binding to cyclins, therefore allowing cell division (Fisher and Morgan, 1994; Larochelle et al., 2007; Olson et al., 2019). CDK5 upregulation is mostly observed in, but not limited to, neurons, and is often correlated to cell apoptosis. Nevertheless, it can also regulate the cell cycle by phosphorylating Rb and interacting with E2F during G1 phase (Zhang et al., 2010; Chang et al., 2012; Futatsugi et al., 2012). CDK8 is a partner of Cyclin C and its expression has been shown to be important in stabilizing Cyclin C activity during cell cycle (Tassan et al., 1995; Barette et al., 2001). Interestingly, CDK8 and Cyclin C, as well as CDK19/Cyclin C complex, are strongly required during p53-dependent p21 transcriptional activation, for cell cycle arrest in response to DNA damage (Donner et al., 2007; Audetat et al., 2017). Last, cyclin-dependent kinases such as CDK9 and CDK13 are not directly controlling cell cycle phase transitions, but are rather involved in transcription mechanisms, by associating with Cyclin T or Cyclin K (Garriga et al., 2003; Yu et al., 2010; Greifenberg et al., 2016).

To summarize, entry into cell cycle depends on mitogenic or growth factor signals. CDK4/6/Cyclin D complex formation promotes Rb phosphorylation and accumulation of free E2F, which ensures progression through G1 phase. CDK5 activity also increases E2F levels during G1. High levels of E2F during late G1 induce CDK2/Cyclin E complex that in return further phosphorylates Rb and promotes G1/S transition. At the beginning of S phase, Cyclin E levels decrease and CDK2 forms a complex with the increasing Cyclin A, which not only ensures progression through S phase, but also transition into G2 phase. CDK2/Cyclin A complex is especially regulated by the CDK7/Cyclin H/Mat1 complex, also described as CAK. CAK also regulates CDK1/Cyclin A complex formation during late G2 and Cyclin B binding to CDK1 during mitosis. Any disturbances to the cell cycle machinery will result in cell cycle arrest. CDK2 and CDK3 are especially important in mediating either quiescence or senescence. Indirectly, CDK8, 9, 13, and 19 also mediate cell cycle, being involved in the transcription machinery, while CDK5 can directly modulate apoptosis as well. A schematic representation of the important role of CDKs in cell cycle is shown in **Figure 1**. While normal cells are able to activate the necessary mechanisms for cell cycle arrest when the DNA is damaged, these pathways are usually suppressed or non-existent in tumor cells, enabling them to continue progression through cell cycle. The following sections will address the CDK's role in the tumor cell division and how therapies targeting CDKs can modulate CRC development.

CYCLIN-DEPENDENT KINASE EXPRESSION IN HUMAN COLORECTAL CANCER

Changes in the regulatory mechanisms that control cell division are often related to accumulation of mutations and/or epigenetic dysregulations of cancer related genes and can contribute to the molecular mechanisms of colorectal cancer (CRC). CRC tissue often shows changes in genes related to cell cycle arrest (p16 and p21), apoptosis (p53) or proliferation (PCNA) (Yue et al., 2003; Kruschewski et al., 2011). Multiple other mutations have also been described to be involved in CRC development. As a result, CDKs expression can be changed in tumor cells.

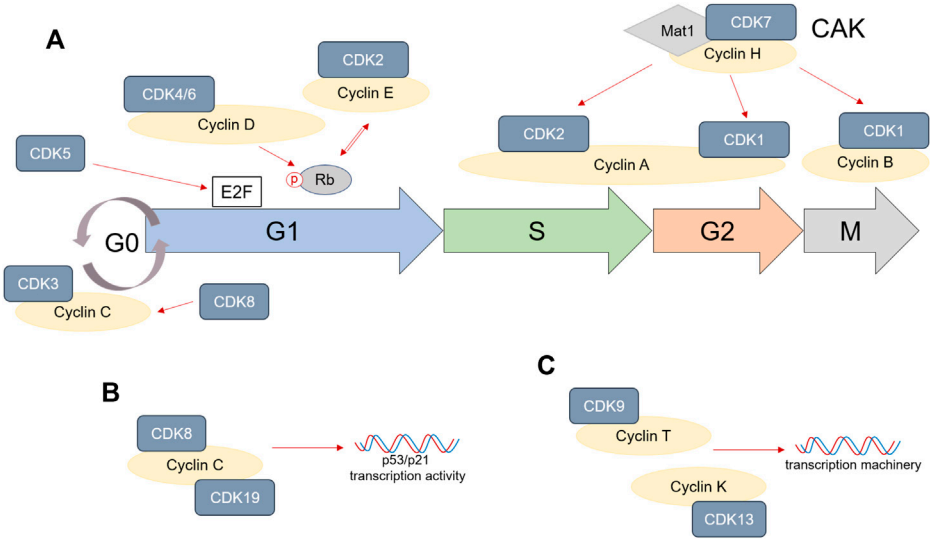

FIGURE 1 | Cyclins and cyclin-dependent kinase (CDK) role in cell cycle. **(A)** CDK1, 2, 3, 4/6, and 7 are directly involved in progression through cell cycle phases by associating themselves with various Cyclins. CDK5 can have a direct impact on E2F accumulation, especially in cancer, while CDK8 activation stabilizes Cyclin C. **(B,C)** CDK8, 9, 13, and 19 are not directly involved in cell cycle progression, but are involved in either p53/p21 transcription (CDK8/19) or the DNA transcription machinery (CDK9/13).

When looking at the signature of differentially expressed genes (DEGs) in patients with CRC compared to normal colon tissue, an upregulation in CDK1 gene expression is often observed (Zhao et al., 2019; Ding et al., 2020; Li et al., 2020). Interestingly, the expression of CDK1 in the nucleus and cytoplasm has been used as a marker to describe patterns in the overall survival of patients with CRC (Sung et al., 2014). Staining of over 164 cancer samples from primary CRC revealed that CDK1 is expressed in both cell nucleus and cytoplasm to a certain degree. The evaluation of nuclear/cytoplasm (N/C) ratio on these samples showed that high N/C expression is often found in patients with overall worse survival and a N/C > 1.5 can be considered a risk factor. Furthermore, high CDK1 expression is predominant in patients with resistance to 5-fluorouracil (5-Fu), a common CRC treatment, and it seems to reduce the effect of chemotherapy (Zhu et al., 2020). An upregulation of CDK1 in CRC has been also observed in response to other drugs such as: betaxol, penbutolol and propofol amongst others (Mastrogamvraki and Zaravinos, 2020).

CDK2, 4 and 6 levels in CRC are closely related to the Rb protein hyperphosphorylation, which seems to promote cancer progression. CDK4/6 is usually amplified in colon tumors compared to healthy epithelium (Mastrogamvraki and Zaravinos, 2020; Jardim et al., 2021). Abundant levels of CDK4 are especially observed in CRC patients with enhanced dysplasia and are correlated to increased tumor cell proliferation (Zhang et al., 1997; Bartkova et al., 2001). Some CDK2 expression is normally found in healthy epithelium. However, its upregulation can be predominantly observed human CRC tissue samples (Yamamoto et al., 1995). Interestingly, CDK2 overexpression in primary CRC tumors is also linked to lymph nodes metastasis, but not liver metastasis (Li et al., 2001; McCurdy et al., 2017). Nevertheless, a certain CDK2 activity has been reported to improve recurrence-free survival (RFS) of patients after surgery (Yamamoto et al., 1995). A similar pattern to CDK2 expression in CRC is observed in CDK3 levels as well. Its overexpression has been linked to metastasis and tumor cell invasion, where it seems to be promoting epithelial to mesenchymal transitions (Lu et al., 2016).

CDK5 expression is also reported to be much higher in CRC cells compared to normal epithelium and it correlates to increased tumor growth and poor prognosis (Zhuang et al., 2016; de Porras et al., 2019). Most important, CDK5 is directly involved in the degradation of the cell cycle inhibitor p21 and can enhance CDK2 activity, which might further promote tumor cell growth (Huang et al., 2016). Decreased survival rates are also observed in CRC patients with high CDK9 and CDK13 levels (Kim et al., 2012; Wang et al., 2019). Interestingly, high CDK9 expression in CRC tissue was negatively correlated with cytotoxic $CD8^+$ T cell infiltration. Furthermore, these infiltrated cells showed increased cell exhaustion in CDK9-high tumors, which might further affect patient outcome (Wang et al., 2019). Last, CDK8 overexpression in CRC is also considered as a marker for poor patient prognosis, being directly linked to β-catenin activation amongst others and therefore promoting cancer growth (Firestein et al., 2008; Firestein et al., 2010; Seo et al., 2010). Overall, cyclin-dependent kinase activation is often observed in colorectal cancer and seems to promote tumor progression and an overall worse survival of patients, as summarized in **Table 1**.

THE FUNCTIONAL ROLE OF CDKS IN CRC

Basic research using murine knockout models or *in vitro* gene silencing in tumor colon cancer cell lines also provided some

TABLE 1 | Effects of increased CDK expression in patients with colorectal cancer.

Gene	Expression in CRC	Patient outcome	References
CDK1	Upregulated in tumor tissue compared to normal tissue	Decreased overall patient survival	Ding et al. (2020)
	Ratio between nuclear and cytoplasmatic expression can be used as an indicator of patient outcome	Interferes with 5-Fu therapy	Sung et al. (2014)
	Medication can further upregulate CDK1 in CRC		Zhu et al. (2020) Mastrogamvraki and Zaravinos (2020)
CDK2	A normal CDK2 expression is also found in healthy colon	Increased expression in normal colon tissue after surgery is correlated to a good prognosis	Yamamoto et al. (1995)
	Upregulated in CRC tissue compared to normal tissue		Li et al. (2001)
	Overexpression correlated to lymph node metastasis		McCurdy et al. (2017)
CDK3	No expression found in normal colonic tissue	Not described	Lu et al. (2016)
	Overexpressed in CRC tissue and metastatic tissue		
CDK4/6	Upregulated in CRC samples compared to healthy tissue	Poor prognosis in patients with strong CDK4 expression in tumors	Jardim et al. (2021) Mastrogamvraki and Zaravinos (2020) Zhao et al. (2003)
CDK5	Upregulated in tumor tissue compared to the adjacent healthy tissue	Increased tumor growth	de Porras et al. (2019)
	Can upregulate CDK2 expression as well	Poor patient prognosis	Zhuang et al. (2016) Huang et al. (2016)
CDK8	Overexpressed in CRC tissue compared to matched healthy tissue	Promotes cancer growth	Firestein et al. (2010)
		Poor patient prognosis	Seo et al. (2010)
CDK9/13	High in CRC tissue	Worse overall patient survival	Kim et al. (2012) Wang et al. (2019)

understanding for the relevance on CDKs in CRC development. Since CDKs are vital components of the cell cycle, creating knockout mouse models is usually unsuccessful. This is because most CDKs (e.g. CDK1, 4, 6, 9, and 13) are critical during embryonic development, as is summarized in (Campbell et al., 2020). Similarly, conditional knockout models often show severe impairments.

Nevertheless, some fundamental research data in regards to the role of CDKs in colorectal cancer are available. For example, it is know that CDK4 activation in $CDK4^{R24C/R24C}Apc^{+/min}$ mice leads to significant increased in tumor vascularity in comparison to $CDK4^{+/+}Apc^{+/min}$ mice or $APC^{+/min}$ mice (Abedin et al., 2010), while knocking out CDK4 in $APC^{+/min}$ mice reduces adenoma development (Karim et al., 2013). CDK5 silencing via transfection can directly reduce the proliferation of human HCT116 and SW480 tumor cell lines (Zhuang et al., 2016). Similarly, knocking down CDK9 in HCT116 and HT29 tumor cell lines induced their apoptosis by Caspase 7 cleavage (Rahaman et al., 2019). Furthermore, it reduced Cyclin D1 protein expression, suggesting cell cycle arrest induction in these cells.

Stable silencing of CDK8 and CDK19 in Colo205 human colon cancer cells reduced β-catenin/TCF-dependent transcription (Dale et al., 2015). A direct link between CDK8 and β-catenin regulation in tumor cell proliferation and death has also been described, where inactivation of CDK8 by siRNA transfection in HCT116 cells significantly reduced the RNA and protein levels of β-catenin (He et al., 2011). Generally, silencing CDK genes in colon cancer cells reduces their proliferation and induces cell death, which makes them an attractive target for the development of new inhibitory therapies.

CDK INHIBITORS AS A POTENTIAL CRC TREATMENT

CDK inhibitors are also often used in basic research to understand molecular mechanisms of CDK activation in cell cycle regulation or tumor cell proliferation. This section describes the current understanding on the potential use of various CDK inhibitors to mediate colorectal cancer development.

CDK7-Specific Inhibitors

Samuraciclib and SY-1365 are inhibitors of CDK7 activity. Interestingly, the colon cell line HCT116 is particulary sensitive to Samuraciclib, which induces their apoptosis and cell cycle arrest (Patel et al., 2018). Its mechanism of action is mostly based on inhibition of phosphorylation of CDK7 substrates like CDK1 and 2. One important advantage of Samuraciclib is its availability as an oral drug that can accumulate at the tumor site upon multiple doses, as shown by the *in vivo* HCT116 murine tumor xenograf model. CDK7 inhibition was also successful when using SY-1365, in more than 26 types of cancer types, including colon cell lines (Hu et al., 2019).

CDK1/2-Specific Inhibitors

SU9516 and CVT-313 are known to directly inhibit CDK2 activity. The use of SU9516 for *in vitro* treatment of HT29, RKO and SW480 human colon carcinoma cell lines revealed that it can successfully induce their apoptosis and cell cycle arrest (Lane et al., 2001; Yu et al., 2002). CDK2 inhibition also significantly decreases free E2F, but increases E2F/Rb complexes, therefore arresting the tumor cells. This effect was

dependent on the duration of the treatment, since more E3F/Rb complexes were observed after 48 h than after 24 h in HT29 cell line. Inhibition of CDK2 in patient-derived human cell lines using CVT-313 has minimal effect on cell death (Somarelli et al., 2020). Nevertheless, combined therapy using CDK2 and 9 inhibitors significantly increased the numbers of cells arrested in G2/M.

RO-3306 is a CDK1-specific inhibitor can be used to induce apoptosis in a specific type of BRAF-mutated colorectal cancer cells (Zhang et al., 2018). Interestingly, this inhibitor induced Caspase 8-regulated cell death when combined with the MEK inhibitor, cobimetinib, while most CDK inhibitors promote apoptosis via Caspase 3 cleavage.

CDK5, 8/19, and 9-Specific Inhibitors

CP668863 or 20-223 is a CDK5 inhibitor whose cytotoxic potential has been evaluated in CRC settings as well (Robb et al., 2018). Interestingly, 20-223 is 65-fold more potent for cell growth inhibition than the pan CDK inhibitor AT7519. Its cytotoxicity has been evaluated on SW620, DLD1 and HT29 tumor cell lines. 20-223 also significantly inhibited tumor growth in xenograf models and reduced the migration of colon cancer cells, which shows its potential for CRC therapy.

The development of MSC2530818 was fine tuned to specifically inhibit CDK8/19 (Czodrowski et al., 2016). This compound can be orally administered and it is well tolerated by mice. Treatment with MSC2530818 of mice subjected to an *in vivo* xenograft model using SW620 human colon cell line showed its potential to reduce tumor growth. CDK8/19 inhibition by MSC2530818 it is known to directly reduce STAT1 phosphorylation, further proving its efficacy.

CDKI-73 is a potent CDK9 inhibitor, which shows increased cytotoxicity against the HT29 and HCT116 human carcinoma cell lines (Rahaman et al., 2019). *In vitro* treatment of these cell lines revealed that CDKI-73 reduces the expression of survival genes. Its effect has also been tested in *in vivo* HT116 xenograf models. CDKI-73 significantly reduced tumor growth without being over toxic to the mice.

Purvanalol and Roscovitine

Purvanalol and Roscovitine (Celiciclib or CYC202) are common CDK inhibitors effective against CDK2, 4, and 5 activity. Purvanalol is known to induce apoptosis and autophagy of HCT116 colon tumor cells by activating endoplasmatic reticulum (ER) stress (Coker-Gurkan et al., 2015). Its effect is nevertheless limited to wildtype HCT116, while Bax-deficient HCT116 cells are resistant against this treatment. This effect can be overcome by combining of Purvanalol with 3-MA, an inhibitor of autophagy, which promotes Purvanalol-induced apoptosis in Bax−/− HCT116 as well (Coker-Gurkan et al., 2014). Roscovitine has a similar effect on apoptosis induction in HCT116 tumor cells, but on a weaker scale than Purvanalol (Gurkan et al., 2013; Coker-Gurkan et al., 2015). Analysis of Roscovitine-induced apoptosis using Raman spectroscopy revealed changes in amide I and III bands, common of protein and DNA alterations (Akyuz et al., 2011). HCT116 cell death in presence of Roscovitine has been shown to be enhanced during polyamine depletion or phosphatase nuclear targeting subunit (PNUTS) knockdown (De Leon et al., 2010; Arisan et al., 2012). More important, the effect of Roscovitine is especially higher in combination to current chemotherapeutic drugs such as 5-Fu or doxorubicine, as shown by the experiments done with SW48, SW116 and SW837 colon cancer cell lines (Abaza et al., 2008).

Wogonin

Wogonin is a flavone isolated from Scutellaria baicalensis known to inhibit CDK2, 4, 8, and 9. Nevertheless, its effect is not specific to only CDKs, but it also downregulates activation of PI3K/Akt and Stat3 signaling pathways (Wang et al., 2014; Tan et al., 2019). Along with its role in inducing apoptosis and autophagy of colorectal tumor cells, Wogonin can also induce cell cycle arrest in both G1 and G2/M cell cycle phases (He et al., 2013; Tan et al., 2019). Interestingly, Wogonin treatment of wildtype mice subjected to AOM/DSS tumor model reduces tumor growth by facilitating nuclear translocation of tumor suppresor p53 (Feng et al., 2018).

Flavopiridol

Flavopiridol or Alvocidib is effective in inhibiting most CDKs: CDK1, 2, CDK4/6 and 9, by inducing cell cycle arrest and apoptosis of human colon tumor cell lines (Sausville et al., 2000; Kim et al., 2003; Okada et al., 2017). Treatment of CRC cell lines with flavopiridol enhances cell death when used in combination with chemotherapeutic agent gemcitabine or γ-radiation (Jung et al., 2001; Jung et al., 2003). Furthermore, a combination of docetaxal, flavopiridol and 5-Fu is described to more effective in inhibiting tumor growth and inducing increased apoptosis in HCT116 tumor cells, than any of the drugs alone (Guo et al., 2006). Phase I and phase II studies in patients with untreated advanced colorectal cancer showed little efficacy and was terminated early (Aklilu et al., 2003). Overall, it appears that Flavopiridol works best when coupled with other chemotherapeutic drugs.

Other Pan CDK Inhibitors

Along with Purvanalol, Roscovitine, Wogonin and Flavopiridol, various other molecules have been described to inhibit multiple CDKs. For example, AT7519 is able to inhibit CDK1, 2, 4/6, and 9 and therefore induce colon cancer cell death. Its potency has been observed in xenograf mouse models using HCT116 and HT29, where tumor regression was observed upon multiple doses (Squires et al., 2009). Nevertheless, other CDK inhibitors such as 20-223 seem to be more effective than AT7519 (Robb et al., 2018). Pan CDK inhibitor AG-012986 has been shown to significantly reduce the colony formation of HCT116 colon carcinoma in a concentration-dependent manner, by inducing arrest into G1 phase (Zhang et al., 2008). Indirubin derivates are also known to reduce proliferation of DLD1 and HT29 tumor cell lines (Kim et al., 2009). Last, SNS-032 or BMS-387032, a specific inhibitor against CDK2, 7, and 9, was used to significantly reduce the intestinal tumor burden of Ink4/Arf-null Min mice (Boquoi et al., 2009). All in all, these data provide important insight on the effectiveness of CDK inhibitors in colorectal cancer therapy.

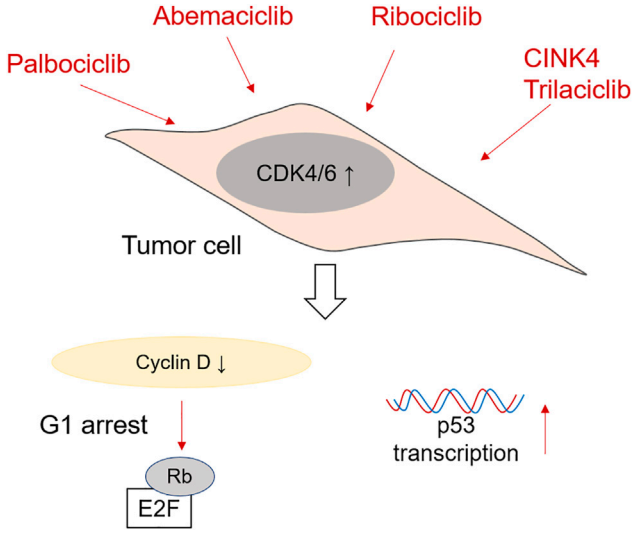

FIGURE 2 | Mechanism of action of CDK4/6 inhibitors in CRC cells. Palbociclib, Ademaciclib, Ribociclib, CINK4 and Trilaciclib are able to prevent the formation of Cyclin D/CDK4/6 complexes, which reduces Retinoblastoma phosphorylation and induces G1 cell cycle arrest. Palbociclib has been shown to be effective in promoting p53 transcription after irradiation as well.

CDK4/6 INHIBITORS USE IN CRC

When thinking about preventing cell cycle progression and proliferation of tumor cells, CDK4/6 inhibitors are very efficient. The most commonly used are Ribociclib, Palbociclib, Abemaciclib and Trilaciclib. CDK4/6 inhibitors are especially effective at treating breast cancer amongst others, many of them being nowadays tested in phase I and II clinical trials (Wu et al., 2020). Nevertheless, they are also being tested as therapy for colorectal cancer. A schematic representation of the mechanism of action of CDK4/6 inhibitors is shown in **Figure 2**.

CINK4 and Trilaciclib

Small molecule CINK4 is a triaminopyrimidine derivative specially designed to inhibit the activity of CDK4 in tumor cells. *In vitro* treatment of HCT116 colon tumor cell line with CINK4 prevented their cell growth by reducing Cyclin D/CDK4 complexes and Rb phosphorylation (Soni et al., 2001). Furthermore, intraperitoneal injection of CINK4 every 12 h was successful in reducing tumor growth in an *in vivo* mouse xenograf model using HCT116 tumor cells. Trilaciclib (CoselaTM) is known to directly induce reversible G1 cell cycle arrest and inhibit the formation of complexes between CDK4/6 and Cyclin D. As of 2021, Trilaciclib is used in a multinational trial (ClinicalTrials.gov Identifier: NCT04607668 in United States) in treating microsatelite stable metastatic CRC, in patients treated with FOLFOXIRI and Bevacizumab (Dhillon 2021). This clinical study has been recently approved and is at the moment recruiting participants in USA, Europe (Hungary, Italy, Poland, Slovakia, Spain, Ukraine, United Kingdom) and China.

Abemaciclib

Patients with advanced and metastatic breast cancer can be treated with the CDK4/6 inhibitor Abemaciclib (also known as LY2835219, Verzenio, Verzenios, Ramiven). This inhibitor is also involved in various clinical trials for treating other advanced solid tumors such as melanoma or lung cancer (Shapiro et al., 2013; Fujiwara et al., 2016). The potential of Abemaciclib to treat colorectal cancer has been tested in mice with human tumor xenographs using Colo205 and A375 (Tate et al., 2014). The mice were treated orally in a concentration-dependent manner. The authors suggest that a constant level of 200 ng/ml Abemaciclib in plasma are necessary to arrest the tumor cells in G1 phase, as shown by Rb phosphorylation data. This shows that treatment using multiple doses might promote tumor cell cycle arrest in humans as well. Indeed Abemaciclib therapy in CRC patient cohort during a clinical trial induced stable disease even in a patient with KRAS and p53 mutated tumor cells (Patnaik et al., 2016). At the moment, Abemaciclib, in combination with LY3214996 (ERK1/2 inhibitor) and Cetuximab (EGFR inhibitor), is undergoing evaluation in Phase I and Phase II clinical trials in patients with metastatic CRC (ClinicalTrials.gov Identifier: NCT04616183). Recruiting phase is set to be completed in December 2021.

Palbociclib

The efficacy of Palbociclib (PD-0332991) in inhibiting CDK4/6 activity has been assessed in human colon carcinoma cell lines as well (Li et al., 2014). Palbociclib successfully arrested various tumor cells (HT29, Colo205 and DLD1 amongst others) in G1 cell cycle phase, by reducing the phosphorylation of Rb. Interestingly, its therapeutic effect does depend on Rb presence (Heijink et al., 2011). Nevertheless, *in vivo* administration of Palbociclib in ApcMin mice successfully reduced tumor cell proliferation without affecting normal epithelial cells. It is very important to remark that Palbociclib mechanism of action directly targets the transcriptional activity of p53 after exposure to radiation and therefore, its efficacy might be limited to p53-expressing CRC tumors (Fernandez-Aroca et al., 2019). Palbociclib is also involved in a phase II clinical trial (ClinicalTrials.gov Identifier: NCT03981614), where it is used in combination with chemotherapeutic drug TAS-102 for KRAS/NRAS metastatic or unresectable CRC. First phase of the study has been recently completed (June 2021), but no data are momentarily available.

Ribociclib

Treatment of HT29 and SW480 colon tumor cell lines with Ribociclib (or LEE011) significantly decreases their viability and induces G1 cell cycle arrest in concentration dependent manner (Lin et al., 2020). Similarly to the other CDK4/6 inhibitors, Ribociclib also reduces the phosphorylation of Retinoblastoma protein. Furthermore, used in combination with 5-FU, it increases significantly p53 phosphorylation. Ribociclib treatment was also used in a study case on a young female diagnosed with desmoid tumors (DT) (Santti et al., 2019). She underwent colectomy and various other surgeries to remove the tumors, as well as irradiation therapy. Unfortunately, the

treatment with cytotoxic drugs usually used to treat these cancers did not reduced the tumors. The addition of Ribociclib, together with goserelin and letrozole therapy, stabilized temporarily the tumors and gave symptomatic relief. A Phase I clinical trial for treating selected malignancies, including CRC, using Ribociclib in combination with TNO155 (SPH2 inhibitor) is currently running (ClinicalTrials.gov Identifier: NCT04000529). Patients are still being recruited in this clinical trial.

FUTURE PERSPECTIVES IN CDK4/6 INHIBITOR THERAPY IN CRC

There is no doubt that targeting cell cycle machinery, and especially cyclin-dependent kinase activity of tumor cells, offers new opportunities to treat patients with advanced colorectal cancer. Nevertheless, cancer itself is a multifactorial disease and therefore the treatment with just one drug is not always successful.

CDK4/6 inhibitor therapy in particular shows promising results in the relief and stabilization of the patients, but its effect is amplified when used in combination with other treatments. More recent studies have focused on evaluating therapeutic potential of CDK4/6 inhibitors when coupled with other drugs in treating CRC. For example, when treating tumors in patient-derived Rb+ colorectal xenograph models, the authors found that a combination of MEK inhibitor Trametinib with Palbociclib significantly reduces tumor volume in comparison to monotherapy. Furthermore, KRAS-mutated cells were especially sensitive to this treatment (Lee et al., 2016; Ziemke et al., 2016). Similiar results were obtained when using a Raf inhibitor (LY3009120) in combination with Abemaciclib, where Ras- and Braf-mutated CRC was especially sensitive to this treatment (Chen et al., 2018). Last, the combination of checkpoint inhibitors like anti-PD1 therapy (SHR-1210) with CDK4/6 inhibitor (SHR6390) is currently evaluated in Phase I and II clinical trial for advanced colorectal cancer (ClinicalTrials.gov Identifier: NCT03601598), but no data have been published yet.

Further studies are necessary for understanding the potential of targeting CDK4/6, together with other genes involved in cell cycle machinery. For example, tumor cells depend on high telomerase activity, which enables them to preserve the telomeres during extensive proliferation. Inducing telomere dysfunctions in tumor cells, using the telomere-specific inhibitor 6-thio-dG, potentiates antitumor responses in mice bearing MC38 tumors (Mender et al., 2020). Therefore, combining CDK4/6 inhibitors for cell cycle arrest and 6-thio-dG might provide a more efficient tumor targeted therapy.

One significant challenge raised by the use of CDK4/6 inhibitors is its effect on normal cells, and especially on the highly proliferating cells, such as activated immune cells found in the tumor microenvironment. Targeting CDKs might disrupt the function of upstream genes involved in the cell cycle, such as sirtuins, in normal cells. Modifications in sirtuin 1 (SIRT1) function are especially important. Even though SIRT1 is also upregulated in the CRC tissue compared to the normal one and it has been linked to tumor size and invasion (Chen et al., 2014; Yu et al., 2016), its function in haematopoiesis is nevertheless crucial (Rimmele et al., 2012). Dysfunctions in SIRT1 in normal cells due to CDK4/6 inhibitor use might therefore potentiate cellular senescence and premature aging in various cellular compartments (Sasaki et al., 2006).

Overall, CDK inhibitors are efficient in preventing colon tumor cells from proliferating by inducing cell cycle arrests, and, in some cases, even apoptosis, making them useful for developing new potential therapeutic strategies for CRC. Nevertheless, a comprehensive analysis on how CDK inhibitors might affect normal cells, as well as the antitumor response of immune cells to CRC, would enhance our understanding on this novel therapy.

AUTHOR CONTRIBUTIONS

O-MT and MW prepared the concept, wrote and reviewed the manuscript. MN provided valuable input during the review process of this manuscript.

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SMAD3 Host and Tumor Profiling to Identify Locally Advanced Rectal Cancer Patients at High Risk of Poor Response to Neoadjuvant Chemoradiotherapy

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Identifying patients at risk of poor response to neoadjuvant chemoradiotherapy (nCRT) is an emerging clinical need in locally advanced rectal cancer (LARC). SMAD3 is a key player in the chemoradio-resistance phenotype and its expression is both constitutive and locally induced. The aim was to investigate both host (genetic polymorphisms) and tumor SMAD3 profiling to predict response to nCRT. In a group of 76 LARC patients, SMAD3 and phosphorylated-SMAD3 expression was assessed by immunohistochemistry in preoperative tumor tissue. In an expanded study group (n = 378), a set of *SMAD3* polymorphisms (rs35874463, rs1065080, rs1061427, rs17228212, rs744910, and rs745103) was analyzed. Association with tumor regression grade (TRG) and patient prognosis (progression-free survival [PFS] and overall survival [OS]) was assessed. Patients with high tumor expression of SMAD3 had a significantly increased risk of poor response (TRG≥2) [cellularity >55% (OR:10.36, p = 0.0004), or moderate/high intensity (OR:5.20, p = 0.0038), or an H-score≥1 (OR:9.84, p = 0.0004)]. Patients carrying the variant SMAD3 rs745103-G allele had a poorer response (OR:0.48, p = 0.0093), a longer OS (HR:0.65, p = 0.0307), and a trend for longer PFS (HR:0.75, p = 0.0944). Patients who carried both high SMAD3 tumor expression and the wild-type rs745103-A allele had an extremely high risk of not achieving a complete response (OR: 13.45, p = 0.0005). Host and tumor SMAD3 status might be considered to improve risk stratification of LARC patients to facilitate selection for alternative personalized neoadjuvant strategies including intensified regimens.

Keywords: rectal cancer, neoadjuvant chemoradiotherapy, 5-fluorouracil, Smad3, immunohistochemistry, polymorphisms, predictive markers

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INTRODUCTION

A combined modality approach with fluoropyrimidine-based neoadjuvant chemoradiotherapy (nCRT) followed by total mesorectal excision represents the standard of care for patients with locally advanced rectal cancer (LARC) (Yoo and Kim, 2019; Roeder et al., 2020). New treatment trends are based on risk stratification and include regimens with intensified pre-operative chemotherapy, such as total neoadjuvant therapy (TNT), in high-risk cases with poor response and recurrence (Rosello et al., 2018; Fokas et al., 2019; Papaccio et al., 2020; Riesco-Martinez et al., 2020; Bahadoer et al., 2021). The burning clinical question is how to better identify high-risk patients, currently defined only by specific clinical criteria such as clinical T and N stages, distance of tumor from anal verge, involvement of mesorectal fascia, and extramural vascular invasion (Glynne-Jones et al., 2018). Therefore, additional molecular predictors to be integrated in the clinical practice are specifically needed.

SMAD family member 3 (SMAD3) is an attractive candidate for a predictive and prognostic marker in cancer (Moon et al., 2015; Jung et al., 2017). It is a major transcription factor in the transforming growth factor-β (TGF-β) downstream signaling pathway, which is critical for the immunosuppressive and radioresistant phenotype associated with TGF-β (Tauriello and Batlle, 2016; Koveitypour et al., 2019). Within the tumor microenvironment, TGF-β is the most potent suppressor of radiotherapy-triggered anti-tumor T-cell responses (Vanpouille-Box et al., 2015; Wennerberg et al., 2017; Farhood et al., 2020; Liu et al., 2021). Following radiotherapy-mediated activation, TGF-β has been shown to upregulate immunosuppressive T regulatory cells and to downregulate anti-tumor effector cells (i.e., $CD8^+$ T lymphocytes and natural killers) (Vanpouille-Box et al., 2015; Wennerberg et al., 2017; Farhood et al., 2020; Liu et al., 2021). The role of SMAD3 in this context is not fully elucidated, although pharmacological approaches targeting SMAD3 have shown some enhancement of the immune response to radiotherapy (Akhurst and Hata, 2012; Rodriguez-Ruiz et al., 2019).

SMAD3 exhibits both constitutive (host-driven) and inducible (tumor-driven) expression in cancer (Jung et al., 2017). Host genetic variations in *SMAD3* can lead to dysregulation of TGF-β signaling. We previously reported a key role of three intronic *SMAD3* germline polymorphisms (rs744910, rs745103, and rs17228212) in predicting response to fluoropyrimidine-based nCRT in LARC patients (Dreussi et al., 2016). On the other hand, SMAD3 expression and activation at the tumor cellular level may serve as a marker for tumor proliferation, metastasis, and patient prognosis. Overexpression of nuclear C-terminal phosphorylated SMAD3 (p-SMAD3) in preoperative tumor samples was indicated to identify LARC patients at higher risk for poor response to fluoropyrimidine-based nCRT (Huang et al., 2015).

This study addresses for the first time both the host and tumor component of SMAD3 profiling through a combined molecular approach. The primary aim of the study was to define the association between the constitutive genetic features of SMAD3, tumor protein expression and their combination, and tumor response to standard nCRT regimens in LARC patients. These findings may improve the upfront identification of high-risk patients who could be proposed for alternative personalized preoperative approaches.

MATERIALS AND METHODS

Study Design and Patient Cohorts

All cases included in the study were selected from a consecutive collection of 617 patients with mid and low (stage II-III) primary adenocarcinoma of the rectum treated with nCRT between March 1994 and November 2015 at Centro di Riferimento Oncologico-IRCCS (CRO) of Aviano, Istituto Oncologico Veneto- IRCCS (IOV) of Padua, and Clinica Chirurgica I of Padua University.

Patients were enrolled in a prospective study protocol with the aim of revealing predictive and prognostic molecular biomarkers. The inclusion criteria were as follows: 1) Histologically confirmed diagnosis of primary resectable LARC; 2) confirmed absence of distant metastases; 3) age ≥18 years; 4) self-reported Caucasian ethnicity; 5) disease stage T3-T4 and N0-N2; 6) performance status (World Health Organization) 0–2; 7) normal bone marrow, renal, and liver function.

The study design (**Figure 1**) included an initial study group of 76 patients with both a formalin-fixed paraffin-embedded (FFPE) pre-treatment tumor biopsy and a peripheral blood sample. In this group, SMAD3 and *p*-SMAD3 expressions were determined by immunohistochemistry (IHC) and tested for association with tumor regression grade (TRG). The *SMAD3* gene was sequenced on germline DNA extracted from peripheral blood.

The initial study group was then expanded by adding 302 patients with an available peripheral blood sample. In the expanded study group (n = 378), the impact of a set of *SMAD3* genetic polymorphisms (rs35874463, rs1065080, rs1061427, rs17228212, rs744910, and rs745103) on TRG and patient prognosis (progression-free survival [PFS] and overall survival [OS]) were investigated.

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. The protocol was approved by the Ethical Committee of all participating institutions, and all patients gave written informed consent for research purposes before participating in the study. All experiments were conducted in accordance with the relevant guidelines and regulations of the Centro di Riferimento Oncologico di Aviano.

Tumor Treatment, Response Evaluation and Follow-Up

All patients received a nCRT based on fluoropyrimidines (either 5-fluorouracil [5-FU] or capecitabine) concomitant with radiotherapy as previously described (Cecchin et al., 2020). A radiation dose of 50.4 Gy, administered in 28 daily fractions over a period of 5.5 weeks, was given as standard treatment in most cases. A subset of patients received nCRT intensification either by a concomitant radiotherapy boost to the bulky tumor (cumulative radiation dose of 55.0 Gy administered by a concomitant boost of 1Gy 2 times/week for 5 weeks) or by concomitant administration of oxaliplatin according to enrollment in an institutional

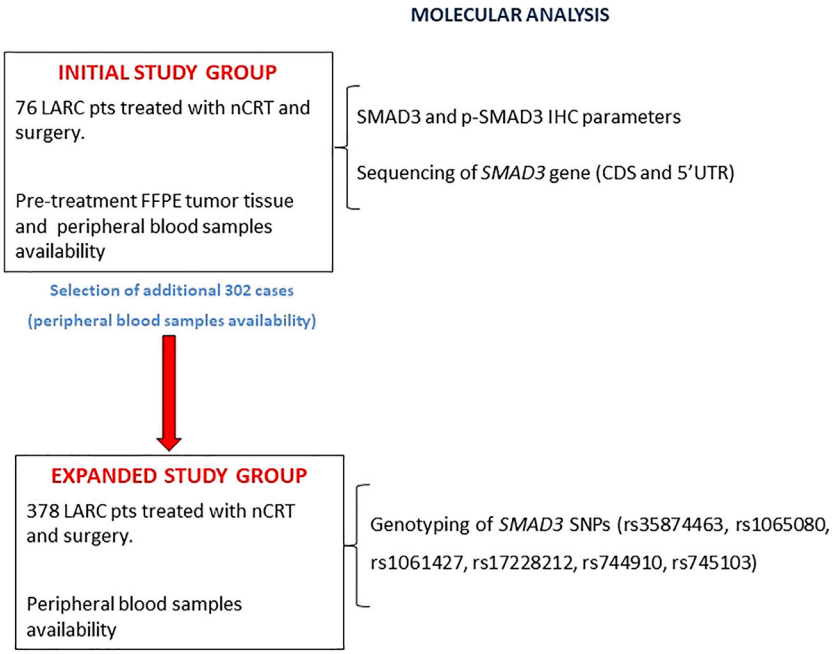

FIGURE 1 | Flowchart of the study design. Abbreviations: CDS, coding sequence; FFPE, formalin-fixed, paraffin-embedded; IHC, immunohistochemistry; LARC, locally advanced rectal cancer; nCRT, neoadjuvant chemoradiotherapy; pts, patients; p-SMAD3, phosphorylated SMAD3; SNP, single nucleotide polymorphism.

randomized clinical trial (Valentini et al., 2019). Six to eight weeks after completion of the chemoradiotherapy program, patients underwent either Total Mesorectal Excision or local excision, depending on clinical response to treatment; adjuvant chemotherapy was optional depending on pathologic stage after surgery.

Pathological tumor staging of the resected specimens was performed according to the American Joint Committee on Cancer TNM classification guidelines (AJCC American Joint Committee on Cancer, 1997). The whole residual tumoral area was sampled for histopathological examination and ypT evaluation, as well as assessment of mesorectal surgical margin status and lymph nodal changes. Pathological response to nCRT of the primary tumor was recorded according to the TRG criteria proposed by Mandard (Mandard et al., 1994). Survival and tumor progression data were obtained by active follow-up.

SMAD3 and p-SMAD3 Protein Expression

Protein expression was assessed by IHC analysis on FFPE samples from tumor biopsies collected during staging colonoscopy prior to nCRT. Three µm-thick sections were serially cut from each FFPE block, one slide was stained with hematoxylin and eosin, and the remaining slides were used for IHC analysis of SMAD3 and p-SMAD3, which were independently reviewed and scored by two trained pathologists blinded to patient clinical information. SMAD3 expression was assessed in the apical part of the cytoplasm using the mouse SMAD3 monoclonal antibody (M01, clone 2C12, and Abnova) (**Figure 2A**). The nuclear phosphorylated form of SMAD3 was evaluated using the rabbit SMAD3 polyclonal antibody (phospho S423/425, Abnova) (**Figure 2B**).

SMAD3 and p-SMAD3 protein expressions were assessed based on IHC staining intensity (0, absent; 1, weak; 3, moderate; 4, strong) and cellularity (from 0 to 100%), which was defined as the percentage of positively stained tumor cells on the total number of visible tumor cells. Staining intensity and cellularity were then combined into an H-score ranging from 0 to 3, as previously described (Huang et al., 2015).

SMAD3 Sequencing

Genomic DNA was extracted from peripheral blood samples using the automated extractor BioRobot EZ1 ("EZ1 DNA Blood Kit 350 µl" kit; Qiagen).

Sequencing of the *SMAD3* gene (ENSG00000166949; reference transcript ENST00000327367.9) was performed using the Sanger method. The assay was designed to map the coding region of the nine exons, their adjacent splice junctions (20 bases upstream and downstream of the exon), and the 5′-untranslated region (approximately 70 bases upstream the start codon AUG). PCR primers were selected using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). PCR reactions were performed in an Eppendorf Mastercycler gradient, using TaqGold DNA Polymerase (ThermoFisher-Applied Biosystems). Purified reactions were sequenced using the Big Dye Terminator kit (ThermoFisher-Applied Biosystems) on an ABI PRISM 3130 capillary sequencer. Both reverse and forward primers were used to sequence the target regions. Chromatograms were visualized using Chromas software version 2.5 and aligned to the human *SMAD3* reference genome sequence through the T-Coffee Multiple Sequence Alignment Server (http://tcoffee.crg.cat/) to identify genetic variants.

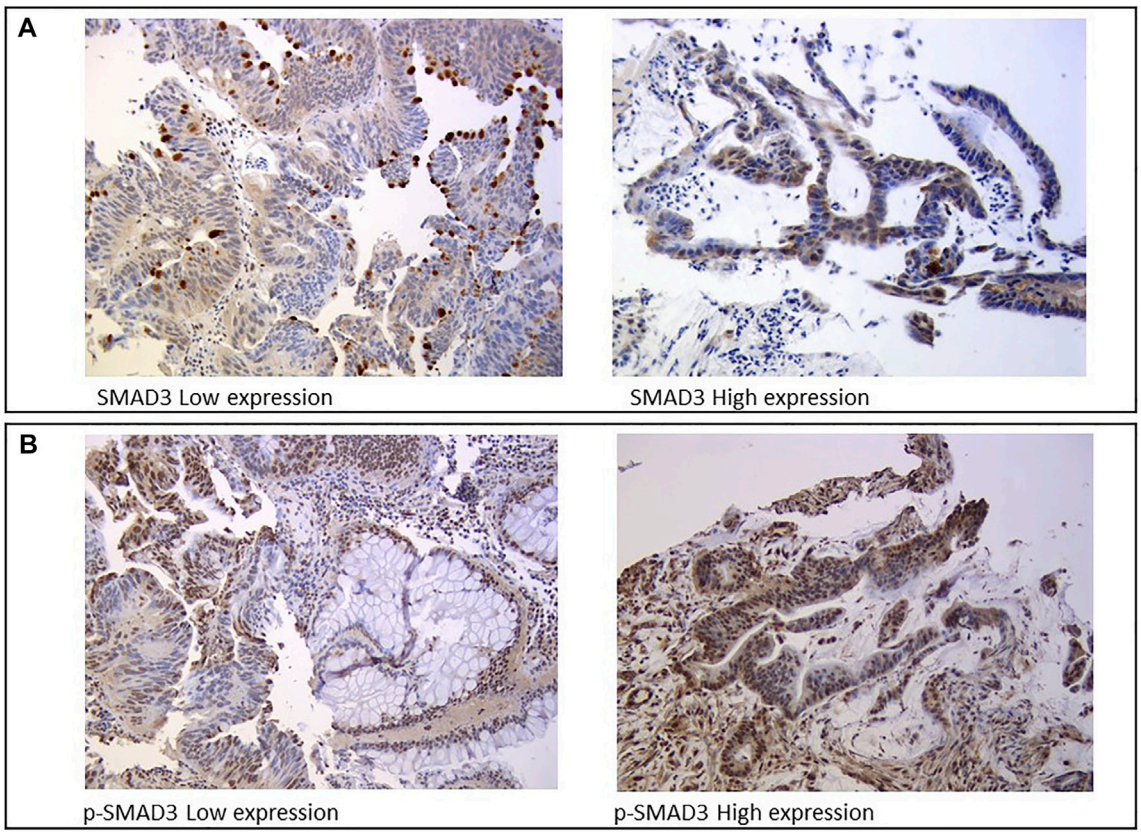

FIGURE 2 | Immunohistochemistry staining of **(A)** SMAD3 and **(B)** and its phosphorylated form (p-SMAD3).

SMAD3 Polymorphisms Genotyping

Six polymorphisms (rs35874463, rs1065080, rs1061427, rs17228212, rs744910, and rs745103) were tested in the expanded study group using a predesigned TaqMan SNP genotyping assay based on the allelic discrimination method using fluorescent probes. All commercial TaqMan assays were purchased from ThermoFisher-Applied Biosystems (https://www.thermofisher.com) and analyses were performed on an ABI 7500 instrument (ThermoFisher-Applied Biosystems) according to the manufacturer's instructions. Positive and negative control samples were included in each analysis. Further details of the analytical procedures are available on request.

Bioinformatic Analysis

Functional prediction of the putative effect of candidate polymorphisms was performed using three online software: HaploReg v4.1 (https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php), RegulomeDB v2.0 (https://regulomedb.org/regulome-search/) and Ensembl's Variant Effect Predictor (VEP) Ensembl release 103, February 2021 (https://www.ensembl.org/info/docs/tools/vep/index.html). For haploblock identification by HaploReg, a stringency of $r^2 = 0.80$ and a 1,000 Genomes Project (EUR) dataset were chosen.

Statistical Analysis

The association between IHC parameters and TRG was investigated in the initial study group: each parameter was dichotomized according to the optimal cut-off from a receiver operating characteristic (ROC) curve discriminating TRG1 from TRG2-4 patients. Odds ratio (OR) for TRG2-4 and corresponding confidence intervals (CI) were calculated using the unconditional logistic regression model, adjusting for sex, age, distance from anal verge, total RT dose, time between RT and surgery, and oxaliplatin use.

The association between *SMAD3* polymorphisms and oncological endpoints was investigated in the expanded study group. Hardy-Weinberg equilibrium was first tested by a permutation procedure based on an exact test. OR for TRG2-4 and the corresponding CI were calculated by an unconditional logistic regression model, considering dominant, recessive, and additive genetic models by combining heterozygous with homozygous genotypes; the best-fitting genetic model was selected according to the Wald chi-square test. Furthermore, the association between *SMAD3* polymorphisms and PFS/OS was evaluated by survival analysis. For each patient, the risk time was calculated from the date of surgery to the date of the event of interest (i.e., progression or death for PFS and death for OS) or the last follow-up, whichever occurred first. The hazard ratio (HR) and corresponding CI were calculated through Cox proportional hazard model, adjusting for study, sex, age, distance from anal verge, total RT dose, time between RT and surgery,

TABLE 1 | Socio-demographic and clinical characteristic of locally advanced rectal cancer patients enrolled in the study.

	Initial study group (*n* = 76)		Expanded study group (*n* = 378)	
	n	(%)	n	(%)
Sex				
Female	28	(36.8)	117	(30.9)
Male	48	(63.2)	261	(69.1)
Age, years (median, range)	62	(24-81)	63	(20-87)
Tumor distance from anal margin (cm)				
<8	54	(71.1)	260	(68.8)
≥8	22	(29.0)	118	(31.2)
Total dose of radiation therapy (Gy)				
50.4	49	(64.5)	278	(73.5)
55.0	27	(35.5)	80	(21.2)
Unknown	0	(0.0)	20	(5.3)
Surgical procedures				
Low anterior resection	42	(55.3)	231	(61.1)
Abdominal perineal resection	9	(11.8)	43	(11.4)
Local excision	17	(22.4)	41	(10.8)
Hartmann's	2	(2.6)	10	(2.6)
Colo-anal anastomosis	0	(0.0)	27	(7.1)
Other	6	(7.9)	23	(6.1)
Unknown	0	(0.0)	3	(0.8)
Preoperative Chemotherapy				
Fluoropyrimidines				
5-Fluorouracil	4	(5.3)	131	(34.7)
Capecitabine	65	(85.5)	205	(54.2)
Unknown	7	(9.2)	42	(11.1)
Association therapy with oxaliplatin				
No	54	(71.1)	284	(75.1)
Yes	22	(29.0)	94	(24.9)
Adjuvant therapy				
Yes	39	(51.3)	191	(50.5)
No	34	(44.7)	162	(42.9)
Unknown	3	(4.0)	25	(6.6)
Tumor Regression grade				
1	24	(31.6)	100	(26.5)
2	11	(14.5)	68	(18.0)
3	36	(47.4)	133	(35.2)
4	5	(6.6)	64	(16.9)
5	0	(0.0)	13	(3.4)

and use of oxaliplatin. Statistical significance was claimed for $p < 0.05$ (two-tailed).

RESULTS

SMAD3 and p-SMAD3 Tumor Expression and Tumor Response (TRG)

The main demographic, clinical and pathological characteristics of the initial study group ($n = 76$) are summarized in **Table 1**.

SMAD3 parameters were significantly associated with TRG (**Table 2**). SMAD3 cellularity above 55% or moderate/high immunostaining intensity were both associated with higher risk of TRG2-4 (OR = 10.36 CI:2.81-38.18, and OR = 5.20 CI:1.70-15.88, respectively). These results were confirmed when H-score (cut-off = 1) (OR = 9.84 CI:2.75-34.40) was taken into account. The association with tumor response was not significant for pSMAD3 cellularity (cut-off = 85%) or immunostaining intensity, while it became significant when H-score (cut-off = 2) was considered (OR = 4.23 CI:1.31-13.64) (**Table 2**).

A similar association trend, though not significant, was observed when focusing on the subgroup of patients ($n = 22$) treated with a combination chemotherapy including fluoropyrimidines and oxaliplatin (data not shown).

SMAD3 Genetic Sequencing

A total of 1,619bp in the *SMAD3* gene was sequenced by Sanger method. Four genetic variants (2.47 variants/kbp) (i.e., rs35874463, rs1065080, rs117185005, and rs1061427) were detected in 76 evaluable patients (**Supplementary Figure S1**). Their main characteristics and genotype frequencies are listed in **Table 3** and are consistent with the 1,000 Genomes Project data for the European population. Further details have been reported below in the results of the bioinformatic analysis.

SMAD3 Polymorphisms and Tumor Response and Prognosis

The main demographic, clinical and pathological characteristics of the expanded study population ($n = 378$) are listed in **Table 1**.

TABLE 2 | Association between immunohistochemistry (IHC) parameters and tumor regression grade (TRG), in the initial study group (*n* = 76). Associations with P-value <0.05 are in bold.

IHC parameters[a]	TRG1		TRG2-4		OR (95% CI)[b]
	n	(%)	n	(%)	
SMAD3 cellularity					
≤55%	19	(50.0)	19	(50.0)	**Reference**
>55%	5	(13.2)	33	(86.8)	**10.36 (2.81-38.18)**
			***p* = 0.0011[c]**		***p* = 0.0004[d]**
SMAD3 intensity					
Low	14	(51.9)	13	(48.1)	**Reference**
Moderate/High	10	(20.4)	39	(79.6)	**5.20 (1.70-15.88)**
			***p* = 0.0090[c]**		***p* = 0.0038[d]**
SMAD3 score (Huang[f])					
0	14	(60.9)	9	(39.1)	**Reference**
≥1	10	(18.9)	43	(81.1)	**9.84 (2.75-34.40)**
			***p* = 0.0009[c]**		***p* = 0.0004[d]**
p-SMAD3 cellularity					
<85%	19	(34.5)	36	(65.5)	Reference
≥85%	5	(23.8)	16	(76.2)	1.84 (0.54-6.31)
			$p = 0.4212^{c}$		$p = 0.3304^{d}$
p-SMAD3 intensity					
Low-Moderate	12	(41.4)	17	(58.6)	Reference
High	12	(25.5)	35	(74.5)	2.56 (0.85-7.76)
			$p = 0.2046^{c}$		$p = 0.0957^{d}$
p-SMAD3 score (Huang[f])					
≤2	17	(44.7)	21	(55.3)	**Reference**
>2	7	(18.4)	31	(81.6)	**4.23 (1.31-13.64)**
			***p* = 0.0253[c]**		***p* = 0.0158[d]**

Abbreviations: 95% CI, 95% confidence interval; OR, odds ratio; p-SMAD3, phosphorylated SMAD3.

[a]Optimal cut-off was calculated by ROC analysis.

[b]Estimated using an unconditional logistic regression model adjusting for sex, age (<60, 60-69, and ≥70 years), distance from anal verge (<5, 5-6, and ≥7 cm), total radiotherapy dose (<55.0, 55.0 Gy), time between radiotherapy and surgery (<60, ≥60 days), and oxaliplatin use (no, yes).

[c]Fisher's exact test.

[d]Wald $\chi 2$ test.

[f]"0", complete absence of staining; "1" weak staining in more than 50% of positive cells or with moderate staining in less than 50% of positive cells; "2", moderate positive staining in more than 50% of cells, or with strong staining in less than 50 %t of cells; "3", strong staining in more than 50% of cells (according to Huang et al., 2015).

TABLE 3 | The main features and genotype frequency of the identified *SMAD3* polymorphisms.

Rs ID	Genomic position (GRCh38)	Nucleotide change	Typology	Location	Amino acid change	MAF, EUR/TSI[a]	Genotypes frequency in study group (*n* = 76)			
							AA	Aa	aa	MAF
rs1065080	chr15:67164997	CT**A**/CT**G**	synonymous	Exon 2	Leu103Leu	A: 0.139/ 0.103	GG: 0.800 (60)	GA: 0.173 (13)	AA: 0.027 (2)	A: 0.113
rs35874463	chr15:67165360	ATC/GTC	missense	Exon 3	Ile170Val	G: 0.053/ 0.037	AA: 0.920 (69)	AG: 0.080 (6)	GG: 0	G: 0.04
rs117185005	chr15:67181452	AT**C**/AT**T**	synonymous	Exon 6	Ile290Ile	T: 0.024/ 0.014	CC: 0.973 (72)	CT: 0.027 (2)	TT: (0)	T: 0.014
rs1061427	chr15:67066140	CCGC**G**CG/ CCGC**A**CG	5′ regulatory region	5′UTR	—	A: 0.246/ 0.206	GG: 0.635 (47)	GA: 0.257 (19)	AA: 0.108 (8)	A: 0.236

Abbreviations: MAF, minor allele frequency.

[a]1000 Genomes Project Phase 3 (32), European (EUR) and Toscany in Italy (TSI) population.

Three variants identified by Sanger sequencing (i.e., rs35874463, rs1065080, and rs1061427) and the three intronic variants (i.e., rs17228212, rs744910, and rs745103) studied in our previous work (Dreussi et al., 2016) (**Supplementary Figure S1**) were tested. The rs117185005 detected by sequencing was not analyzed in the expanded study group due to its very low frequency.

The predictive effect we previously observed for rs744910 and rs745103 on TRG (Dreussi et al., 2016) was validated (**Table 4**). Patients carrying the rs744910-GG (p = 0.0176) or rs745103-GG (p = 0.0093) genotype had a significantly reduced risk of poor response (TRG≥2). Only a trend was observed for rs17228212, with the C-allele being associated with an increased risk of getting TRG2-5

TABLE 4 | (A) Odds ratio (OR) and 95% confidence interval (CI) for tumor regression grade (TRG) and (B) Hazard ratios (HR) and 95% CI for progression-free survival (PFS) and overall survival (OS) in the expanded study group (n = 378) according to gene polymorphisms (SNP). Associations with P-value < 0.05 are in bold.

(A)

SNP	Base change	Genotype frequency						Genetic model	OR (95% CI)[a]	p-value
		TRG1			TRG2-5					
		AA	Aa	Aa	AA	Aa	aa			
rs17228212	T > C	0.632	0.327	0.040	0.544	0.365	0.091	Additive	1.42 (0.95–2.12)	0.0848
rs744910	A > G	0.202	0.475	0.323	0.256	0.542	0.202	Recessive	0.52 (0.31–0.89)	**0.0176**
rs745103	A > G	0.222	0.475	0.303	0.272	0.544	0.184	Recessive	0.48 (0.28–0.83)	**0.0093**
rs1065080	G > A	0.765	0.214	0.020	0.803	0.182	0.015	Recessive	0.71 (0.12–4.12)	0.7009
rs35874463	A > G	0.969	0.031	0.000	0.919	0.078	0.004	Dominant	2.74 (0.79–9.52)	0.1138
rs1061427	G > A	0.563	0.354	0.083	0.629	0.309	0.062	Additive	0.79 (0.54–1.16)	0.2267

(B)

SNPs	Base change	PFS			OS		
		Genetic model	HR (95% CI)[a]	p-value	Genetic model	HR (95% CI)[a]	p-value
rs17228212	T > C	Recessive	0.75 (0.30–1.88)	0.5365	Recessive	0.57 (0.18–1.84)	0.3486
rs744910	A > G	Recessive	0.66 (0.37–1.18)	0.1614	Recessive	0.86 (0.45–1.45)	0.6536
rs745103	A > G	Additive	0.75 (0.54–1.05)	0.0944	Additive	0.65 (0.44–0.96)	**0.0307**
rs1065080	G > A	Dominant	1.19 (0.71–2.01)	0.5092	Additive	1.28 (0.74–2.19)	0.3762
rs35874463	A > G	Dominant	1.00 (0.39–2.51)	0.9919	Dominant	0.99 (0.35–2.79)	0.9838
rs1061427	G > A	Additive	0.70 (0.47–1.03)	0.0693	Additive	0.71 (0.45–1.12)	0.1418

[a]*Estimated from Cox proportional hazards model, adjusted for sex, age (<60, 60-69, and ≥70 years), distance from anal verge (<5, 5-6, and ≥7 cm), total radiotherapy dose (<55.0, 55.0 Gy), time between radiotherapy and surgery (<60, ≥60 days), and oxaliplatin use (no, yes).*

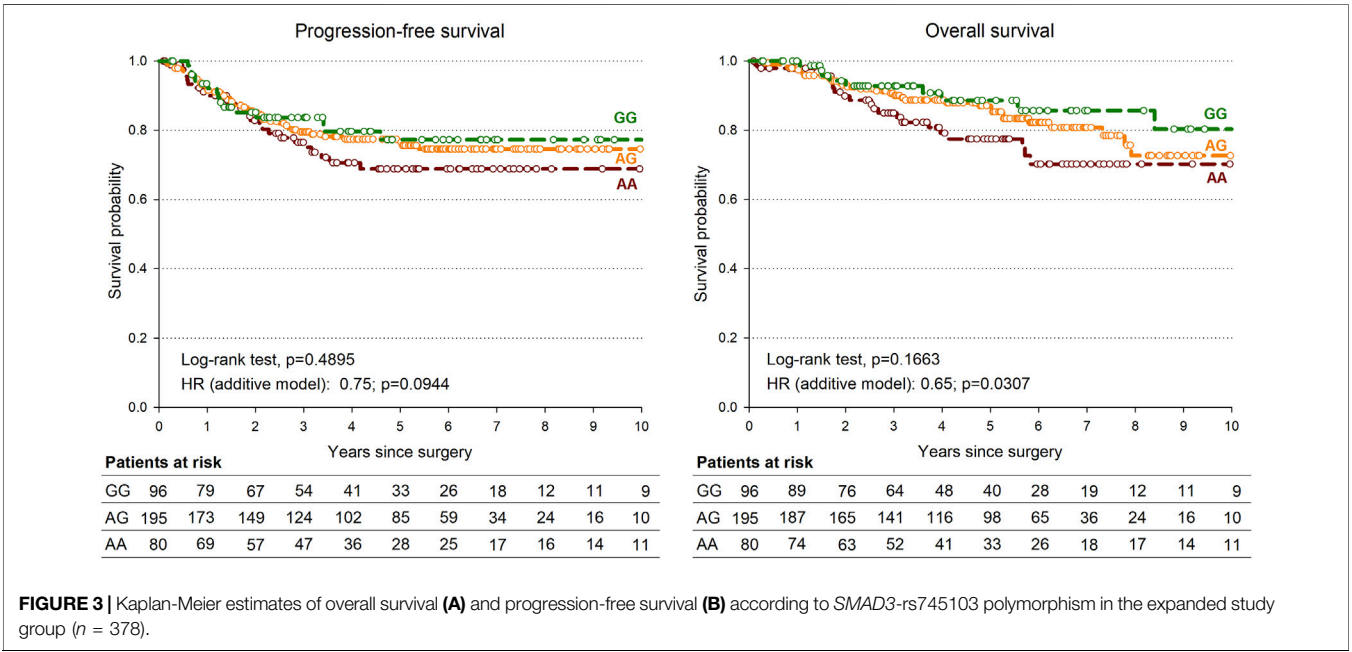

FIGURE 3 | Kaplan-Meier estimates of overall survival **(A)** and progression-free survival **(B)** according to *SMAD3*-rs745103 polymorphism in the expanded study group (n = 378).

(p = 0.0848), consistent with our previous results (Dreussi et al., 2016).

During a median follow-up of 56 months, 62 patients (16.4%) died, while 89 patients (23.5%) had disease recurrence. The five-years OS was 84.8% (80.2–88.4%), while the 5-years PFS was 74.8% (69.6–79.3%).

Only the rs745103 variant was significantly associated with patient survival (**Table 4**), with the G-allele associated with longer OS (p = 0.0307). A non-significant association trend was observed for longer PFS (p = 0.0944) (**Table 4**). The log-rank test was not significant, but the Kaplan-Meier curves presented in **Figure 3** showed a trend consistent with the

TABLE 5 | Combined predictive effect of SMAD3 tumor expression, SMAD3-rs745103 and SMAD3-rs744910 on tumor response. Associations with *P*-value < 0.05 are in bold.

IHC parameters	Risk	Expression Level[a] (*n* = 76)	OR (95% CI)[b]	Expression Level[a] and rs745103 (*n* = 74)	OR (95% CI)[b]	Expression Level[a], rs745103, and rs744910 (*n* = 74)	OR (95% CI)[b]
SMAD3 cellularity	Low High	≤55% >55%	**Reference** **10.36 (2.81-38.18)** ***p* = 0.0004[c]**	≤55% or GG >55% and AA/AG	**Reference** **13.45 (3.14-57.57)** ***p* = 0.0005[c]**	≤55% or GG or GG >55% and AA/AG and AA/AG	**Reference** **10.15 (2.39-43.19)** ***p* = 0.0017[c]**
SMAD3 intensity	Low High	Low Moderate/High	**Reference** **5.20 (1.70-15.88)** ***p* = 0.0038[c]**	Low or GG Moderate/High and AA/AG	**Reference** **8.17 (2.35-28.40)** ***p* = 0.0010[c]**	Low or GG or GG Moderate/High and AA/AG and AA/AG	**Reference** **5.83 (1.74-19.53)** ***p* = 0.0043[c]**
SMAD3 score (Huang)[d]	Low High	0 ≥1	**Reference** **9.84 (2.75-34.40)** ***p* = 0.0004[c]**	0 or GG ≥1 and AA/AG	**Reference** **11.41 (3.19-40.79)** ***p* = 0.0002[c]**	0 or GG or GG ≥1 and AA/AG and AA/AG	**Reference** **6.85 (2.07-22.67)** ***p* = 0.0016[c]**
p-SMAD3 cellularity		<85% ≥85%	Reference 1.84 (0.54-6.31) *p* = 0.3304[c]	<85% or GG ≥85% and AA/AG	Reference 1.98 (0.52-7.56) *p* = 0.3195[c]	<85% or GG or GG ≥85% and AA/AG and AA/AG	Reference 1.53 (0.39-5.97) *p* = 0.5370[c]
p-SMAD3 intensity	Low High	Low-Moderate High	Reference 2.56 (0.85-7.76) *p* = 0.0957[c]	Low-Moderate or GG High and AA/AG	Reference 2.87 (0.90-9.09) *p* = 0.0739[c]	Low-Moderate or GG or GG High and AA/AG and AA/AG	Reference 2.06 (0.68-6.28) *p* = 0.2033[c]
p-SMAD3 score (Huang)[d]	Low High	≤2 >2	**Reference** **4.23 (1.31-13.64)** ***p* = 0.0158[c]**	≤2 or GG >2 and AA/AG	**Reference** **4.90 (1.27-18.99)** ***p* = 0.0214[c]**	≤2 or GG or GG >2 and AA/AG and AA/AG	Reference 3.39 (0.92-12.51) *p* = 0.0670[c]

Abbreviations: 95% CI, 95% confidence interval; OR, odds ratio; p-SMAD3, phosphorylated SMAD3.

[a]*Optimal cut-off was calculated by ROC analysis.*

[b]*ORs, for TRG2-4 vs TRG1 were estimated from an unconditional logistic regression model adjusting for sex, age (<60, 60-69, and ≥70 years), distance from anal verge (<5, 5-6, and ≥7 cm), total radiotherapy dose (<55.0, 55.0 Gy), time between radiotherapy and surgery (<60, ≥60 days), and oxaliplatin use (no, yes).*

[c]*Wald $\chi 2$ test.*

[d]*"0", complete absence of staining; "1" weak staining in more than 50% of positive cells or with moderate staining in less than 50% of positive cells; "2", moderate positive staining in more than 50% of cells, or with strong staining in less than 50 %t of cells; "3", strong staining in more than 50% of cells (according to Huang et al., 2015).*

results of the Cox analysis. The effect of rs745103 on OS and PFS was consistent with that observed for tumor response to treatment.

Combined Predictive Effect of SMAD3 Tumor Expression, *SMAD3*-rs745103 and *SMAD3*-rs744910, on Tumor Response

Patients who were part of the initial study group characterized for both SMAD3 tumor expression and *SMAD3*-rs745103 and rs744910 genotypes (74/76 patients) were considered for the combined analysis.

We started splitting patients into high- and low-risk groups incomplete response based on tumor IHC expression parameters (**Table 5**). We then performed a stepwise forward regression analysis by including patient's genotype for *SMAD3*-rs744910 and *SMAD3*-rs745103 in the model. Patients carrying both the IHC and genetic unfavorable features were considered at "high-risk" and compared with the others. Inclusion of *SMAD3*-rs745103 information in the IHC score improved identification of patients at increased risk (i.e., OR) of not completely responding to treatment (TRG2-4) compared to using only IHC features. The addition of *SMAD3*-rs744910 information did not further improve the model's capacity to identify patients at high-risk of poor response.

Results of the Bioinformatic Analysis and Association of polymorphisms With Tumor Expression

The *in silico* prediction of the possible functional effect of the four genetic variants identified by Sanger sequencing is shown in **Supplementary Table S1A**. One missense polymorphism was detected by Sanger Sequencing, the rs35874463 (Ile170Val, and exon 3). This variant is located in the linker region of SMAD3, that is required for TGFbeta-mediated transcriptional activity and acts synergistically with the MH2 domain. Despite its location in a critical region, all *in silico* tools predicted that rs35874463 has a tolerated or benign im-pact on protein functionality. However, an impact of this variant on SMAD3 expression through alteration of transcriptional regulation or epigenetic control could not be ruled out (RegulomeDb score of 2b). Two synonymous polymorphisms were found, the rs1065080 (Leu103Leu, exon 2), and rs117185005 (Ile290Ile, exon 6). Rs1065080 is located in the MH1 domain required for DNA binding; MH1 domain also binds zinc ions, which are necessary for its function. Rs1065080 was predicted to have a regulatory effect by potentially altering the binding site for some transcriptional regulators including the CCCTC-binding factor (CTCF). Rs117185005 is located in the MSH2 domain, which is required for both homomeric and heteromeric interactions, and transcriptional regulation and nuclear import. This

polymorphism, situated one base upstream of the end of exon 6, may alter the splicing pattern of the gene. Rs117185005 was also found to change the consensus motif for the CRCF regulator. A variant was identified in the 5′UTR region, the rs1061427. This polymorphism is thought to have a moderate effect on gene expression by broadly altering regulatory chromatin states and the consensus motif for transcriptional factors.

A summary of the available in silico functional data for the *SMAD3* intronic variants (rs745103, rs744910, and rs17228212) is presented in **Supplementary Table S1B**. The rs745103 polymorphism could have a moderate impact on gene functionality and/or expression, as it broadly alters regulatory chromatin states (i.e., 3 promoter histone marks, 21 enhancer histone marks, 16 DNAse items), proteins bound (i.e., 1 hit) and motifs (i.e., 4 motifs changed), according to the prediction of the HaploReg tool. This effect was summarized by a RegulomeDB rank score equal to 4 (i.e., transcription factors binding + DNase peak data) and a probability score equal to 0.60906. The VEP tool showed a CADD score of 0.780 and a conservation GERPP score of −3.18. HaploReg detected no other polymorphisms in the *SMAD3*-rs745103 haploblock ($r^2 > 0.8$). Similar results were obtained for rs744910 and rs17228212. Rs744910 could potentially affect chromatin architecture and DNA methylation pattern (10 enhancer histone marks), and ultimately DNA accessibility for gene transcription. This variant also resulted in DNAse hypersensitivity (7 DNAse items) and is located in a transcriptional binding element (2 altered motifs) with a result-ing impact on the regulation of protein expression (NHGRI-EBI GWAS and eQTL hits). These effects were globally summarized with a RegulomeDB rank score equal to 3a (i.e., transcription factors binding, + any motif + DNase peak data) and a probability score equal to 0.85505. The VEP tool indicated a CADD score of 5.153 and a conservation GERPP score of 0.22. Rs17228212 was predicted to have an impact on *SMAD3* gene functionality and/or expression by potentially altering the chromatin architecture, nucleosomal positioning, and DNA methylation pattern (i.e., 2 promoter histone marks, 15 enhancer histone marks, and 6 DNAse items). Furthermore, this polymorphism is located in a transcriptional binding element (2 altered motifs) with a consequent effect on protein ex-pression (NHGRI-EBI GWAS and eQTL hits). RegulomeDB provided a rank score equal to 3a (i.e., transcription factors binding, + matched transcription factors motif + matched DNase Footprint + DNase peak) and a probability score equal to 0.47489. The VEP tool indicated a CADD score of 4.384 and a conservation GERPP score of -3.02. Use of HaploReg revealed that 2 and 10 additional genetic variants are tagged by *SMAD3*-rs744910 and rs17228212 (r2>0.8) respectively.

The association between these seven polymorphisms and the baseline expression level of SMAD3 in tumor tissue was tested, but no statistically significant association was found (data not shown).

DISCUSSION

Selection for innovative intensified nCRT programs, such as TNT, of LARC patients at high risk for poor clinical outcome is currently based solely on clinical parameters. The integration of new predictive markers could improve existing clinical risk algorithms to achieve a precision medicine approach.

Our most important finding was the identification of some host *SMAD3* genetic polymorphisms (rs744910, rs745103) and SMAD3 protein expression in pre-treatment tumor tissue as predictive markers of response to neoadjuvant treatment in LARC. For the first time, we demonstrated that the combination of SMAD3 tumor expression level with host *SMAD3*-rs745103 genotype could identify smaller groups of patients at significantly higher risk of not responding to nCRT treatment compared to individual molecular parameters. This preliminary result highlights the advantage of integrating multiple molecular markers (host- and tumor-related) for predicting the likelihood of response to treatment. It also suggests that they could independently account for the constitutive (host) and inducible (tumor) SMAD3 effect on the treatment outcome.

SMAD3 is a key transcription factor in the TGF-β signaling pathway and could contribute to determine the immunosuppressive phenotype associated with TGF-β activation and to counteract the ability of radiotherapy to induce an effective antitumor immune response (Vanpouille-Box et al., 2015; Tauriello and Batlle, 2016; Wennerberg et al., 2017; Farhood et al., 2020; Liu et al., 2021). SMAD3 could also reduce DNA damage response and promote cell survival, invasion, migration, and epithelial-mesenchymal transition (Choi et al., 2016; Lee et al., 2016; Li et al., 2017; Jiang et al., 2019; Niu et al., 2020). In the present work, high SMAD3 expression was associated with poorer response to nCRT. Accordingly, recent *in vitro* studies have reported that silencing of SMAD3 resulted in increased sensitivity to radiotherapy (Jiang et al., 2019; Niu et al., 2020) and that higher SMAD3 expression is associated with shorter survival and higher risk of recurrence after radiotherapy (Niu et al., 2020). Huang and colleagues (Huang et al., 2015), reported that high preoperative p-SMAD3 tumor expression could be a potential predictor of poor response to nCRT in LARC patients.

SMAD3-rs745103 and rs744910 proved to be a predictive marker of poor response to treatment, confirming in this larger and prospective population our previous pharmacogenetic analyses (Dreussi et al., 2016). Furthermore, a prognostic impact of *SMAD3*-rs745103 on OS and PFS was highlighted. It could be hypothesized that the genetic variant might affect the constitutive expression/activity of SMAD3, which in turn modifies the TGF-β-related transcriptional response and influences the antitumor efficacy of nCRT. *SMAD3*-rs745103 is an intronic variant and our bioinformatic prediction analysis indicated that it could moderately affect gene functionality and/or expression.

Beyond the effect on radiotherapy, it should be noted that the TGF-β/SMAD3 pathway was also reported to be involved in the mechanism of resistance to chemotherapeutics, including 5-FU, in colorectal cancer by modulating TGF-β downstream effectors with pro-proliferative, and pro-metastatic and anti-apoptotic effects (Moon et al., 2015; Romano et al., 2016). On the other hand, suppression of the TGF-β/SMAD3 cascade was shown to inhibit 5-FU-induced gene transcription and restore the sensitivity of 5-FU chemoresistant cells (Romano et al., 2016).

Based on our gene sequencing results, only four variants were identified, corresponding to a variation rate of 2.47 variants/kbp. This is significantly lower compared to the ExAc project data (1 variant/8bp within exome intervals) (Lek et al., 2016). All four

variants were predicted by *in silico* analysis to have minimal impact on SMAD3 functionality and/or expression. *SMAD3* was found to be a highly conserved gene, consistent with its basic biological role, and including regulation of immune response.

The study was not devoid of limitations. First, it was performed on a large series of prospectively enrolled LARC patients, diagnostic biopsy was only available for a subset to allow an integrated molecular approach focusing on both host and tumor. In any case, the rarity of the pathology and the novelty of the results warrant attention, although further validation in independent groups of patients is needed. Second, even if preliminary *in silico* results support a possible phenotypic impact, the precise functional significance of *SMAD3*-rs745103 is still unknown, and confirmatory functional analyses are required. Third, considering the effect of SMAD3 on the tumor aggressiveness phenotype, regardless the impact on chemoradiotherapy (Liu et al., 2015; Romano et al., 2016; Tauriello and Batlle, 2016), a non-treated control group would have been helpful to clarify the contribution of SMAD3 on tumor response to treatment.

This study demonstrates that tumor SMAD3 protein expression and germline genotype could predict response to fluoropyrimidine-based nCRT. Its findings suggest the relevance of the TGF-β/SMAD3 pathway in determining the success of nCRT and may help elucidate the molecular mechanisms underlying response to chemoradiotherapy. The crucial role of TGFβ in determining the sensitivity to radiation therapy is well-recognized and the inhibition of TGFβ signaling by emerging pharmacological interventions (i.e., receptor kinase inhibitors, TGFβ-directed monoclonal antibodies, TGFβ ligand traps, antisense oligonucleotides, and vaccine-based approaches) has been reported by pre-clinical and clinical studies to reverse radioresistance of irradiated cells and boost the immune system against cancer (Formenti et al., 2018; Farhood et al., 2020; Chen et al., 2021; Kim et al., 2021; Liu et al., 2021). The present study identified SMAD3 as an additional key player in the TGFβ-related molecular cascade (Millet and Zhang, 2007) that determines the response to nCRT. It could be hypothesized that high SMAD3 expression enhances the activation of TGFβ-related genes with proliferative, anti-apoptotic, and immune suppressive effects, globally increasing the risk of not responding to nCRT. Hence, SMAD3 could be a further druggable target and SMAD3 blocking by pharmacological strategies could represent an additional promising approach to improve the tumor radiosensitivity. Preliminary data supporting the effectiveness of targeting SMAD3 for enhancing the response to radiotherapy have been published (Akhurst and Hata, 2012; Rodriguez-Ruiz et al., 2019). However, the usefulness of those pharmacological approaches should be better investigated through appropriate clinical trials prior to enter into clinical practice.

In conclusion, the significant role of SMAD3 in identifying LARC patients who are at higher risk of not responding to nCRT treatment may be critical to improve treatment strategies. SMAD3 status in LARC at diagnosis could be considered for integration into the already known clinical risk algorithms to identify patients at high risk of poor response to the combination of chemotherapy with radiotherapy. Those patients might therefore be selected for alternative personalized neoadjuvant treatments including currently available schemes, as TNT, and with intensification of preoperative chemotherapy.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because it is currently in use for additional analyses. Requests to access the datasets should be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Committee of the "Ospedale Universitario di Padova". The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization, EM and EC; validation, EM and EC; formal analysis, JP; investigation, VC, SM, CF, ED, and RR; resources, VC, RI, CB, SP, AP, and EP; data curation, JP; writing—original draft preparation, EM; writing—review and editing, EC; visualization, EM; supervision, GT; project administration, EM and EC; funding acquisition, GT All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.778781/full#supplementary-material

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