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Recent advances in enhancing chemotherapeutic efficacy of colorectal cancer

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Recent advances in enhancing chemotherapeutic efficacy of colorectal cancer

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REVIEW
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Role of Ginseng, Quercetin, and Tea in Enhancing Chemotherapeutic Efficacy of Colorectal Cancer

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As the most common gastrointestinal malignancy, colorectal cancer (CRC) remains a leading cause of cancer death worldwide. Although multimodal chemotherapy has effectively improved the prognosis of patients with CRC in recent years, severe chemotherapy-associated side effects and chemoresistance still greatly impair efficacy and limit its clinical application. In response to these challenges, an increasing number of traditional Chinese medicines have been used as synergistic agents for CRC administration. In particular, ginseng, quercetin, and tea, three common dietary supplements, have been shown to possess the potent capacity of enhancing the sensitivity of various chemotherapy drugs and reducing their side effects. Ginseng, also named "the king of herbs", contains a great variety of anti-cancer compounds, among which ginsenosides are the most abundant and major research objects of various anti-tumor studies. Quercetin is a flavonoid and has been detected in multiple common foods, which possesses a wide range of pharmacological properties, especially with stronger anti-cancer and anti-inflammatory effects. As one of the most consumed beverages, tea has become particularly prevalent in both West and East in recent years. Tea and its major extracts, such as catechins and various constituents, were capable of significantly improving life quality and exerting anti-cancer effects both in *vivo* and in *vitro*. In this review, we mainly focused on the adjunctive effects of the three herbs and their constituents on the chemotherapy process of CRC.

Keywords: colorectal cancer, ginseng, quercetin, tea, chemotherapy, chemoresistance

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer globally and one of the leading causes of health burden on society (1). The latest epidemiological data show that the incidence of CRC is rapidly increasing year by year, and the number of young patients aged 20–40 years old has increased quickly (2). For early-stage CRC, surgical resection of the primary tumor is the main treatment method, and adjuvant chemotherapy can prolong the survival times of patients (3, 4). In terms of advanced CRC or metastatic CRC, the survival rate is less than 10%, and the primary treatment strategies include radiotherapy and chemotherapy. CRC represents a heterogeneous disease with distinct disease mechanisms and prognoses.

It has been confirmed that multiple factors were involved in CRC development and progression, such as genetic alterations, gut microbiota, chronic inflammation, environmental influence, and others (1, 3). However, the exact mechanisms underlying the onset of colorectal cancer are still unknown. With the development of precision medicine and personalized medicine, chemotherapy plays an increasingly important role in CRC administration. Especially for advanced patients, chemotherapy offers the only possibility of a cure. However, the clinical application of chemotherapeutic regimens is mainly limited by their side effects and toxicity. Therefore, urgent research is needed to discover more adjuvant chemotherapy compounds to enhance the tumoricidal effects at low doses (5).

Over the last decades, traditional herbal medicines have been widely utilized for modern drug development. More and more studies have indicated that a daily intake of these herbal products could improve the life quality of patients (6, 7). Notably, a growing body of research suggests that traditional Chinese herbal can be regarded as effective adjuvant chemotherapy agents for improving the efficacy of cancer chemotherapy. In this review, ginseng, quercetin, and tea are the main research objects. The reasons why we have focused on these herbs are described as follows. First, these herbs are the most widely used traditional herbal medicines both in the East and West, and their beneficial effects have been extensively advertised. Another reason that has led us to pick these phytochemicals is that they are common in dietary supplements and have been confirmed to improve the life quality of hosts. The last and most important reason is that their multiple pharmacological properties, such as anti-oxidant, anti-inflammatory, and anti-cancer properties, have been widely recognized. Based on the above findings, we reviewed a large body of literature and concluded that all these herbs could effectively improve the effects of CRC chemotherapy. It should be noted that the chemical and pharmacological properties of ginseng, quercetin, and tea are completely independent of each other. Therefore, to avoid confusion, we discussed their properties and functions in great detail separately, as shown in **Figure 1**.

CHEMOTHERAPY OF CRC

With improvements in CRC treatment, multiple chemotherapeutic agents have been used in routine clinical practice; the chemotherapeutic agents, mainly including 5-fluorouracil (5-FU), irinotecan, oxaliplatin, and capecitabine, can be used either alone or in combination with each other, (8). Among them, 5-FU has historically been considered the foundation of the therapy for CRC, which has been used in clinical treatment for more than 60 years (9). An increasing number of clinical trials have demonstrated that 5-FU administered alone or in combination with other chemotherapeutic agents can significantly improve the survival rate of patients with CRC (10, 11). The response rate of 5-FU administered alone is only approximately 10–15% (12). However, combining 5-FU with other chemotherapeutic agents can effectively enhance curative effects and has been regarded as the first routine clinical practice. For example, leucovorin, a folinic acid derivative, can enhance the therapeutic response rate to 37% by suppressing the activation of thymidylate synthase (13). However, like many other common chemotherapy drugs, 5-FU also has many side effects, mainly including leukopenia, nausea, vomiting, hematopoietic depression, bone marrow suppression, neurotoxicity, and cardiotoxicity (14). In particular, leukopenia has been reckoned major dose-limiting toxicity of 5-FU administration occurs in approximately 93% of patients (15). In recent years, with a deepening understanding of drug properties, 5-FU has also shown stronger anti-cancer efficacy in clinical combinations with new-generation chemotherapy drugs. In conclusion, although 5-FU is an essential agent for treating both advanced and early-stage patients with CRC, its side effects cannot be ignored. Therefore, it is necessary to overcome these therapeutic challenges.

Capecitabine, an oral 5-FU prodrug that has been used in treating CRC for 20 years, can be enzymatically transformed into 5-FU at colorectal tumor sites after oral administration (16). Moreover, it has been demonstrated that even along administration of capecitabine exerts stronger chemotherapy effects and lower incidence of side effects than combined administration of 5-FU and leucovorin (17). However, capecitabine also has deficiencies, in particular the significantly increased incidence of the hand-foot syndrome and hyperbilirubinemia (18). As the most commonly used chemotherapy drugs for various malignant diseases, platinum-based agents have also been used in CRC treatment. Oxaliplatin, a third-generation platinum anti-cancer agent, is also a novel first-line treatment for metastatic CRC. It can inhibit the growth of tumor cells by inducing the formation of platinum-DNA adducts and eliciting a DNA damage response (19). The typical side effects include hematologic toxicity, gastrointestinal symptoms, and peripheral neuropathy (20).

Irinotecan is approved as second-line therapy for treating advanced/metastatic CRC, especially for patients who do not respond to the first-line 5-FU therapy (21). Its active metabolite SN-38, a camptothecin-based agent, can promote DNA damage and tumor cell apoptosis by binding with topoisomerase I, an important mediator of DNA transcription (22). The most common side effects of irinotecan treatment include myelosuppression, delayed-type diarrhea, cholinergic syndrome, vomiting, constipation, and neutropenia (23, 24). As shown above, each chemotherapeutic agent has its own properties and side effects. Over the past decade, sequential combination therapy with multiple chemotherapeutics has been the most standard chemotherapeutic treatment for CRC management; this therapy can promote the synergy of different agents and improve chemotherapy resistance using different action mechanisms (8, 25). For instance, the combined administration of oxaliplatin and irinotecan can be used as a salvage therapy for patients failing to respond to single-agent 5-FU treatment and is a first-line sequential treatment option for advanced CRC (26, 27). Although

Abbreviations: CRC, colorectal cancer; 5-FU, 5-fluorouracil; PD, panaxadiol; PPD, protopanaxadiol; EGCG, (−)-epigallocatechin-3-gallate; EGC, (−)-epigallocatechin; ECG, (−)-epicatechin-3-gallate.

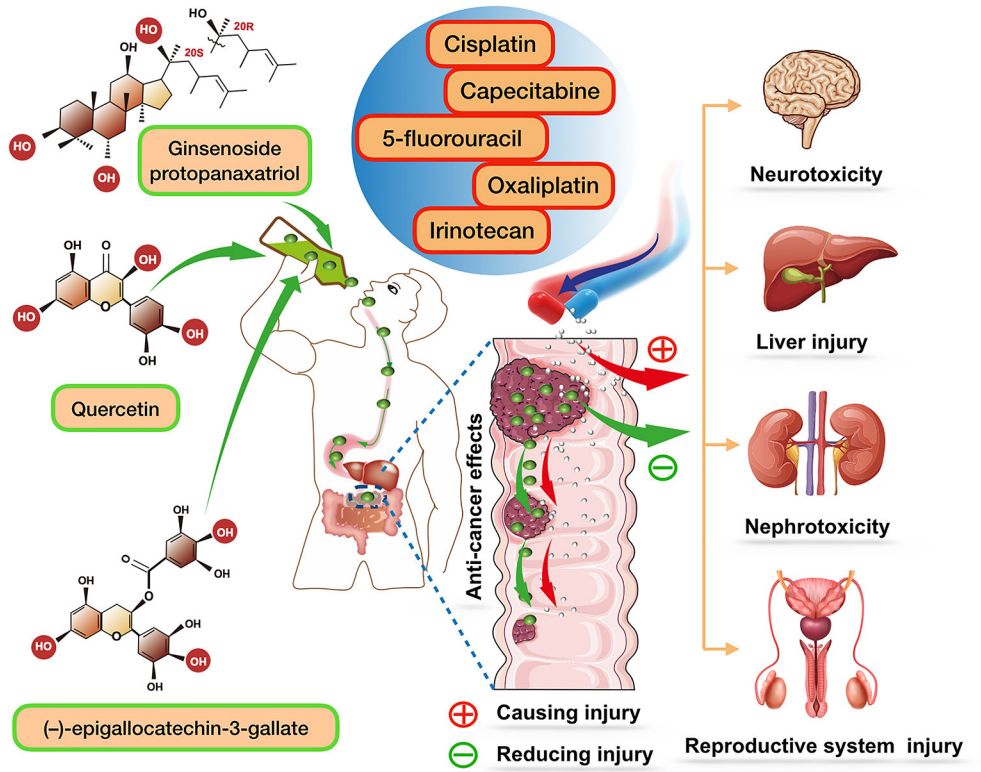

FIGURE 1 | The role of ginsenoside protopanaxatriol (one major extract of ginseng), quercetin, and (–)-epigallocatechin-3-gallate (one major extract of tea) in enhancing chemotherapeutic efficacy of various chemotherapy drugs, and together reducing their side effects. After oral administration of these compounds, they can be biotransformed to stronger components and play a synergistic role with various chemotherapy drugs.

chemotherapeutic therapies have greatly improved the outcomes of patients with CRC, serious side effects and drug resistance are still major clinical challenges. In recent years, more and more drugs, especially traditional Chinese medicines, have been used to alleviate various side effects and improve chemoresistance.

GINSENG

Ginseng is one of the most common traditional herbal medicines, which has been discovered in both East (Asian ginseng) and West (American ginseng) (28, 29). With the recent developments in the extraction process, multiple active components have been isolated from ginseng, mainly including ginsenosides, ginseng polysaccharides, flavonoids, polysaccharides, and ginseng polypeptides (30). Since ancient times, ginseng has been found to possess multiple pharmacological effects and has been used to treat various diseases, such as inflammation, cancers, metabolic syndromes, and autoimmune diseases. The anti-cancer effect of ginseng has attracted increasing interest and attention in the fields of various cancers, including ovarian cancer, CRC, breast cancer, lung cancer, prostate cancer, and liver cancer (31–34). Many in *vitro* and in *vivo* studies have demonstrated that ginseng or its extracts could significantly decrease the incidence of CRC and inhibit tumor growth (35). For example, Rg3, one of the most abundant and active ginsenosides can effectively inhibit the proliferation of CRC cells by suppressing the activity of the C/EBPβ/NF-κB signaling pathway (36). Similarly, another study also reported that ginsenoside Rg3 could inhibit the proliferation, migration and invasion of CRC cells and promote the apoptosis of these tumor cells by downregulating the expression of lncRNA CCAT1 (37). Other chemical compounds extracted from ginseng, such as flavonoids and polysaccharides, have been confirmed to have anti-CRC effects (38, 39). Recent studies further proposed that ginseng and its various constituents could improve the status of patients with CRC by increasing the efficiency of chemotherapy drugs (40, 41).

Asian Ginseng, American Ginseng, and Panax Notoginseng

Ginseng and its extracts have great potential as chemotherapy adjuvant agents due to their low toxicity and strong anti-cancer properties (42). In particular, ginseng or its active components can enhance the sensitivity of chemotherapy and reduce its side effects. For instance, Fishbein et al. proposed that Asian ginseng could improve the anti-cancer function of 5-FU on HCT-116 human CRC cells (43). In addition, Panax notoginseng root extract, a remedy anti-cancer medicine, can also improve the chemopreventive functions of 5-FU and irinotecan in experiments *in vitro* (SW480) (44). These results are consistent with previous studies that notoginseng can enhance tumor radiosensitivity to the cytotoxic effect of ionizing radiation (45).

In addition, another study reported that Panax notoginseng could increase the anti-proliferative ability of 5-FU on HCT-116 cells and significantly decrease the dosage of 5-FU required by CRC administration (46). Moreover, Li et al. reported that American ginseng berry extract could enhance the chemopreventive effect of 5-FU during CRC treatment both *in vivo* and *in vitro* (SW480, HCT-116 and HT-29), possibly by increasing cell arrest at S and G2/M phases (47).

Nausea and vomiting may be the most common adverse events in cancer chemotherapy treatment. For patients with oxaliplatin-based regimens, the incidence of nausea and vomiting is more than 70% (48). Previous studies reported that Korean red ginseng total extract could effectively attenuate cisplatin-induced nausea and vomiting in a ferret model (49). Further studies proposed that the anti-emetic effect of ginseng or its extracts was achieved by the antagonism of the 5-HT 3A receptor (50, 51). In a recent clinical trial, scholars investigated the curative effect of ginseng on nausea and vomiting induced by oxaliplatin-based regimens during CRC treatment, and they found that the administration of ginseng combined with some traditional medicines was capable of suppressing nausea and vomiting (52). In a randomized clinical phase III trial, Kim et al. proposed that Korean red ginseng administration could alleviate cancer-related fatigue in CRC patients with chemotherapy (53). Cancer-related fatigue, a common side effect of cancer chemotherapy treatment is a subjective physical feeling and can interfere with the sleep, mood, concentration, work, and daily life quality of patients (54). In this trial, 219 patients with mFOLFOX-6 administration chemotherapy were included in the Korean red ginseng treatment group, and other 219 patients treated with placebos were included in the control group. After 16-week administration, the results showed that Korean red ginseng treatment effectively improved fatigue, inhibited deterioration of fatigue-related life quality, and reduced the stress of these CRC patients receiving chemotherapy.

Ginsenosides

Panaxadiol (PD), a diol-type ginsenoside derived from Panax ginseng or Panax pseudoginseng can also enhance the anti-cancer effects of 5-FU on CRC (55). The results showed that the combined administration of 5-FU and PD significantly exerted stronger anti-proliferative and pro-apoptotic abilities in the HCT-116 human CRC cell line than treatment with 5-FU alone. These results are consistent with a previous clinical study (56). Moreover, another *in vitro* study (HCT-116 and SW480) showed that PD could also enhance the anti-cancer effects of irinotecan, which might be achieved *via* inducing tumor cell apoptosis (57). This synergistic administration can effectively reduce the dose of irinotecan and the rate of side effects, indicating that some natural products are beneficial for CRC chemoadjuvant treatment.

Ginsenoside Rg3, a tetracyclic triterpenoid saponin with strong anti-cancer properties can inhibit the proliferation, invasion and migration of various tumors (58). For instance, one study reported that Rg3 could block the progression of colon cancer and promote the apoptosis of HT-29 colon cells by inhibiting the stemness of cancer stem cells, reducing tumor angiogenesis, and upregulating the AMPK pathway (59). In recent studies, scholars further proposed that Rg3 administration could significantly enhance the anti-cancer function of 5-FU both *in vivo* and *in vitro* (SW620 and LOVO) (60). After treatment with Rg3 and 5-FU together, this synergistic therapy was found to effectively suppress the proliferation, development and metastasis of tumors by activating the PI3K/Akt signaling pathway.

Protopanaxadiol (PPD), a secondary ginsenoside induced by a gut microbiome, can be bio-transformed by intestinal flora from ginseng extracts such as Rb1 and compound K (61, 62). According to a recent study, in addition to being able to inhibit tumor development directly, PPD can effectively enhance the effects of 5-FU on patients with CRC (62). It was found that the co-administration of PPD and 5-FU exerted stronger anti-proliferative and pro-apoptotic effects on HCT-116 human CRC cells than PPD or 5-FU alone treatment. A further *in vivo* experiment also confirmed that this co-administration could markedly reduce the tumor size in a dose-related manner.

QUERCETIN

Quercetin (3, 3′, 4′, 5, 7-pentahydroxyflavone), a well-studied flavonoid in various vegetables and fruits is easily dissolved in the glacial acetic acid and aqueous solution (63). Hydrophilic glycoside, one of the most common constituents of quercetin extracts, cannot be directly absorbed by the host body and has to be transformed into quercetin metabolites by interacting with intestinal flora and key enzymes in digestive systems (64). Multiple pharmacological effects, including anti-inflammatory, anti-oxidative, anti-atherosclerosis, and anti-cancer effects, have been discovered in quercetin or its extracts (65). Further study demonstrated that quercetin could exert anti-cancer effects through various mechanisms, including inhibiting the activity of tyrosine kinase, regulating pathways involved in tumorigenesis, and interacting with specific proteins or receptors (66). It was found that quercetin and its derivatives could effectively inhibit tumor initiation and progression in both *in vivo* and *in vitro* CRC models (67). The molecular mechanisms are very complex and incompletely understood. According to previous studies, multiple signaling pathways were involved in the anti-cancer processes, such as Wnt/β-catenin, MAPK/JNK, NF-κB, and other related pathways (67). For instance, quercetin was reported to suppress the growth of multiple CRC cell lines (such as HT-29, Caco-2, DLD-1, and HCT-15) by blocking the activity of the AKT pathway (68–70). Another study further indicated that 3, 4-dihydroxyphenylacetic acid, a major derivative of quercetin, was capable of exerting CRC protective effects by reducing reactive oxygen species responses (71). In addition, recent studies reported that quercetin could be used as an effectively adjuvant chemotherapy agent for various cancer administration (65, 72–74). Especially in CRC chemotherapy, the synergistic effect of polyphenols has achieved relatively good potentiating effects.

A study in 1994 first reported that the combined administration of quercetin and 5-FU could significantly

inhibit the growth of CRC cell line COLO 320DM cells (75). Recent studies further indicated that quercetin could increase the bioavailability of drugs by regulating the expression of key proteins associated with the development of drug resistance (76). Based on the above findings, Atashpour et al. proposed that quercetin treatment could enhance the cytotoxicity and apoptosis induction of doxorubicin in CRC stem cells and HT-29 cells by arresting tumor cells at the G2/M phase (77). Moreover, Han et al. reported that quercetin pretreatment could significantly promote the apoptosis of HT-29 cells induced by cisplatin, thus improving the anti-cancer functions of cisplatin during CRC administration (78). Further studies found that the combination of quercetin and cisplatin could directly activate the NF-κB signaling pathway to suppress cell proliferation and induce apoptosis (78). A recent study suggested that the combination of quercetin and luteolin, a member of the flavone group of flavonoids, could effectively increase the anti-cancer functions of 5-FU in HT-29 cells (79). Compared with the control group, this combination exerted stronger anti-proliferative and pro-apoptotic effects. This phenomenon was caused by suppressing angiogenesis and vasculogenesis. This combination modulated the apoptotic pathways and minimized the toxic effects of 5-FU.

P-glycoprotein-mediated multidrug resistance has been considered one of the most fundamental factors of cancer chemotherapy. Quercetin has been regarded as an inhibitor of P-glycoprotein-mediated multidrug resistance, which can overcome CRC resistance to chemotherapy *via* molecular mechanisms. For example, Zhou et al. reported that quercetin could effectively increase the cytotoxicity of doxorubicin to P-glycoprotein-overexpressed SW620/Ad300 cells by blocking D-glutamate metabolism and reducing the solute carrier family 1 member 5 (80). The CRC with microsatellite instability is resistant to 5-FU administration, which remains a clinical challenge. Xavier et al. first reported that quercetin treatment could effectively enhance the sensitivity of 5-FU on CO-115 and HCT-15 cells (81). After treatment with quercetin and 5-FU together, they found that the ratio of apoptotic cells significantly increased, which might be caused by special activation of the mitochondrial pathway.

Quercetin has been recognized as the most representative drug of flavonoids. In addition to quercetin, other flavonoids, such as epigallocatechin-3 gallate and isoflavone genistein, also possess chemopreventive properties (82, 83). Howells et al. further investigated whether the chemical modification of flavonol structures could enhance the pharmacological and toxicological properties of other flavonoids. They hypothesized that a flavonol molecule had no hydroxyl group on the A ring and only methoxyl groups on the B ring, which might possess cancer chemopreventive efficacy (84). To test this hypothesis, they produced a new compound, 3′, 4′, 5′-trimethoxyflavonol, a quercetin analog. Then, they compared the preclinical cancer chemopreventive properties of the new compound with those of two naturally flavonol congeners, quercetin and fisetin, *in vivo* (human-derived HCT-116 adenocarcinoma-bearing nude mice) and *in vitro* (APC10.1 cells derived from adenomas of Apc^{Min} mice). The result showed that the synthesized 3′, 4′, 5′-trimethoxyflavonol could significantly inhibit tumor proliferation and promote apoptosis by increasing wild-type p53 expression in two mouse models. The above studies also demonstrated that chemical modification might be an effective way to generate safe and efficacious cancer chemopreventive agents.

Moreover, one study reported that quercetin treatment could also effectively enhance the radio sensitivity of CRC in addition to improving the chemotherapy sensitivity. They found that the pretreatment of quercetin enabled colorectal cells to be more sensitive to radiotherapy by downregulating the ataxia–telangiectasia-mutated-related signaling pathways and promoting irradiation-induced γ-H2AX and 53BP1 focus formation (85). A recent study indicated that the combination of quercetin and ionizing radiation could have greater therapeutic potential for CRC, which is consistent with the above results (86). The detailed mechanism included directly suppressing the Notch-1 signaling pathway and targeting colon cancer stem cells, one group of rare immortal cells involved in radiation therapy resistance.

TEA

Tea is a commonly consumed beverage derived from the leaves and leaf buds of the Camellia sinensis. Tea has been studied extensively in health and disease fields, such as preventing hypertension and cardiovascular diseases, reducing obesity, treating metabolic syndromes, mediating gut microbiotas, and preventing and treating cancers (87). There are many types of tea, such as black tea, green tea, Pu-erh tea, white tea, yellow tea, oolong tea, and dark tea, all of which were produced *via* different methods (88). For instance, green tea, also named non-fermented tea is produced from dried green tea leaves. Black tea also named most or fully fermented tea is obtained from extensively solid-state fermentation involving microorganisms. The partially fermented tea is named oolong tea (89). In the last few years, various chemical components, mainly including catechin derivatives, polysaccharides, pigments, theophylline, glycosides, phenolic acids, and alkaloids have been isolated from tea. Catechins, such as (–)-epigallocatechin-3-gallate (EGCG), (–)-epigallocatechin (EGC), (–)-epicatechin-3-gallate (ECG), and (–)-epicatechin, have been studied extensively in cancer prevention and administration. Previous studies reported that tea and its components could exert anti-cancer effects through various signaling and metabolic mechanisms, such as inhibiting tumorigenesis, promoting apoptosis, regulating proliferation transformation, and targeting key transmembrane receptors or kinases (87). It has been confirmed that EGCG could inhibit CRC initiation and progression by reducing oxidative reaction and promoting tumor cell apoptosis (90). Some meta-analyses also showed that tea consumption was closely associated with CRC risk (91, 92). Based on these results, some scholars further proposed that tea and tea polyphenols could be used as promising chemopreventive agents for CRC treatment (93).

EGCG and EGC

(–)-epigallocatechin-3-gallate is a major green tea polyphenol and is regarded as an important tumor inhibitor in various

cancers (94). It has been shown that the combinations of EGCG and other catechins can exert relatively strong anti-cancer effects in both *in vitro* and *in vivo* experiments (95). Some studies indicated that EGCG could improve chemoresistance and reduce tumor recurrence. For instance, Toden et al. found that EGCG treatment could sensitize chemoresistant CRC cells (HCT-116 and SW480) to standard 5-FU administration. Specific chemopreventive activities include increasing 5-FU-induced cytotoxicity and suppressing the growth of tumor cells by triggering apoptosis and promoting cell cycle arrest (96). La et al. reported that EGCG administration could effectively increase the chemosensitivity of 5-FU in HCT-116 and DLD1 cell lines by suppressing tumor growth, promoting apoptosis, and causing DNA damage, which is consistent with the above results (97). According to further mechanistic studies, EGCG can upregulate the expression of NF-κB and miR-155-5p by blocking GRP78 activity, further suppressing the protein expression of MDR1 and increasing the 5-FU accumulation in CRC cells. In another study, Shimizu et al. proposed that EGCG could exert chemopreventive effects by inhibiting the activity of signaling pathways related to receptor tyrosine kinases, such as EGFR, IGF-1R, and VEGFR2 signaling pathways (98). Moreover, combining EGCG with cisplatin or oxaliplatin could significantly inhibit the proliferation of DLD-1 and HT-29 cells and reduce cytotoxic effects by regulating autophagy-related signaling pathways (93).

Irinotecan is a common DNA-damaging chemotherapeutic agent for CRC treatment, the use of which is limited by its low solubility and high toxicity. Combined with the previous studies, they found that the co-administration of EGCG and Gefitinib or Bleomycin could reduce their dose and resistance (99, 100). Wu et al. further investigated the synergy of EGCG and irinotecan on CRC treatment (101). They treated CRC cells RKO and HCT116 with EGCG and irinotecan together, and the results showed that the combined administration exerted relatively strong inhibitory effects on the proliferation, migration, and invasion of tumor cells. The specific molecular mechanism includes inducing S- or G2-phase arrest and causing more extensive DNA damage. Moreover, a study reported that EGCG and EGC could increase the chemosensitivity of low-dose doxorubicin both *in vivo* and *in vitro* (SW620) by blocking the activation of protein kinase C, a drug resistance-related protein (102). According to some studies, in addition to directly improving chemotherapy responses, tea nanoparticles can be used to deliver chemotherapeutic agents for cancer treatment. For instance, Wang et al. proposed that tea nanoparticles, a safe nanocarrier with good biocompatibility and low toxicity could load doxorubicin into tumors, thus enhancing its intertumoral accumulation and improving its chemotherapy efficacy in an animal study (103).

Chemopreventive Effects

The view that drinking tea can prevent cancer has been proposed for many years. For instance, Shimizu et al. reported that consuming proper green tea every day could inhibit the recurrence of CRC (104). Another study showed that

TABLE 1 | The synergistic effects of ginseng, quercetin, and tea on chemotherapy treatment of colorectal cancer.

Herbs or their composition	Studied objects	Chemotherapeutics	Effects	Refs.
Asian ginseng	HCT-116	5-fluorouracil	Improving efficacy of chemotherapy.	(43)
Panax notoginseng root extract	SW-480	5-fluorouracil Irinotecan	Improving efficacy of chemotherapy.	(44)
Panax notoginseng	HCT-116	5-fluorouracil	Improving efficacy of chemotherapy.	(46)
American ginseng berry extract	SW-480, HCT-116 HT-29, Animal model	5-fluorouracil	Improving efficacy of chemotherapy.	(47)
Korean red ginseng	Animal model	Cisplatin	Reducing side effects of chemotherapy.	(49)
Asian ginseng	Clinical trial	Oxaliplatin	Reducing side effects of chemotherapy.	(52).
Korean red ginseng	Clinical trial	mFOLFOX-6	Reducing side effects of chemotherapy.	(53)
Panaxadiol	HCT-116	5-fluorouracil	Improving efficacy of chemotherapy.	(55)
Panaxadiol	HCT-116, SW-480	Irinotecan	Improving efficacy of chemotherapy.	(57)
Ginsenoside Rg3	SW620, LOVO, Animal model	5-fluorouracil	Improving efficacy of chemotherapy.	(60)
Protopanaxadiol	HCT-116	5-fluorouracil	Improving efficacy of chemotherapy.	(62)
Quercetin	COLO 320 DM	5-fluorouracil	Improving efficacy of chemotherapy.	(75)
Quercetin	HT-29	Doxorubicin	Improving efficacy of chemotherapy.	(77)
Quercetin	HT-29	Cisplatin	Improving efficacy of chemotherapy.	(78)
Quercetin	HT-29	5-fluorouracil	Improving efficacy of chemotherapy.	(79)
Quercetin	SW620, Ad300	Doxorubicin	Increases sensitivity to chemotherapy	(80)
Quercetin	CO-115, HCT-15	5-fluorouracil	Increases sensitivity to chemotherapy	(81)
(–)-epigallocatechin-3-gallate	HCT116, SW480	5-fluorouracil	Increases sensitivity to chemotherapy	(96)
(–)-epigallocatechin-3-gallate	HCT-116, DLD1	5-fluorouracil	Increases sensitivity to chemotherapy	(97)
(–)-epigallocatechin-3-gallate	DLD-1, HT-29	CisplatinOxaliplatin	Reducing side effects of chemotherapy.	(93)
(–)-epigallocatechin-3-gallate	RKO and HCT-116	Irinotecan	Improving efficacy of chemotherapy.	(101)
(–)-epigallocatechin-3-gallate	SW620	Doxorubicin	Increases sensitivity to chemotherapy	(102)
Tea nanoparticle	Animal model	Doxorubicin	Improving efficacy of chemotherapy.	(103)

green tea catechins could prevent CRC through multiple molecular mechanisms, including decreasing detergent-insoluble membrane domain, inhibiting the activity of the specific receptor tyrosine kinases (such as EGFR, IGF-1R, and VEGFR-2), and reducing the expression of hypoxia-inducible factor 1a (HIF1a), IGF-1, IGF2, and EGF (98, 105). In a randomized controlled trial, Henning et al. proposed that tea polyphenols could be transformed into phenolic metabolites by the colonic microflora, thus playing a significant role in CRC prevention (106). Ku-jin tea, a very popular beverage in the world, is an essential anti-inflammatory and anti-oxidative regulator and can also play chemopreventive effects on CRC. In a CRC rat model induced by azoxymethane, Bi et al. found that long-term treatment with Ku-jin tea could significantly decrease the number of aberrant crypts, aberrant crypt foci (ACF), and crypts/foci in rats through regulating metabolism-associated pathways, further indicating that Ku-jin tea can be used as a promising chemopreventive agent for CRC chemoprevention (107).

PERSPECTIVES AND CONCLUSIONS

The synergistic therapy of herbal medicines combined with chemotherapy may revolutionize cancer treatment. With the development of precision medicine, chemotherapy has played an increasingly important role in clinical cancer treatment (108, 109). Especially for CRC, various chemotherapeutic regimens have been proposed and have achieved remarkable clinical efficacy. For example, for lymph node-positive patients, the FOLFOX regimen (5-FU, leucovorin, and oxaliplatin) is recommended (110). In terms of locally advanced rectal cancer, neoadjuvant chemoradiation therapy with 5-FU and radiation therapy should be considered for the patients. For patients with metastatic CRC, FOLFOX, or FOLFIRI (5-FU, leucovorin, and irinotecan) regimens are recommended as standard first-line treatment choices (111).

Traditional herbal medicines, exerting huge therapeutic potential in various diseases, are promising adjuvant chemotherapy agents. In this review, to allow readers to quickly know this field, we selected the three most studied herbs (ginseng, quercetin, and tea) as representative drugs to conclude their synergies in CRC chemotherapy administration (**Table 1**). By summing up the points, we discovered that most studies were focused on investigating the synergistic effects of the three herbs on 5-FU, the most commonly used chemotherapy drug for CRC treatment. As expected, we found that all the three herbs and their major extracts could significantly enhance the chemopreventive functions of 5-Fu and reduce its side effects. In terms of ginseng, there are three most common species, including Asian ginseng, American ginseng, and Panax notoginseng, all of which have been confirmed to possess synergistic effects. Their major components, such as ginsenoside Rg3, PPT, and PPD, also possess stronger pharmacological and biological effects. In addition, the synergistic effects of them and other chemotherapy drugs, such as irinotecan, have also been studied both *in vivo* and *in vitro*. Quercitrin is commonly found in plant foods used to treat various diseases, especially, which has been identified to be an effectively antitumor agent. Herein, we have summarized that quercetin treatment could enhance the cytotoxicity of 5-FU, cisplatin, and doxorubicin by regulating different molecular mechanisms. The research on tea was mainly focused on EGCG, a major tea polyphenol, which can improve the chemotherapy efficacy of 5-FU, irinotecan, cisplatin, and oxaliplatin and play chemopreventive effects both *in vivo* and *in vitro*. However, its detailed mechanisms, mainly including promoting proliferation and inhibiting apoptosis by regulating related signaling pathways, are not fully understood.

Although many preclinical studies have been performed, the clinical applications of these herbs are still limited due to too many unknown variables. In the future, an increasing number of studies should be performed to clarify specific mechanisms and develop more effective chemopreventive agents for CRC administration.

AUTHOR CONTRIBUTIONS

LZ drafted the review. HZ and MS generated the graphs. NL guided the construction of the manuscript. YZ edited the review. KZ, JL, and PL provided input on the scope and content of the review. All the authors contributed to the article and approved the submitted version.

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The treatment effects of Trametes Robiniophila Murr against colorectal cancer: A mini-review

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Colorectal cancer (CRC) is a worldwide disease threatening people's lives. Surgery and chemotherapy are still the main methods for CRC treatment. However, the side effects and chemotherapeutic drug resistance restrict the application of chemotherapy. Trametes Robiniophila Murr, also known as Huaier, is a traditional Chinese medicine that has been used for more than 1,600 years. Huaier extracts have promising anti-cancer effects on hepatoma, breast cancer, and gastric cancer. Nowadays, the tumor inhibition of Huaier on CRC has attracted more and more attention. This review mainly provides the possible anti-tumor mechanisms of Huaier for CRC treatment in apoptosis and inhibiting proliferation of tumor cells, preventing epithelial-mesenchymal transformation (EMT), weakening proliferation and differentiation of CRC stem cells, decreasing the vessel density in tumor tissues, and enhancing the immune system and chemotherapeutic efficacy. Huaier extract may be a good candidate for CRC treatment, especially when combined with other chemotherapeutic agents.

KEYWORDS

colorectal cancer, Trametes Robiniophila Murr, Huaier, mechanisms, anticancer

Introduction

Colorectal cancer (CRC) is a common malignant tumor in the digestive tract, and its incidence is increasing. The mortality of CRC is on the rise and ranks third among all malignancies (1) that seriously endanger the health of people. Surgery is still the most important current treatment method for CRC, but the average 5-year survival rate is < 50%, and about 30% of patients may develop tumor recurrence after surgery (2). Chemotherapy and radiotherapy are still the most commonly used methods to prevent the post-surgical tumor recurrence or treat the advanced CRC that is unsuitable for surgical resection. However, the side effects of chemotherapy and radiotherapy cannot be restricted nowadays. Therefore, developing new anti-cancer drugs with low

toxicity and drug resistance is urgent. More and more researchers are focusing on the effects of Chinese medicine in postoperative adjuvant therapy for many years. Post-surgical adjuvant therapy by Chinese medicine can effectively enhance the effect of chemotherapy, reduce the toxic side effects of chemotherapy and adverse reactions caused by surgery, etc., and improve the survival rate and the quality of life of CRC patients (3, 4).

Trametes Robiniophila Murr, also known as Huaier, has a history of more than 1,600 years as traditional Chinese medicine. Huaier granules are common clinical pharmaceutical agents. Huaier is a medicinal fungus that grows on acacia, locust tree, sandalwood and many other trees, and it contains various organic components and more than 10 minerals. The main ingredient of Huaier is the fungal matter, which includes polysaccharides, proteins, ketones, and alkaloids, and the active ingredient is polysaccharide-protein (5). Huaier granules have shown promising tumor inhibitory effects on many kinds of cancers, including hepatic cancer (6), breast cancer (7), and gastric cancer (8). It is reported that Huaier extracts can effectively inhibit the proliferation of colon cancer cells (9) and prevent the progression of colon tumors in nude mice (10). These findings may provide new aspects for CRC treatment. However, the concrete mechanisms of Huaier against CRC are not clear. As a result, this review mainly provides the possible anti-tumor mechanisms of Huaier for CRC treatment (Figure 1).

The role of Huaier in CRC cells

Promoting apoptosis and inhibiting proliferation of tumor cells

Huaier can inhibit the proliferation of CRC cells by inducing tumor apoptosis. It is reported that Huaier extract can induce G0/G1 and S phrases arrest, and the proliferation ability of tumor cells is weakened and the ability of apoptosis is enhanced (11, 12). Studies have shown that wild-type p53 protein encoded by the p53 gene inhibits cell growth, induces apoptosis, and repairs damaged cells. These functions are related to genomic stability, cell cycle progression, apoptosis, and DNA damage repair (13). Bcl-2 can regulate tumor cell apoptosis and inhibit cancer cell proliferation through the mitochondrial pathway (14). Lin *et al.* found that Huaier extract can improve the severity of inflammatory bowel disease-related tumors, induce apoptosis of related tumor cells, and inhibit tumor cell proliferation in CRC mouse model. In addition, the apoptosis-associated protein levels, including p53 and Bcl-2, show significant differences when treated with Huaier in CRC cell lines (15). Therefore, Huaier may possess good properties to induce apoptosis of CRC cells by activating and upregulating p53 and downregulating Bcl-2/Bax genes. Sun *et al.* have reported that Huaier granules can significantly decrease the tumor development in nude mice transplanted with HT-29 colon carcinoma cell line by downregulating the expression of PI3KR1, Akt, Wnt1, CTTNB1, and Notch genes (9). It is reported that chromosomal maintenance protein is a target for Huaier in hepatocellular carcinoma, and Huaier inhibits the cell cycle of liver cancer cells by regulating chromosomal maintenance proteins, thereby inhibiting tumors (11). In addition, Huaier extracts can inhibit proliferation and induction of apoptosis in two tuberous sclerosis complex cell models by inhibiting JAK2/STAT3 and MAPK signaling pathways (16). However, the above mechanisms of Huaier should be further investigated in CRC cell lines or animal models.

Inhibiting epithelial-mesenchymal transformation

Epithelial-mesenchymal transformation (EMT) refers to the biological process by which epithelial cells are transformed into cells with interstitial phenotypes through specific procedures (17). EMT is always associated with tumor invasion and distant metastasis (18). Matrix metalloproteinase (MMP) plays an essential role in the EMT by influencing the degradation and remodeling of the extracellular matrix, increasing local invasion of tumor cells, and improving distant metastasis (19). Researchers found that Huaier can decrease the expressions of Bcl-2, MMP-2, and MMP-9 in the MKN-45 cell line, thus inducing apoptosis and preventing the invasion of tumor cells (20). Huaier may reduce the invasion and metastasis of CRC cells by inhibiting EMT. The effects may be associated with the regulation of the expression of messenger RNA and the transcription factors. Furthermore, Huaier extracts can slow the growth of pancreatic cancer and decrease the invasion, migration, and EMT of pancreatic cancer cells by suppressing Wnt/beta-catenin pathway (21). However, the inhibitory effect of Huaier on EMT in CRC treatment remains to be further studied.

The role of Huaier in CRC stem cells

CRC stem cells have a stronger ability to proliferate, infiltrate, and metastasize than CRC cells (22). Inhibiting the proliferation, invasion, and migration of tumor stem cells can decrease the proportion of stem cells transformed into cancer cells, thus preventing the recurrence and migration of tumors (23). The proliferation, differentiation, and self-renew ability of CRC stem cells are regulated by multiple signaling pathways. The expression of messenger RNA associated with tumor proliferation in CRC stem cells treated with Huaier granules is significantly downregulated, and the proliferation of stem cells is inhibited (9). Huaier extracts inhibit the migration of CRC and may be achieved by down-regulating the expression

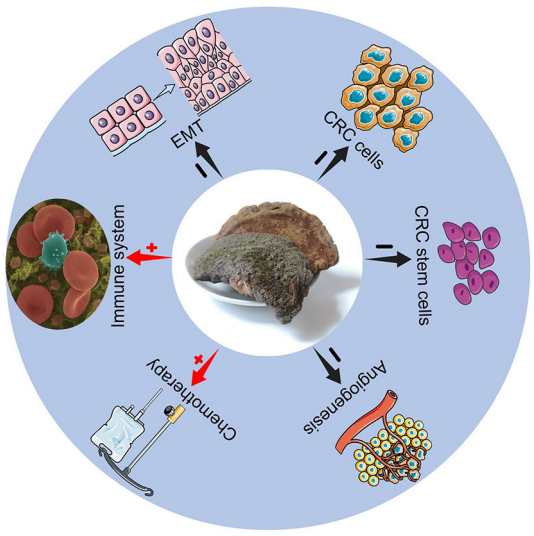

FIGURE 1
Mechanisms of Trametes Robiniophila Murr for CRC treatment.

of messenger RNA of key genes or proteins to attenuate the properties of cancer stem cells. Detailed mechanisms about the effect of Huaier on CRC stem cells need to be further verified.

The role of Huaier in tumor angiogenesis

Neovascularization is a typical feature of tumors and is a necessary process for tumor invasion and distant metastasis (24, 25). Neogenesis in tumors is regulated by various angiogenesis factors, such as vascular endothelial growth factor (VEGF) and its regulatory gene hypoxia inducing factor 1α, human macrophage metal elastase (26, 27). The overexpression of VEGF is strongly associated with poor treatment outcomes and reduced survival rates in cancer patients (28, 29). Therefore, inhibiting the proliferation of vascular endothelial cells induced by VEGF is the key to preventing tumor invasion and migration. Zheng *et al.* found that Huaier polysaccharide (TP-1) can reduce the expression of hypoxia-inducible factor and VEGF in tumor tissues in mice bearing hepatocellular carcinoma SMMC-7721 tumors model (30). Huaier extracts can not only decrease VEGF levels in mouse mammary tumor cells but also decrease microvessel density in tumor tissues (31). Huaier granules can suppress the infiltration of tumor-associated macrophages, and inhibit the angiogenesis of macrophages, thereby inhibiting tumor progression in RAW264.7 murine macrophage cell line (32). However, this effect has not been tested in CRC.

Huaier extracts can inhibit the invasion and migration of CRC by inhibiting neovascularization in the tumor, which may be the focus of future clinical research and can be applied in the clinical treatment of CRC. However, more experiments are needed to validate and explore the possible molecular mechanisms of the effects of Huaier on tumor angiogenesis.

The role of Huaier in the immune system

The balance between tumor-specific immunity and tolerance affects the health of the host. Under the protection of the immune system, a normal organism can prevent the deterioration of mutated tumor cells. However, cellular immune function in CRC gradually decreases and continues to deteriorate with tumor progress, recurrence, and distant metastasis (33, 34). Therefore, it is of great significance to protect and enhance the immune defense ability to increase the efficacy of anti-tumor therapy for CRC patients. As a traditional Chinese medicine, Huaier extracts can act as an effective immune enhancer and modulator by fully mobilizing cellular and humoral immunity, thus reducing the cachexia caused by chemotherapy (5, 35). Huaier granules can enhance the phagocytosis of macrophages, increase the number and activity of natural killer cells, and enhance the body's immunity to effectively induce the death of tumor cells (19, 36). Huaier granules can promote the maturation of dendritic cells, and the dendritic cells treated by Huaier can significantly stimulate the proliferation of CD4+T cells and promote their differentiation into the Th1 subgroup (37). In one study, the researchers found that Huaier polysaccharides can decrease the nephrotoxicity caused by cisplatin chemotherapy and protect renal function by regulating PI3K/Akt/mTOR signaling pathway *in vitro*, thus enhancing the immune ability of patients (38). Huaier extracts can perform the anti-tumor effects by enhancing the immune system, but detailed mechanisms still need further study.

Enhancing chemotherapeutic efficacy

Clinically, the main causes of tumor deaths are metastasis and recurrence (39, 40). Tumor metastasis is a multistep process in which tumor cells penetrate stromal tissue, blood, or lymph node metastasis, adhere to the basement membrane, and invade the target organ (41, 42). Although chemotherapy, radiotherapy, and targeted therapy can decrease the recurrence and metastasis of cancer, there are still some problems that need to be solved, such as insensitivity to chemotherapy drugs and drug resistance. Chemotherapy still plays a dominant role in the comprehensive treatment of CRC, so it is significant to enhance chemotherapy sensitivity. The inhibition of EMT can increase the chemotherapy sensitivity of CRC to oxaliplatin (43). It is reported that Huaier extracts can prevent EMT (21), which may be used to increase

chemotherapy sensitivity. Studies have shown that Huaier plays an effect on the reversal of chemotherapeutic agents resistance, thus increasing the chemotherapy effects (44, 45). The efficacy of Huaier combined with paclitaxel in treating BT474 and MDA-MB-231 breast cancer-bearing mice is superior to that of paclitaxel alone. The combination of Huaier and paclitaxel can reduce the levels of PI3K and p-AKT (46). In one meta-analysis, the researchers concluded that Huaier granules can enhance the chemotherapeutic efficacy of gastric cancer (8). Huaier granules can increase the sensitivity of chemotherapy and inhibit the recurrence and metastasis of tumors. However, a large number of basic and clinical studies need to confirm its effect on CRC and its specific mechanism.

Conclusions and perspectives

There are still some problems that need to be solved to be widely applied in CRC treatment. (1) The active ingredient of Huaier for tumor treatment has not been determined. (2) Although increasing studies have focused on the direct effects of Huaier on cancer cells, few studies have explored its molecular and immunomodulatory mechanisms. (3) Whether the inhibitory effect of Huaier granules on the invasion and migration of CRC is related to the influence of intestinal flora needs further study. (4) More basic and clinical studies should be performed to provide a more convincing basis for applying Huaier in the treatment of CRC.

In conclusion, Huaier extracts may inhibit the progression of CRC in various ways, include inducing apoptosis and inhibition of tumor cell proliferation, blocking epithelial mesenchymal transition (EMT), attenuating proliferation and differentiation of CRC stem cells, reducing vascular density in tumor tissue, and enhancing the immune system. Therefore, Huaier may be an excellent candidate to enhance the sensitivity of chemotherapy while enhancing the immune system and decreasing side effects. The genes, signaling pathways, and related molecular mechanisms involved in the interaction between Huaier extracts and CRC are the focuses of future research.

Author contributions

BL drafted the review. QC generated the graph and guided the construction of the manuscript. ZL edited the review. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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Anti-colorectal cancer effects of seaweed-derived bioactive compounds

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Seaweeds are classified as Chlorophyta, Rhodophyta, and Phaeophyta. They constitute a number of the most significant repositories of new therapeutic compounds for human use. Seaweed has been proven to possess diverse bioactive properties, which include anticancer properties. The present review focuses on colorectal cancer, which is a primary cause of cancer-related mortality in humans. In addition, it discusses various compounds derived from a series of seaweeds that have been shown to eradicate or slow the progression of cancer. Therapeutic compounds extracted from seaweed have shown activity against colorectal cancer. Furthermore, the mechanisms through which these compounds can induce apoptosis *in vitro* and *in vivo* were reviewed. This review emphasizes the potential utility of seaweeds as anticancer agents through the consideration of the capability of compounds present in seaweeds to fight against colorectal cancer.

KEYWORDS

colorectal cancer, therapeutic compounds, Chlorophyta, Rhodophyta, Phaeophyta

Introduction

Colorectal cancer (CRC) is the most common type of cancer throughout the world, accounting for approximately 10% of all new cancer cases and mortality, as projected in GLOBOCAN 2020 (1). The prevalence rates of CRC are increasing among nations with a medium human development index, such as Brazil, Russia, and countries of Latin America (2). The pathology of CRC includes carcinogenesis of the rectum, colon, appendix, and anus (3). Familial and environmental factors contribute to the risk of CRC from two well-defined causes particularly amenable to dietary influence (4). In the clinical situation, chemotherapy is a common treatment modality for CRC (5). Nevertheless, the majority of current chemotherapeutic drugs for the therapy of advanced-stage CRC, for instance cisplatin, have been repeatedly reported to elicit adverse side effects and are comparatively less effective (6). Several lines of scientific evidence, from molecular mechanisms to clinical trials, show that herbal medicines have anti-CRC potential and have been used for therapy and recovery (7).

Seaweeds have been utilized for food and medicinal herbs since ancient times in Asia (8). It has been consumed as a food for over 1,700 years, which can be dated back to Japan in the fourth century and China during sixth century. Particularly, people

living long in coastal areas frequently used seaweed as a main dish, side dish, or soup (9). Consumption of seaweed supplies sufficient macro and micronutrients, which are essential to maintaining human health (10). Besides nutritional effects, seaweed has long been adopted as a drug in Traditional East Asian Medicine to alleviate the progression of multiple cancers (11). Seaweeds as large multicellular marine organisms are classified into three major groups based on their pigments and the origin of sulfated polysaccharides: green (Chlorophyta), red (Rhodophyta), and brown (Phaeophyta) (12, 13). They represent a main source of bioactive compounds, yielding primary metabolites essential for natural growth and many secondary metabolites, which include polysaccharides, polyunsaturated fatty acids, phenolics, vitamins, pigments, minerals, terpenes, and phytosterols (14). Due to their various constituents, seaweeds have shown diverse biological activities, including anticancer activity (15).

Seaweeds have long been recognized as a therapeutic option of cancer (16). Accumulating evidence advocates that the anticancer effects of bioactive ingredients extracted from seaweed are produced *via* multiple mechanisms of action, including inhibition of growth, invasion, and metastasis of cancer cells, and through the stimulation of apoptosis in cancer cells (17). Among the East Asian population, people who regularly consumed seaweed reduced their risk of CRC development by half (10). Several researches have suggested that CRC can be effectively treated with marine natural products (18). According to one such report, brown seaweed *Turbinaria decurrens* has the potential as an anti-CRC agent (19). The highly cytotoxic and antiproliferative activities of seaweeds from the Portuguese coast have been proven in a model of Caco-2 CRC cells *in vitro* (20). *Sargassum oligocystom* significantly decreased cell viability in SW742, HT-29, WiDr, and CT-26 CRC cells through activation of the APC gene (21). Although several studies have reported the therapeutic properties of seaweed in CRC, its mechanism of action and active ingredients are still unclear and unclassified. In this review, we summarize the various effects of diverse compounds derived from seaweed on CRC.

Categorization of anti-CRC compounds isolated from seaweeds

Polysaccharides

The polysaccharides present in seaweeds are many and diverse (22). They are hydrophilic molecules with high solubility in water and a repeating structure (23). The polysaccharides in seaweed are divided into sulfated (fucoidan, carrageenan, and ulvan) and non-sulfated (agarose and laminarin) (24). Chlorophyta, Phaeophyta, and Rhodophyta contain polysaccharides of varied chemical composition and structure (25).

Previous studies suggested that polysaccharides from seaweed showed strong anti-CRC and preventive activities. They can either directly inhibit cancer cells or affect various phases of carcinogenesis and the progression of tumor through the regulation of the balance between proliferation and programmed cell death and can also be potentially used for cancer prophylaxis (26). Three polysaccharide fractions isolated from *Porphyra haitanensis* exerted inhibitory effects on growth in the HT-29, LoVo, and SW-480 colon cancer cell lines (27). Other active components contained in seaweed that exhibit similar effects against CRC are shown in Table 1. Another study reported on an evaluation of the anti-CRC activity of sulfated glucuronorhamnoxylan polysaccharides from *Capsosiphon fulvescens* (28). Polysaccharides from *Jania rubens* upregulated the gene expression of Bax, caspase 8, and P53 in human colon cancer Caco-2 cells (29). A summary of mechanisms for other active components from seaweed on anti-CRC is shown in Table 2.

Agarose

Red algae cell walls mainly consist of agarose which is composed of alternative units of D-galactose and 3,6-anhydro-L-galactose (AHG) linked by alternating α-1,3- and β-1,4-glycosidic bonds (62). After being consumed, agarose is digested, fermented, and metabolized by intestinal microbiota in the human large intestine, which makes it unique among red algal polysaccharides (63). Clinical trials have suggested that people in Asia who regularly consume red seaweeds are at a lower risk of CRC, which is relevant to their daily intake of seaweeds (64). It is speculated that this effect may be related to biologically active agarose components enriched from red seaweed. Upregulation of caspase-3, Bax, and caspase-9 expression and downregulation of Bcl-2 and Bcl-xL were observed in HCT-116 cells after AHG treatment (30). Therefore, the growth of human colon cancer HCT-116 cells was effectively suppressed by AHG, indicating that AHG is a potential alternative as an anti-CRC agent.

Ulvan

The sulfated polysaccharide known as "ulvan" is extracted from green algae of the ulva species (35). Ulvan mainly consists of cellulose, xyloglucan, and glucuronan with various other types of sugars (65). It is reported to possess diverse physiological and bioactive activities, including anticancer activity (66). Ulvan has been demonstrated to decrease viability in cancer cells while leaving healthy cells unaffected (65). The current study has categorically proven that biogenic silver nanoparticles (AgNP), which were generated *via* an extract of the marine alga *Ulva lactuca*, can induce p53-dependent apoptosis in colon cancer HCT-116 cells (31, 32).

TABLE 1 The effects of active components isolated from seaweeds on colorectal cancer.

Seaweed	Division	Therapeutic ingredients	Cell line	IC_{50}	References
Sphaerococcus coronopifolius	Rhodophyta	Dichloromethane extract	Caco-2	21.3 μg/mL	(20)
Sargassum oligocystom	Phaeophyta	Hydroalcoholic extract	CT-26	-	(21)
Porphyra haitanensis	Rhodophyta	PHP-F1, PHP-F2 and PHP-F3	HT-29	664.4 μg/mL, 575.1 μg/mL and 578.3 μg/mL	(27)
Capsosiphon fulvescens	Chlorophyta	SPS-CF	HT-29	-	(28)
Jania rubens	Rhodophyta	*J. rubens* polysaccharide	Caco-2	20 mg/mL	(29)
Red Seaweeds	Rhodophyta	AHG	HCT-116	-	(30)
Ulva lactuca	Chlorophyta	AgNP	HCT-116	142μM	(31)
Ulva lactuca	Chlorophyta	Ulvan polysaccharide	HCT-116	22.65 μg/mL	(32)
Fucus evanescens	Phaeophyta	Laminarin	HCT-116	200 μg/mL	(33)
Kappaphycus alvarezii	Rhodophyta	κ-carrageenan	HCT-116 HT-29	- 73.87 μg/mL	(34, 35)
Fucus vesiculosus	Phaeophyta	Fucoidan	HT-29 HCT-116	200 μg/mL -	(36–38)
Fucus evanescens	Phaeophyta	Fucoidan	HCT-116	-	(39)
Sargassum mcclurei	Phaeophyta	SmF1, SmF2 and SmF3	DLD-1	-	(40)
Sargassum glaucescens	Phaeophyta	SG4	HT-29	272 μg/mL	(41)
Sargassum cinereum	Phaeophyta	Fucoidan	Caco-2	250 μg/mL	(42)
Halimeda opuntia	Chlorophyta	Carotenoids, chlorophyll a	HT-29	45.23 μg/mL	(43)
Laminaria japonica	Phaeophyta	Fucoxanthin	HCT-116	-	(44)
Sargassum angustifolium	Phaeophyta	Fucosterol	HT-29	70.41 μg/mL	(45)
Pterocladiella capillacea	Rhodophyta	Mertensene	HT-29	56.5 μg/mL	(46)
Cystoseira usneoides	Phaeophyta	Meroterpenoids	HT-29	7.8–36.9 μg/mL	(3)

IC_{50}, the half-maximal inhibitory concentration.

Laminarin

Laminarin, sometimes referred to as laminaran, which is an essential biodegradable and non-toxic polysaccharide isolated from the cell wall reservoirs of brown algae, has caught the interest of researchers (67). Laminarins are essentially a group of low-molecular-weight storage β-glucans consisting of (1,3)-β-D-glucan (68). (1,3)-β-D-glucopyranose residues with a few 6-O-branching on the main chain, and also several β-(1,6)-intra-chain links, which are abundant in their structures (69). β-glucans can promote cell apoptosis of colon cancer, and they may be beneficial natural agents for colon cancer treatment and chemoprevention (70). Additionally, certain studies have suggested that the biological activity of laminarin can be strengthened with particular chemical modifications (71). For example, Ji et al. (71) demonstrated that laminarin treated with sulfated provided a stronger antitumor effect compared with unmodified laminarin in human colorectal adenocarcinoma cells. The cell survival rate was significantly decreased after culturing with sulfated laminarin in LoVo cells. Apparently, peculiarities of the polysaccharide structure and sulfation contribute to the anticancer activity of laminarins. Malyarenko et al. (33) found that the antiproliferative activity of laminarins from *Fucus evanescens* was comparable to that of their sulfated derivatives. The anticancer effect of laminarin isolated from *F. evanescens* was stronger than that of its sulfated derivatives in HCT-116 colon carcinoma cells. Ji et al. (72) proved that laminarin increased the intracellular reactive oxygen species (ROS) level, increased intracellular Ca^{2+}, decreased intracellular pH, and induced LoVo apoptosis through a mitochondrial pathway. A further study revealed that the expression of procaspase-8 and−3 was downregulated and the activity of caspase-8,−3,−6, and−7 was increased in human colon cancer LoVo cells through the TRAIL/DR pathway after treatment with laminarin (73). Thus, laminarin induces apoptosis in human colon cancer *via* the mitochondrial and DR pathways, indicating that laminarin is a potent anticancer agent.

Carrageenan

A set of sulfated polysaccharides generically described as carrageenan is present in red algae, which is the major ingredient of cell walls and interstitial spaces, acting as

TABLE 2 Properties of active components isolated from seaweed against colorectal cancer.

Therapeutic ingredients (Seaweed)	Cell line	Mechanism	Cell cycle arrest	References
Hydroalcoholic extract (*Sargassum oligocystom*)	CT-26	Upregulate APC and P53	+	(21)
PHP-F1, PHP-F2 and PHP-F3 (*Porphyra haitanensis*)	HT-29	Induce oxidative stress and apoptosis	G0–G1	(27)
SPS-CF (*Capsosiphon fulvescens*)	HT-29	Upregulate caspase-8,−9,−3 and cleavage of poly (ADP-ribose) polymerase (PARP), induce DNA fragmentation, disrupt MMP	G2/M	(28)
Polysaccharide (*Jania rubens*)	Caco-2	Upregulate Bax, caspase 8 and P53	+	(29)
AHG (Red Seaweeds)	HCT-116	Upregulate Bax, caspase-3,−9 and P53, downregulate Bcl-2 and Bcl-xL	+	(30)
AgNP (*Ulva lactuca*)	HCT-116	Upregulate Bax, P53 and P21, downregulate Bcl-2	+	(31)
Ulvan polysaccharide (*Ulva lactuca*)	HCT-116	Upregulate P53, downregulate Bcl-2	+	(32)
Laminarin (*Fucus evanescens*)	HCT-116	Anti-Proliferation, inhibit MMP-2 and MMP-9 activity	-	(33)
κ-carrageenan (*Kappaphycus alvarezii*)	HCT-116	Induce apoptotic cell death, nuclear fragmentation and apoptosome formation, downregulate XIAP and PARP-1	G1	(34)
Fucoidan (*Fucus vesiculosus*)	HT-29 HCT-116	Increase Bax, caspase-3, PARP-1 and P21, decrease Bcl-2, Cyclin D1 and E, CDK2 and CDK4	G1	(36, 38, 47)
Fucoidan (*Fucus evanescens*)	HCT-116	Decrease TOPK kinase activity, inhibit phosphorylation of TOPK (Thr 9)	-	(39)
SG4 (*Sargassum glaucescens*)	HT-29	Increase cytochrome c release, caspase-9,−3 and DNA fragmentation, disrupt MMP	sub-G1, S, and G2/M	(41)
Fucoidan (*Sargassum cinereum*)	Caco-2	Increase ROS, induce chromatin condensation	-	(42)
Fucoxanthin (*Undaria pinnatidfida*)	Caco-2 DLD-1 HT-29	Upregulate apoptosis, downregulat DNA fragmentation	-	(48)
Fucoxanthin (*Laminaria japonica*)	WiDr HCT-116	Upregulate cell cycle arrest and apoptosis, up-regulation of p21WAF1/Cip1, downregulat proliferation	G0/G1	(44)
Fucoxanthin (Marine algae)	HCT-116 HT29	Upregulate DNA damage	+	(49)
Fucoxanthinol (Brown algae)	DLD-1 HCT-116	Upregulate anoikis and integrin β1, downregulat PPARγ, Akt activation	G1	(50)
Astaxanthin (Marine source)	WiDr	Downregulat proliferation, inhibiting the MYC-mediated downregulation of microRNA-29a-3p and microRNA-200a	-	(51)
ω-3 PUFAs	LOVO	Anti-Proliferation, induce phosphorylation of YAP	-	(52)
EPA	HCT-116	Suppress EGFR and VEGFR activation pathways, downregulate VEGF and HIF1α	-	(53)
DHA	HCT-8 HT-29 HCT-116 SW480	Upregulate TNFα, ERdj5 and caspase-4, downregulate microRNA-21, inhibit RIP1 kinase and AMP-activated protein kinase α	-	(54, 55)
ARA	HT-29	Induce ER stress and apoptosis, inhibit SREBP-1 activity and DNA replication	G1/S	(56, 57)
LA	LOVO CT-26	Upregulate microRNA-494, cytochrome c release, caspase-9,−3 and ROS, downregulate MYCC and PGC1α	S and G2/M	(58, 59)
Fucosterol	HT-29	Anti-Proliferation, upregulate P53, decrease cell viability	+	(60)
Fucosterol (*Sargassum angustifolium*)	HT-29	Induce cytotoxicity	-	(45)
Mertensene (*Pterocladiella capillacea*)	HT-29	Upregulate caspase-3 and cleavage of poly (ADP-ribose) polymerase (PARP), inhibit phosphorylation of P53, Rb, cdc2 and chkp2	G2/M	(46)
Meroterpenoids (*Cystoseira usneoides*)	HT-29	Inhibit phosphorylation of ERK, JKN and AKT	G2/M	(3)
Phloroglucinol	HT-29 HCT-116	Upregulate caspase-3 and caspase-8, inhibited the expression of Ras, Raf, mitogen-activated protein kinase, extracellular-signal regulated kinase phosphorylation, PI3K and Akt	G0/G1	(61)

+, effects reported; −, no effects reported.

structural compounds and supplying intercellular adhesion and signaling. The structural units of these natural polysaccharides are a mixture of sulfated linear galactans, which consist of disaccharides of α-(1,4)-linked D-galactopyranose (D) residues or 3,6-anhydrogalactopyranose (DA) and β-(1,3)-linked D-galactopyranose (G) residues (74). According to the concentration, position, and sulfation of 3,6-anhydrogalactose, they are categorized into κ, λ, ι, ν, m, and θ types (75). Polysaccharides with a molecular weight ranging from 500 to 1,000 kDa are present in most of them; however, up to 25% of them may contain polysaccharides at a molecular weight of <100 kDa (76). The significant anticancer and antitumor activities were found in the low-molecular-weight κ- and λ-carrageenan, probably attributed to their antiviral and antioxidant effects as well as the stimulation of immunity against tumors (77). Some studies have reported that the risk of colon cancer appears to be minimized with low-molecular-weight carrageenan, a type of functional food ingredient (34). Carrageenans from *Gigartina pistillata* (78), *Apostichopus japonicus* (79) and *Kappaphycus alvarezii* (80) have an anti-CRC effect on the colon cancer HT-29 cell line. Native carrageenan exerted high cell suppressive activity in colon cancer cells compared with commercial carrageenan. Raman et al. (34) examined the role of the κ-carrageenan-containing soluble dietary fiber fraction of red algae in human colon cancer HCT-116 cells.

However, conflicting studies have suggested that colitis and inflammation may be induced by carrageenan (81). Wei et al. (82) suggested that the existing intestinal inflammation was magnified and TNBS-induced intestinal inflammation was aggravated by κ-carrageenan *via* activating the TLR4-NF-κB and MAPK/ERK1/2 pathways, which indicates it might act as a potential pro-inflammatory factor. In addition, further studies from their group revealed that the LPS-induced inflammation can be synergistically activated by κ-carrageenan through the Bcl10-NF-κB pathway, as illustrated by the aggravation of *Citrobacter freundii* DBS100-induced colitis in mice treated with it (83). Mi et al. (84) investigated the effectiveness of the carrageenan intake form and host intestinal microecology on toxicity in C57BL/6J mice. The severity of colitis in high-fat diet-fed mice could be increased by native carrageenan from drinking water *via* decreasing the abundance of the anti-inflammatory bacterium *Akkermansia muciniphila* and increasing that of harmful bacteria. The inflammatory effect and secretion of proinflammatory cytokines in HT-29 cells can be increased and promoted by using the fermentation supernatants of κ-carrageenan oligosaccharides (85). The inflammatory property of κ-carrageenan oligosaccharides in the context of gut microbiota was evidenced by these results.

Fucoidan

Sulfated L-fucose present in the fibrous cell walls and intercellular spaces of brown seaweeds is a major component of fucoidan, which belongs to a large family of marine sulfated polysaccharides (86, 87). Fucoidan is a heparin-like molecule with a simple chemical structure composed of a repeating unit of disaccharides containing α-1,3-linked fucose and α-1,4-linked fucose with branches linked at the C2 positions (86). Generally, L-fucose polymerized with sulfated ester groups is present substantially in fucoidan, while galactose, glucose, mannose, xylose, and glucuronic acid residues are found in only a small proportion (88). Derivatives of fucoidan with a molecular weight varying from 40 to 330 kDa have been examined for their anticancer activities (89). Numerous experiments have shown that tumor cell proliferation and its growth or metastasis can be counteracted by fucoidan *via* eliciting cell apoptosis and suppressing angiogenesis (90). Health can be promoted and gut dysbiosis can be treated by fucoidan, a potential intestinal microbiota modulator. In addition, the effects of induced apoptosis in CRC cells by fucoidan have been evaluated (91).

In HT-29 colon cancer cells, cell viability was reduced by fucoidan in a dose- and time-dependent manner through reducing the expression of CDK2, CDK4, and Cyclin D1 (36). Apoptosis was also mediated by fucoidan *via* inhibition of the IGF-I/IGF-IR/IRS-1/PI3K/AKT (92) and PI3K-Akt-mTOR signaling pathways (37). A further study found that cell cycle arrest at G1-phase was induced by fucoidan *via* the upregulation of p21WAF1 and downregulation of Cyclin D1/CDK4 and Cyclin E/CDK2 expression (47).

In HCT-116 human colon cancer cells, CDK activity was suppressed by fucoidan *via* combining the CDK inhibitor proteins p21 and p27 with the Cyclin/CDK complexes (38). EGF-induced neoplastic cell transformation was significantly inhibited by fucoidan from *F. evanescens via* suppressing the TOPK/ERK1/2/MSK 1 signaling axis (39). In colon cancer cells DLD-1, fucoidan from *Sargassum mcclurei* was observed to be less cytotoxic and inhibited colony formation (40).

Fucoidan strongly regulated the mitochondrial membrane in cancer cells. The evidence found that apoptosis was caused by fucoidan through MMP loss, an increase in cytochrome c release and DNA fragmentation, activated caspase-9 and−3, and an increasing percentage of early and late apoptotic cells in HT-29 cells. Other biological studies indicated that apoptosis was induced by SG4 *via* involvement of the Akt/mTOR/S6 pathway in HT-29 cells (41). Another report showed that fucoidan from *Sargassum cinereum* suppressed the proliferation of Caco-2 cells in a dose-dependent manner, increased ROS production, and augmented mitochondrial membrane permeability (42).

In an animal model, tumor morbidity and average tumor weight were reduced and cellular apoptosis was increased by the treatment of dietary fucoidan in 1,2-

dimethylhydrazine (DMH)-induced colorectal carcinogenesis in rats. The expression of β-catenin, C-Myc, Cyclin D1, and Survivin was reduced by treatment with fucoidan, whereas the Hippo pathway was highly activated and the phosphorylation levels of Mst1, Mst2, LATS1, LATS2, and YAP were significantly upregulated (91).

Pigments

Generally, marine seaweed and animals are rich in pigments, which are widely used in functional food and pharmaceutical industries (93). There are mainly three types of pigments in seaweed involving chlorophylls, carotenoids, and phycobiliproteins (94). The seaweed color was determined by the content and type of pigments (95). For instance, chlorophylls a and b contribute to the green color in green seaweed, whereas allophycocyanin and c-phycoerythrin are responsible for the red color in red seaweed. β-carotene and fucoxanthin (Fx) are commonly observed in brown seaweed (96).

Tumor cells from CRC patients grow uninhibited in the body and enter the blood vessels to spread systemically (97). The apoptosis mechanism is strongly associated with antioxidant properties. Hence, anticancer compounds generally have antioxidant, antiangiogenic, and anti-inflammatory effects to regulate tumor development (98). A recent report showed that the strong antioxidant activity of carotenoids and chlorophyll a from green seaweed *Halimeda opuntia* against HT-29 human colorectal adenocarcinoma was investigated. The finding suggests that natural pigments are potential anticancer ingredients (43).

Carotenoids give seaweed colors from yellow to orangish (99), which of them possess strong activities involving Fx, astaxanthin (AXT), and violaxanthin (100). The evidence showed that carotenoids inhibited the PI3K/Akt apoptosis pathway, eventually integrating the mitochondrial membrane (101).

Fx is one of the most famous lipophilic carotenoids in brown algae, which is responsible for the strong antitumor property. Clinical trials reported that Fx decreased the causing risk of CRC, which has been found to Fx possess potential anti-CRC activity *via* downregulation of tumor-related proteins (102).

In cell experiments, Fx caused a markably decrease of the survival rate in Caco-2, HT-29, DLD-1 (48), and HCT-116 colorectal carcinoma cells (103). It was found that the activation of apoptosis and fragmentation of DNA contributes to the anticancer effect of Fx. Fx showed a significant antiproliferative effect by controlling the level of signaling proteins such as MAPK, NF-κB, and caspase family (99, 104). The disruption of cell cycle arrest causes cell apoptosis. Researchers found that Fx regulated sub-G1 cell cycle arrest in WiDr colon cancer cells (44). Furthermore, Fx stimulated cell cycle arrest at the G0/G1 or G2/M phases and caused programmed cell death (104). It is deduced that Fx induced cell cycle arrest and caused programmed cell death through enhancing intercellular communication between tumor cells.

The derivative compound of Fx was found that exists stronger anticancer activity than Fx. Chemical structural factors significantly influence the antiproliferative properties of Fx. The anti-CRC effect of Fx-degrading compounds was evaluated in Caco-2 cells and its activity may result in partial structures (105). The 13-cis and 13′-cis isomers of Fx showed a markably cancer-preventive effect compared to other derivatives (106). In another study, the antiproliferative effects of 5-fluorouracil (5-Fu) and Fx were determined and compared in HCT-116 and HT-29 cells (49). Fucoxanthinol (FxOH) is the deacetylated type of Fx, which can induce DLD-1 cell apoptosis into anoikis-like changes through the distribution of FAK and integrin β1 expression (50). The mechanism of the anti-CRC effect of FxOH was evaluated *via* regulation of MAPK and STAT apoptosis pathways in HT-29 and HCT-116 cell lines (107), and through inhibiting NF-κB activation in CRC cells (108). According to the previous reports, we found that the anti-CRC activity of FxOH is stronger than Fx, and FxOH induced cancer cells through downregulation of MAPK, STAT, and NF-κB apoptosis pathways. The anticancer effects of Fx and FxOH on six types of CRC cell lines and twenty kinds of tissues from surgically resected clinical CRC specimens were determined as well (109).

In a CRC model animal experiment, a continuous 5-week oral administration of Fx-rich fraction strongly inhibited the number of colorectal adenocarcinomas in DSS-treated male mice (110). Additionally, Fx significantly suppressed colon cancer in azoxymethane-dextrane sodium sulfate (AOM/DSS) carcinogenic model mice (111). In an inflammation-associated CRC mouse model, after a 4-month period of Fx administration, the multiplicity of colorectal adenocarcinoma was strongly decreased *via* upregulated anoikis-like integrin $\beta1^{low/-}$/cleaved caspase-3^{high} cells in colonic mucosal crypts (112). Additionally, Fx also markedly decreased HSP70 protein in colorectal mucosal crypts for 15-week administration (113). In a 14-week administration in a CRC mouse model, Fx markedly decreased CCR1, pAKT(Ser473), Cyclin D1, and pSmad2 compared with untreated mice (114). Therefore, Fx had chemopreventive potency and therapy ability in the progression of colorectal carcinogenesis in mice.

AXT is mainly contained in seaweed, and showed anti-metastatic activity through inhibiting microRNA-29a-3p and microRNA-200a, thereby downregulating MMP2 and ZEB1 (51). Natural AXT isolated from *Haematococcus pluvialis* showed a significantly anti-CRC effect in a dose-dependent manner in HCT-116 cells by regulating the ratio of Bax/Bcl-2 and upregulating the phosphorylation of p38, JNK, and ERK1/2 (115). AXT induced programmed cell death in DMH-induced rat colon carcinogenesis by modifying NF-κB, COX-2, MMPs-2/9, Akt, and ERK-2 expressions (116). A finding observed that AXT inhibits the progression of colonic premalignant lesions in

an obesity-associated colorectal carcinogenesis animal model by attenuating oxidative stress, reducing inflammation and NF-κB activation in the colonic mucosa (117). Hence, AXT is a potential cancer-preventive compound in the therapy of CRC.

Polyunsaturated fatty acids

Seaweeds are known as low-energy food. Despite their low lipid content, seaweeds contain ω-3 and ω-6 polyunsaturated fatty acids (PUFAs) as a significant portion of their lipids (118). PUFAs are the precursors of eicosanoids and all cell membrane components, which can effectively reduce the risk of cancer (119). Several studies have demonstrated that the colorectal tissue distribution of PUFAs is associated with CRC prognosis (120). There are studies suggesting the potential use of some oxidized metabolites of PUFAs as biomarkers of CRC (121).

ω-3 PUFAs

PUFAs in seaweeds contain a substantial amount of ω-3 fatty acids as major components. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in seaweed are important ω-3 fatty acids in the marine environment (122). At present, more and more epidemiology and experiments have verified the antitumor activity of ω-3 PUFAs (123). Consumption of diets rich in ω-3 PUFAs not only inhibits the proliferation of CRC cells, but also can be combined with chemotherapy to enhance their sensitivity (124).

PUFAs have pro-apoptotic and growth-inhibitory effects on cancer cells. In LoVo and RKO colon cancer cells, PUFAs can reduce the synthesis of PGE2 and LTB4, inhibit the expression of ALOX5, LTB4, mPGES, COX-2, and PGE2, and increase the expression of LXA4, thereby promoting apoptosis and inhibiting the growth of LoVo and RKO colon cancer cells (125).

In an animal model, dietary supplementation of ω-3 PUFAs increased CRC cell apoptosis and decreased the tumor incidence in AOM/DSS-induced CRC in mice. ω-3 PUFAs treatment activated the hippo pathway, with increased cytoplasmic retention and phosphorylation of YAP (mediated by LATS1 and MST1/2) and the levels of epoxydocosapentaenoic acids (52). Notably, dietary ω-3 PUFAs treatment suppressed the growth of MC38 colorectal tumors. In C57BL/6 mice, ω-3 PUFAs modulate eicosanoid and fatty acid metabolite profiles (126). Huang et al. (127) demonstrated that ω-3 PUFAs reduced tumor incidence in rats by regulation of the DNA methylation process. Together, these results support the notion that ω-3 PUFAs might contribute to the anti-CRC effects of seaweed.

EPA

There is evidence of the utility of ω-3 PUFA EPA in the treatment of CRC (128). In a clinical study, patients with familial adenomatous polyposis (FAP) were randomized to receive free fatty acid (FFA). Experimental data proved that EPA 2 g daily in the form of FFA has chemo-preventive efficacy in FAP patients (129). Recently, it has been shown that EPA can prevent FAP-related CRC by acting on several molecular mechanisms (129, 130).

The increased risk of colitis-associated colorectal cancer (CAC) is strongly associated with inflammatory bowel disease, but the effectiveness of dietary EPA-FFA in anti-inflammatory and anticancer activities is unclear. In EPA-FFA-treated AOM-DSS mice, Piazzi et al. (131) found an enrichment of *Lactobacillus species* in the gut microbiota, as well as restored Notch signaling and decreased nuclear β-catenin expression, while tumor cell apoptosis increased. Morin et al. (53) reported that the eicosapentaenoic acid monoglyceride (MAG-EPA) treatment increased HCT-116 cell apoptosis and decreased the tumor of a mouse xenograft model of HCT-116 *via* activating the vascular endothelial growth factor (VEGF) receptor pathway and decreasing the epidermal growth factor receptor (EGFR).

DHA

As an ω-3 PUFA, DHA has various biological properties, including anticancer activity (132). The anticancer effect of DHA might be a consequence of its ability to regulate the production of proinflammatory mediators in cancer cells and/or host cells, changing the inflammatory status of the systemic or tumor microenvironment (54, 133). Numerous studies have demonstrated that in CRC cells, the activity of DHA-triggered caspase family members is associated with apoptosis (134). Mechanisms including DHA-induced cellular protein expression explain the antitumor activity of DHA (55).

Fluckiger et al. (54) reported that the TNFα-dependent manner triggers apoptosis in HCT-116, HCT-8, and human CRC cells in DHA-mediated, and cells induced with DHA increased TNFα mRNA content by downregulating microRNA-21 expression, stating that the effect of TNFα on DHA-mediated apoptosis of colon cancer cells. Sarabi et al. (135) demonstrated that DHA could specifically attenuate promoter DNA methylation and VEGF protein levels of microRNA-126 in HCT-116, Caco-2, and CRC cells. Fasano et al. (55) reported that DHA treatment induced apoptosis in HT-29, HCT-116, and SW480 colon cancer cell lines and inhibited their total and surface GRP78 expression, suggesting that pERK1/2 could be the first upstream target of DHA. These studies offer insight into the epigenetic mechanisms by which DHA influences gene expression regulation in CRC cells.

ω-6 PUFAs

The ω-6 PUFAs contained in seaweeds include mostly linoleic acid (LA) and arachidonic acid (ARA) (136). Previous studies have suggested that, unlike ω-3 PUFAs, the ω-6 PUFAs, especially ARA, are generally associated with many adverse effects on the human body, including the promotion of multiple

cancer types, such as CRC (137, 138). However, there is little *in vitro* evidence to show that ARA exerts anticancer activity in CRC cells. Bae et al. (56) and González-Fernández et al. (57) evidenced that ARA may inhibit DNA replication and G1/S cell cycle transition and induce endoplasmic reticulum stress in HT-29 CRC cells, thereby suppressing cell viability and inducing apoptosis.

Research evidence shows that LA may be involved in both pro- and anticancer activities (137). Lu et al. (139) and Ohmori et al. (140) reported that LA at low concentrations (100-200 μM) reduced caspase-3 activation in CRC cells and promoted tumor cell proliferation. LA at high concentrations (above 300μM) enhanced ROS generation, caused mitochondrial dysfunction and inhibited tumor cell growth (58). A recent report from Ogata et al. (59) suggested that LA induced quiescence by promoting microRNA-494 expression, resulting in the dormancy of CT-26 CRC cells. Therefore, it is speculated that LA can inhibit the growth of CRC cells.

Phytosterols

Phytosterols are the major nutritional components of seaweed and the most important chemical constituents of algae (141). Phytosterols are the major lipid components of plant cell biofilms. In the marine environment, brown algae are a major source of phytosterols, including brassicosterols with a small amount of plant cholesterol and fucosanols (142). Not only can phytosterols lower cholesterol, they also have strong anticancer activity, and several studies have shown that foods rich in phytosterols may help control the growth of many types of tumors (143).

β-Sitosterol

β-Sitosterol, isolated from seaweed, is the most common dietary phytosterol and has a proven potential role in the treatment of CRC (144, 145). Shathviha et al. (146) reported on the evaluation of AgNP synthesized using β-sitosterol and its cytotoxic potential in HT-29 human colon cancer cells. β-Sitosterol-mediated AgNP treatments induced p53 expression and early apoptosis in HT-29 cells. Arul et al. (147) investigated the β-Sitosterol significantly reduces fecal bacteria and colonic bioconverting enzymes in mice with DMH-induced colon cancer, thereby preventing colon cancer development. Amplified activities of colonic biotransformation enzymes are considered hallmarks of colon carcinogenesis. Hence, β-sitosterol is a potential chemopreventive agent in colon carcinogenesis.

Fucosterol

The major phytosterol in brown seaweed is fucosterol, which has various biological activities, including anticancer activity (148). A previous study indicated that oxygenated fucosterol inhibited HCT-116 human colon cancer cell growth with higher cytotoxicity than commercial cytotoxic drugs (149). Ramos et al. (60) reported that the combination of fucosterol with 5-Fu can enhance the toxic effect in HT-29 cells. Furthermore, fucosterol is not toxic to normal cells, indicating specificity for cancer cells. The hexane fraction of fucosterol produced by *Sargassum angustifolium* confirmed its cytotoxic activity against HT-29 (45).

Terpenes

Within the marine environment, terpenes synthesized by algae and secreted to the outside of cells to resist environmental insults are major secondary metabolites from seaweeds (150). It is a chemical compound that contains one or more isoprene units with strong anticancer activity (15). Epidemiological and experimental studies suggest that terpenes may be helpful in curbing the growth of a variety of cancer cells, including colon cells, and provide additional opportunities for cancer therapy (151). Terpenes can modulate pathophysiological processes such as the cell cycle, invasion, migration, proliferation, and apoptosis in different types of tumor cells (152), exhibiting a wide spectrum of antitumor activities (153).

Previous studies have reported that a pentahalogenated monoterpene, halomon, isolated from the red seaweed *Portieria hornemannii*, exhibited strong anticancer activity (154). The halogenated monoterpene mertensene from the red seaweed *Pterocladiella capillacea* (S.G. Gmelin) Santelices & Hommersand inhibited LS174 and HT-29 human colorectal adenocarcinoma cell lines by activating caspase-3 and NF-κB, Akt, and MAPK ERK-1/-2 pathways vitality (46). Terpenes from the brown seaweed *Cystoseira usneoides* have anticancer effects on HT-29 colon cancer cells by reducing the phosphorylation levels of JNK and ERK and inhibiting the ERK/JNK/AKT signaling pathway (3). These reports demonstrate the potential of terpenes as drug candidates for the treatment of colon cancer.

Phenolics

Phenolic agents are one of the most active compounds in seaweed. Polyphenols with their high molecular weight, such as phlorotannins, specifically exist in brown algae (155). Phlorotannins are composed of a number of phloroglucinol (Ph) monomeric units (156). Those active compounds play a pivotal role in anti-CRC effects, for instance, as apoptotic, anti-metastatic, and antiangiogenic proprieties. They inhibited CRC cell growth directly or indirectly through attenuated inflammatory cytokines and oxidative stress (157). In other reports, the anticancer effects of Ph on insulin-like growth factor-1 receptor signaling in HT-29 human colon cancer cells

have been investigated. In addition, Ph inhibited the levels of Ras, mitogen-activated protein kinase, and mTOR (158). The polyphenol-rich agent showed a lower survival rate in CRC cells than the non-polyphenol-rich agent from seaweeds involving *Laminaria japonica*, *U. lactuca*, and *Porphyra tenera*. Additionally, the polyphenol-rich agent caused G0/G1 cell cycle arrest in HCT-116 cells (159). Phlorofucofuroeckol A (PFF-A) (160) and Ph (161), isolated from brown seaweed, decreased survival rates via activating the apoptosis pathway in CRC cells. Further, Ph decreased the survival rate dose-dependently and induced apoptosis in HT-29 cells, altering Bcl-2 and caspase family proteins (61). The evidence proved that phenolic agents play an important role in understanding the development of colon CRC.

Vitamins

Seaweed is an important source of various vitamins, among which vitamin C and vitamin D have strong anticancer activity (162). A previous study indicated a potential interplay of vitamin D and immune cells in the tumor microenvironment reduces CRC risk (163). Moreover, some research groups have reported an inverse association between vitamin D3 levels and CRC incidence, and that higher vitamin D3 levels reduce polyp recurrence and improve overall survival in CRC patients (164, 165). Tumor migration and proliferation were inhibited by vitamin D and its analogs in the colon of C57Bl/6 mice (166). Rawson et al. (167) suggested that vitamin D may alter CRC risk by mediating extracellular inhibition. Ferrer-Mayorga et al. (168) indicated that the vitamin D metabolite calcitriol promotes vitamin D receptor expression and inhibits fibroblasts (associated with colon cancer), resulting in anti-CRC effects. There is also a study on vitamin C from Yun et al. (169), who found that cultured CRC cells harboring KRAS or BRAF mutations were selectively killed when exposed to high levels of vitamin C.

Minerals

The minerals in seaweed are 10–20 times those of land plants and are easily bio-accumulated from seawater (12, 170). The macronutrients (e.g., magnesium, potassium, calcium, and sodium) and microelements (e.g., selenium, iodine, iron, manganese, and zinc) contained in seaweed are important for normal physiological functioning of the body and have potential relevance in cancer treatment (171, 172). Numerous clinical and epidemiological studies indicated that the risk of CRC may be reduced with a higher intake of calcium and magnesium (173). Ali et al. (174) evaluated the antitumor effect of selenium against DMH-induced CRC in BALB/C mice and its effect on apoptosis and angiogenesis. The group treated with DMH plus selenium exhibited significantly lower expression of cloned caudal-type homeobox gene-2 and VEGF but a higher caspase-3 expression level than the DMH-treated group.

Conclusions

Many studies have demonstrated the effect and mechanism of red, green, and brown seaweeds in CRC-prevention and therapy. Various therapeutic compounds from seaweed involving large molecular polysaccharides and small molecular pigments, fatty acids, phytosterols, terpenes, phenolics, vitamins and minerals induce programmed cell death *via* various signaling pathways. Such treatments can alter the protein expression of Bax, caspases, Bcl-2, MAPK, NF-κB, VEGF, DNA methylation, and CDK inhibitor and induce changes in the cell cycle and the CRC cellular functions of adhesion, migration, and invasion. Important anti-CRC agents such as polysaccharides and fatty acids existed for their antiproliferative and anti-metastatic effects *in vivo*. Furthermore, terpenes and pigments inhibited CRC cell survival rate and induced programmed cell death *via* regulation of the Akt signaling pathway.

A few molecular alterations in human CRC cells were correspondingly observed in CRC animal models treated with seaweed. Consecutive studies *in vitro* will be important as the basis for clarifying the molecular mechanisms underlying cancer prevention in humans with CRC and CRC animal models. CRC animal models, AOM-DSS, BALB/C, and C57BL/6 mice, have been the vehicles for many discoveries concerning the anti-CRC effects of seaweed. The oxidation, inflammation and gut microbiota, which are significant factors associated with colorectal carcinogenesis, have been reported to be prime targets of various therapeutic compounds from seaweed. In addition, the administration of Fx induced anoikis in CRC animal models. However, the detailed molecular mechanisms underlying the cancer chemopreventive effect in animals remain poorly understood.

Finally, this review highlights the importance of seaweed as a potential agent candidate for preventing CRC. However, the underlying mechanisms remain elusive. Further clinical investigations are needed to assess the anticancer effect of seaweed in humans.

Author contributions

YD and ZP conceptualized the idea and scope of the review. YZ, XZ, HY, and KZ both performed a literature review. YF and DX wrote the manuscript and created the figures. All authors critically revised and edited the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Mechanisms on chemotherapy resistance of colorectal cancer stem cells and research progress of reverse transformation: A mini-review

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Tumor recurrence and chemotherapy resistance are mainly responsible for poor prognosis in colorectal cancer (CRC) patients. Cancer stem cell (CSC) has been identified in many solid tumors, including CRC. Additionally, CSC cannot be completely killed during chemotherapy and develops resistance to chemotherapeutic drugs, which is the main reason for tumor recurrence. This study reviews the main mechanisms of CSC chemotherapy resistance in CRC, including activation of DNA damage checkpoints, epithelial-mesenchymal transition (EMT), inhibition of the overexpression of antiapoptotic regulatory factors, overexpression of ATP-binding cassette (ABC) transporters, maintenance of reactive oxygen species (ROS) levels, and the dormant state of CSC. Advances in research to reverse chemotherapy resistance are also discussed. Our study can provide the promising potential for eliminating CSC and preventing tumor progression for CRC treatment.

KEYWORDS

colorectal cancer, cancer stem cell, chemotherapy resistance, stem cells, reverse

Introduction

Colorectal cancer (CRC) is a worldwide disease, with 2.2 million CRC patients and 1.1 million deaths expected by 2030 (1, 2). Additionally, CRC is the most common malignant tumor of the lower digestive tract, with distinct genetic, epigenetic and phenotypic heterogeneity of tumor cells (3). Despite rapid advances in diagnosis methods, surgery, and chemotherapeutic agents, the prognosis of CRC patients remains poor (4). Tumor recurrence and cancer chemotherapy resistance are leading causes of poor prognosis (5). Inhibition of tumor apoptosis, changes in targeted sites of chemotherapeutic agents, tumor cell heterotrophy, and cancer stem cells (CSCs) can lead to chemotherapy resistance, while CSCs are the key factor for chemotherapy resistance (6). Accounting for about 5% of total tumor cells, CSC is a special cell population capable of self-renewal, multi-lineage differentiation, cloning, tumor initiation, maintenance of tumor characteristics, metastasis, and proliferation (7). In addition, CSC has been

identified in various cancers, including breast, colorectal, pancreatic, lung, prostate and brain cancers (8, 9). Chemotherapy resistance in the CRC stem cell (CRCSC) have diverse mechanisms (Figure 1), mainly including activation of DNA damage checkpoints, epithelial-mesenchymal transition (EMT), inhibition of the overexpression of antiapoptotic regulatory factors, overexpression of ATP-binding cassette (ABC) transporters, and maintenance of reactive oxygen species (ROS) levels. In recent years, more and more researchers have focused on natural drug extracts and CSC-related inhibitors, which can effectively remove CSC and reverse chemotherapy resistance (10). This study reviews the mechanism and the reversal of CRCSC chemotherapy resistance.

Mechanisms of CRCSC chemotherapy resistance

Activation of DNA damage checkpoints

CSC can activate DNA damage checkpoints preferentially in response to DNA damage caused by DNA toxic drugs, thereby improving DNA repair. For example, $CD133^+$ hepatic stem cells preferentially express survival proteins related to the Akt/PKB and Bcl-2 pathways, thereby leading to the chemotherapy resistance of cancer cells to adriamycin and 5-fluorouracil (5-FU) (11, 12). Additionally, DNA damage response may act as a target for sensitizing CSC to overcome chemotherapy resistance (13). Another study suggested that 70% of ovarian cancer patients developed relapse and resistance after platinum-based chemotherapy (14). Methoxyphenyl chalcone can play a role in DNA damage signal-evoking potential that can reverse the chemotherapy resistance. Moreira et al. found that induction of DNA double-strand breaks can effectively kill CSCs, which is vital for overcoming multiple conventional chemotherapy resistance in CRC (15).

EMT

The association between EMT and chemotherapy resistance has been discussed for a long time, but the mechanism is still elusive. Some researchers hold the opinion that cells undergo EMT process have a stem-cell like property, thus sharing the key signaling pathways and drug resistance characteristics with CSC (16, 17). Other important mechanisms related to EMT-induced drug resistance mainly include the gain of cellular resistance to drug-driven apoptosis (18) and associated tumor microenvironment (19). For example, cancer-associated fibroblasts and hypoxia can activate the EMT process of cancer cells and induced drug resistance (18). EMT plays an important role in epigenetic changes in CRC cells and is also associated with the self-renewal ability, tumor heterogeneity, and chemotherapy resistance of CSC. A study showed that hyaluronic acid synthases led to the loss of epithelial traits in tumor cells, which further induced malignant tumors and created a suitable niche for CSC generation (20). The study indicated the fundamental role of EMT in tumor progression and chemotherapy resistance of CSC. Pathak et al. found that downregulation of the mitochondrial $Na^+/Ca^{2+}/Li^+$ exchanger (NCLX) facilitated the metastasis of CRC cells, EMT changes, hypoxia, chemoresistance, and stem cell pathways (21). However, the study did not reveal a direct mechanism between EMT changes and chemoresistance of CSC.

Inhibition of the overexpression of antiapoptotic regulatory factors

When CSC is exposed to chemotherapeutic agents *in vivo*, some special regulatory mechanisms will be activated; pi3-K, MAPK and other pathways will be cascade-activated, and then the anti-apoptotic protein myeloid cell leukemia-1 (McL-1) will increase to inhibit the apoptosis of tumor cells (22). Many studies have suggested that inhibiting the activity of CSCs can increase the apoptosis of tumor cells (23–25). In another study by Zhang et al., CRCSCs were inhibited by pitavastatin, and the apoptosis of colon carcinoma cells was increased (26). However, detailed mechanisms of CSC against the expression of antiapoptotic regulatory factors remain unknown.

Overexpression of ABC transporters

Another potentially important mechanism of CSC leading to chemotherapy resistance is the high expression of ABC transporters. In CSC, the high expression of the ATP-dependent efflux pump ABCG2 enabled it to effectively extract Hoechst 33342, which is a kind of DNA minor groove binder used fluorochrome for visualizing cellular DNA, from cells (27, 28). This export ability is provided by the ABC transporter, which helps to resist cytotoxic drugs. The ABC transporter can export certain chemicals and drugs from cells, resulting in multidrug resistance (29). Stem-like side population tumor cells are key to the cause of drug resistance. A study indicated that the expression of ABCG2 in side population cells within tumors with stem-like properties was higher than that in non-side population tumor cells. Glucose in the microenvironment could further up-regulate the expression of ABCG2 (30). Because the high expression of ABC transporter allowed cells to effectively pump traditional chemotherapeutic drugs out of cells, the high ABCG2 level in the side population made it resistant to many chemotherapeutic drugs (30). Therefore, inhibition of ABC pump function may be a potential strategy to overcome CSC resistance. Although many ABC transporter inhibitors have been shown to sensitize cancer cells to chemotherapeutic drugs

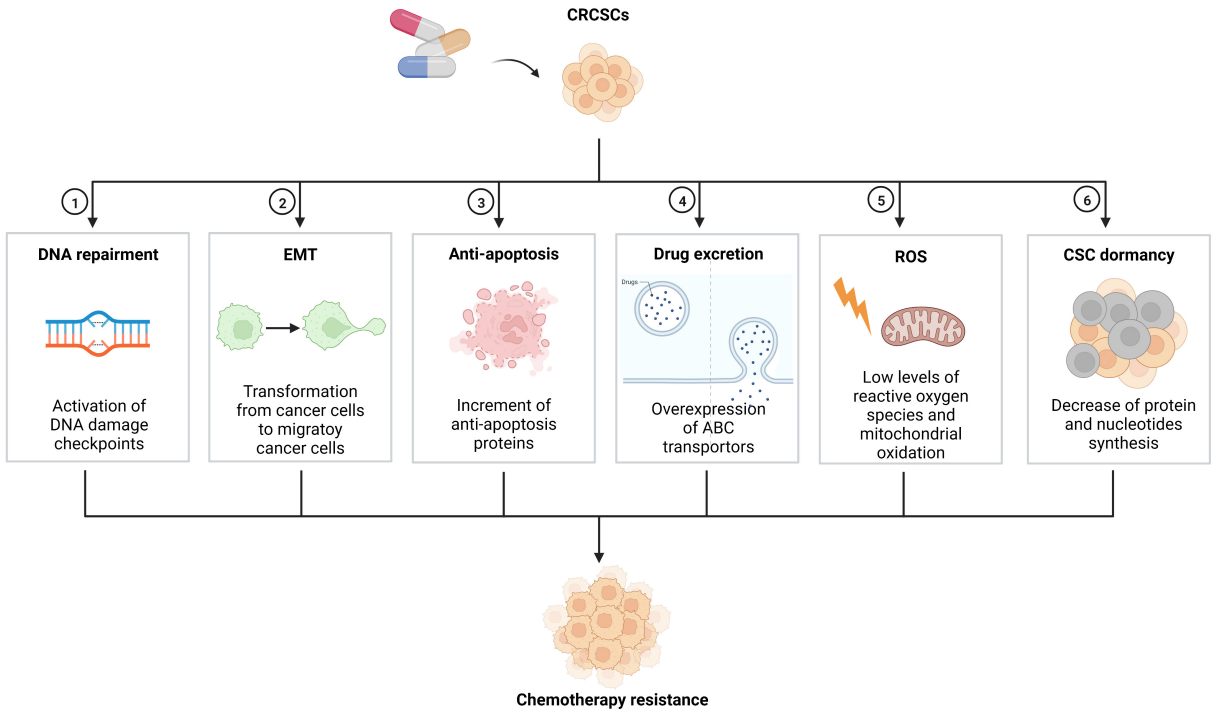

FIGURE 1
Mechanisms on chemotherapy resistance of CRCSCs. CRCSCs, colorectal cancer stem cells; EMT, epithelial-mesenchymal transition; ABC, ATP-binding cassette; ROS, reactive oxygen species; CSC, cancer stem cell.

in vitro, their effectiveness has failed to demonstrate in most clinical trials (31).

Maintenance of ROS levels

ROS, such as superoxide anion and hydrogen peroxide, is the product of normal oxidative metabolism and is involved in many cellular signaling processes. High ROS levels can promote cell migration and differentiation, which impairs the long-term reproduction and survival of tumor cells (32). Normally, ROS maintains at a low level in CSC by increasing glycolysis and reducing mitochondrial oxidation and ROS production (33, 34). Chemotherapy resistance caused by overexpression of multidrug resistance molecules can be overcome by inhibiting glycolytic consumption of cellular ATP (35). Low ROS levels in CSC can support its survival; conversely, excessively high ROS levels may trigger the death of CSC (36). The low ROS level in CSC is partly due to the high expression of free radical scavenger molecules such as glutathione (37). Glutathione participates in cell detoxification by binding to toxic chemicals and certain chemotherapeutic agents (such as cisplatin) and facilitating their export from cells (38, 39). A recent study indicated that intravenous vitamin C combined with traditional cancer treatment significantly decelerated cancer progression (40). Vitamin C promoted oxidation by increasing intracellular ROS levels, inducing endoplasmic reticulum stress, and inhibiting the production of angiogenic factors and insulin-like growth factors. As a result, high-dose vitamin C alone or in combination with chemotherapy (e.g., paclitaxel, cisplatin, carboplatin, and azacytidine) may increase ROS levels in CRCSC and CRC to inhibit tumor.

The dormant state of CSC

Another factor contributing to the drug resistance of CSC is the quiescent or dormant state (41, 42). A metabonomics analysis of CRC showed a significantly down-regulated synthesis of protein in colo205 $CD133^{+}$ CRC cells compared with CD133 cells and the reduced synthesis of nucleotides such as cholesterol and glucose-dependent lipid (43). The unique metabolic characteristics of CRCSC exhibit a slow circulation property that leads to chemotherapy resistance. Because many chemotherapeutic drugs preferentially kill fast-growing cells, tumor cells are more active in DNA replication and are highly sensitive to DNA damage agents. In contrast, relatively dormant CRCSCs are unlikely to induce non-replicating functional DNA in non-circulating cells, so they are insensitive to DNA damage agents (44). Moreover, CSC will have more time to repair DNA

damage and stay alive. Therefore, even though most circulating tumor cells can be killed by chemotherapy, residual CSCs can enter the cell cycle and cause tumor recurrence (45).

The research progress of reverse transformation

Natural agents

Curcumin, a plant polyphenol, is the most important component of ginger (46). Recent studies have not only proved the effect of turmeric in traditional Chinese medicine but also suggested some new pharmacological effects, such as anti-inflammatory, antioxidant, oxygen free radical scavenging, anti-human immunodeficiency virus, liver and kidney protection, anti-fibrosis and anti-cancer effects (47). In recent years, the chemotherapy resistance reverse effect of curcumin in CRC has attracted increasing attention. Kanwar et al. confirmed that curcumin combined with traditional chemotherapeutic agents 5-FU and oxaliplatin reduced the expression of CD44 and CD166 in chemo-resistant colon cancer cells, inhibited tumor growth, and promoted apoptosis in tumor tissue (48). Detailed results of mechanisms revealed that curcumin combined with 5-FU and oxaliplatin could prevent the growth of CSC-enriched chemo-resistant CRC cells by inhibiting epidermal growth factor receptor (EGFR) and insulin-like growth factor 1 receptor (IGF-1R) signaling pathways. Curcumin has been proved to enhance the sensitivity of drug-resistant CRC cells to many traditional chemotherapeutic agents by eliminating CSC (49, 50). Another study demonstrated that curcumin could enhance the chemotherapy efficacy of 5-FU on HCT116 cells, indicating that curcumin may help to treat CRC and overcome chemotherapy resistance (51). Further insights into the mechanism demonstrated that curcumin could inhibit multiple CSC pathways, suggesting its anti-CSC potential in CRC treatment (49).

Salvianolic acid B (SALB), a water-soluble phenolic compound extracted from Salvia miltiorrhiza, can reverse chemotherapy resistance and improve the clinical treatment effect of CRC. Guo et al. developed a nude mouse model bearing human colon CSCs and investigated the effect of SALB on chemotherapy resistance reversal and related mechanisms (52). The nude mice were transplanted with LoVo and HCT-116 colon CSCs to establish an animal model that could exhibit chemotherapy resistance. The results revealed that SALB reversed chemotherapy resistance to 5-FU and oxaliplatin and inhibited tumor growth by suppressing the expression of stemness markers, such as CD44, CD133, and the transcription factor sox-2 (SOX2). In addition, SALB has been proven to target CSCs *in vitro* and *in vivo* and prevent tumor progression by modulating the IL-6/STAT3/NF-κB signaling pathway (53).

Aloysia polystachya (AP) is a medicinal plant extract widely used to treat various diseases. Additionally, CSC is highly associated with tumor invasiveness, chemotherapy resistance and cell death. It was found that AP significantly reduced the invasiveness of HCT116 and CT26 cell lines and the number of tumorspheres compared with the control group (54). When HCT116 and CT26 cells were treated with 5-FU and AP, their sensitivity to low concentrations of 5-FU was increased by AP. These results suggested that the inhibition effect of AP on CSC might be one of the mechanisms to reverse 5-FU resistance.

Inhibitors

Regorafenib is an approved specific multikinase receptor inhibitor for the treatment of metastatic CRC. Cai et al. developed two 5-FU resistance CRC cell lines, HCT-116R and DLD-1R, to evaluate regorafenib inhibition of CRCSCs (55). Combined with 5-FU, regorafenib suppressed tumorigenesis and stemness markers in DLD-1R cell lines. Moreover, regorafenib increased the miR-34a levels and induced the reverse transformation of drug resistance. In another study, researchers implanted human colon cancer cells KM12SM and mesenchymal stem cells (MSCs) into the cecal wall of nude mice, which could provide tumors with abundant stromal components and improve invasion and metastasis ability and drug resistance (56). The results indicated that regorafenib could affect the interaction of tumor cell-MSCs and further inhibit CRC progression.

Conclusion

CSC has been found in many solid tumors, such as CRC, breast cancer, pancreatic cancer, and lung cancer, and is considered a promising target for cancer treatment (7, 8). It cannot be completely killed during chemotherapy and develops resistance to chemotherapeutic drugs, which is mainly responsible for tumor recurrence, metastasis and poor prognosis. This study reviews the main mechanisms of CSC chemotherapy resistance in CRC, including activation of DNA damage checkpoints, EMT, inhibition of the overexpression of antiapoptotic regulatory factors, overexpression of ABC transporters, maintenance of ROS levels, and the dormant state of CSC. Natural plant extracts (e.g., curcumin, SALB, and AP) and specific multikinase receptors (e.g., regorafenib) exhibit promising potential in eliminating CSC and preventing tumor progression.

More work is needed for CRCSC chemotherapy reverse transformation. First, researchers should find more specific markers to distinguish CSC from normal stem cells because CSC shares molecular similarities with embryonic stem cells and

MSCs, which limits the potential for targeted therapy. Second, more research should be performed to significantly improve the delivery efficiency of effective drugs to targeted cells and reduce the side effects of chemotherapy, which provides a new direction for targeted therapy of CRC. Third, more natural drugs and their extracts should be studied to screen out the best natural drug, drug dosage and delivery mode and use in the modern treatment of CRC patients. Fourth, studies on inhibitors of related enzymes for treating CRC should receive more attention. Fifth, more animal and clinical studies should be performed to provide a theoretical basis for the reverse transformation of chemotherapy resistance of CRCSC using specific natural agents and inhibitors.

Author contributions

LC drafted the review. FY and SC generated the graph and guided the construction of the manuscript. JT edited the review. All the authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Predicting lymph node metastasis and recurrence in patients with early stage colorectal cancer

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Tumor budding (TB), a powerful, independent predictor of colorectal cancer (CRC), is important for making appropriate treatment decisions. Currently, TB is assessed only using the tumor bud count (TBC). In this study, we aimed to develop a novel prediction model, which includes different TB features, for lymph node metastasis (LNM) and local recurrence in patients with pT1 CRC. Enrolled patients (n = 354) were stratified into training and validation cohorts. Independent predictors of LNM and recurrence were identified to generate predictive nomograms that were assessed using the area under the receiver operating characteristic (AUROC) and decision curve analysis (DCA). Seven LNM predictors [gross type, histological grade, lymphovascular invasion (LVI), stroma type, TBC, TB mitosis, and TB CDX2 expression] were identified in the training cohort. LNM, histology grade, LVI, TBC, stroma type, and TB mitosis were independent predictors of recurrence. We constructed an LNM predictive nomogram with a high clinical application value using the DCA. Additionally, a nomogram predicting recurrence-free survival (RFS) was constructed. It presented an AUROC value of 0.944 for the training cohort. These models may assist surgeons in making treatment decisions. In the high-risk group, radical surgery with a postoperative adjuvant chemotherapy was associated with RFS. Postoperative chemotherapy can be better for high-risk patients with pT1 CRC. We showed that TB features besides TBC play important roles in CRC pathogenesis, and our study provides prognostic information to guide the clinical management of patients with early stage CRC.

KEYWORDS

colorectal cancer, tumor budding, lymph node metastasis, predictive nomogram, recurrence-free survival, CDX2, risk stratification

Introduction

Owing to recent advances in diagnosis and treatment techniques, endoscopic resection has become the first choice of treatment for early stage colorectal cancer (CRC). However, the optimal management of such excisable tumors is still undefined because of potential metastases; thus, additional surgical resection is necessary to assess nodal status, but the frequency of lymph nodal metastasis (LNM) is relatively low (1). Previous studies have presented guidelines and proposed specific indicators for recommending completion surgery after endoscopic excision to prevent LNM or recurrence (2, 3). However, only approximately 10% (4–6) of patients who were referred for additional surgery based on these guidelines required it. Traditional pathological indicators are not sufficient to identify the need for additional surgery (7). Therefore, reliable criteria to assess patients requiring surgery are crucial.

Besides the resection margin, other promising indicators for additional surgical intervention include the tumor grade, lymphovascular invasion (LVI), and tumor budding (TB). The latter is characterized by the dissociation of small tumor complexes containing up to four cells that "bud" into the intratumoral or peritumoral stroma. TB is associated with a high risk of LNM in patients with pT1 CRC. Consequently, patients with pT1 CRC marked by prominent TB may benefit from additional surgical resection (8, 9). TB assessed in pre-operative biopsies could predict tumor regression for neo-adjuvant chemotherapy (10). Furthermore, high-level TB is a high-risk factor for patients with stage II CRC, and thus can warrant the consideration of adjuvant chemotherapy. Therefore, further studies are needed to determine whether TB assessments can help guide high-risk patients with pT1 CRC to undergo postoperative adjuvant chemotherapy to improve outcomes. The International Tumor Budding Consensus Conference (ITBCC) guidelines provide a standardized counting system for routine reporting. However, several factors should be considered when using the ITBCC TB scoring system in routine practice. First, the current ITBCC three-tier system (Bd1, Bd2, and Bd3) is the same for all stages of CRC. Both Bd2 and Bd3 are considered high-risk factors for LNM in pT1 CRC, whereas in stage II CRCs, only Bd3 is a risk factor for poor survival. Second, reporting of the absolute number of tumor buds is recommended, although inconsistency in tumor bud counts among pathologists may lead to differences in clinical management. Finally, the current TB assessment system focuses only on the tumor bud count and does not account for other features of TB, including structure, location, cell atypia, stroma type, tumor bud cell mitosis, and the immunohistochemical phenotype of the tumor bud cells. Including these other parameters in predictive models could improve the risk stratification power and prognostic value of TB and its various features.

In this retrospective study, we aimed to analyze the clinicopathologic characteristics to evaluate the risk stratification utility of TB features in early stage CRC. Furthermore, we developed a novel nomogram, including different characteristics of TB, to guide adjuvant chemotherapy in patients with early stage CRC. This approach could be combined with traditional clinicopathological indicators to assist surgeons in choosing the most suitable operation for patients with early stage CRC.

Materials and methods

Patients

This retrospective study included 354 consecutive patients who were pathologically diagnosed with pT1 CRC and who underwent radical surgery between January 2010 and December 2018 in the First Hospital of Jilin University (Changchun, China). Sixty patients received chemotherapy with fluorouracil plus oxaliplatin after surgery. We excluded patients who (i) underwent only endoscopic excision; (ii) with missing follow-up data; (iii) with specific histological subtypes of adenocarcinomas, such as poorly cohesive carcinoma, signet-ring cell carcinoma, micropapillary adenocarcinoma, mucinous adenocarcinoma, and medullary adenocarcinoma; and (iv) with more complicated or advanced CRC, higher than stage T1. The study protocol was approved by the institutional ethics committee of Jilin University First Hospital. The need for written informed consent was waived because of the retrospective nature of the study.

Histology

Hematoxylin and eosin-stained slides were reviewed by two pathologists. All slides were reviewed in a double-blinded manner, without knowledge of the corresponding pathological diagnoses. The initial clinical and pathological stages of the disease in all patients were revised according to the American Joint Committee on Cancer staging system (eighth edition). Histological type and grade were defined according to the latest World Health Organization classification system. In all specimens, the following histological features were evaluated: the LVI, predominant structure of tumor bud (cluster or single-cell), predominant location of TB (peritumoral or intratumoral budding), and tumor bud cell atypia (non-specific or anaplasia-like; anaplasia was defined as any $\times$ 400 magnification field with $\geq$ 3 nuclei with diameters equal to or greater than 5 lymphocyte nuclei) (11), stroma type (inflammatory, fibrotic, or myxoid; the predominant feature was recorded), mitosis in tumor bud cells, and tumor bud count. TB was defined as the dissociation

of small tumor complexes containing more than five cells that "budded" into the intratumoral or peritumoral stroma. TB was scored by two independent pathologists according to the ITBCC guidelines (12). Hematoxylin and eosin-stained sections were evaluated at medium magnification (× 10) to determine the densest area of TB at the invasive tumor front ("hotspot"). To reduce interobserver variability, TB features were independently evaluated by two single-blinded pathologists. The final classification of TB features was determined based on agreement among at least two pathologists.

Immunohistochemistry

Immunohistochemistry was performed as described previously (13). Tissue sections were stained using the following primary antibodies: rabbit monoclonal CDX2 (EP25; Zhongshan Golden Bridge Biotechnology LLC, Beijing, China; ready-to-use); Ki-67 (30-9; Ventana, Tucson, AZ, United States; ready-to-use), epidermal growth factor receptor (EGFR; EP22; Zhongshan Golden Bridge Biotechnology LLC; ready-to-use), p53 (4A4 + UMAB4; Zhongshan Golden Bridge Biotechnology LLC; ready-to-use), BRAF V600E (VE1; Ventana; ready-to-use), and microsatellite instability (MSI) proteins, including MLH1 (ES05), PMS2 (EP51), MSH2 (RED2), and MSH6 (EP49) (Zhongshan Golden Bridge Biotechnology LLC; ready-to-use).

CDX2 and EGFR immunohistochemical staining were performed as described previously (14). The extent of tumor bed cell staining (0–100%) and the staining intensity (0, negative; 1, weak brown; 2, brown; and 3, dark brown) were evaluated. The final scores were defined as the product of the extent and intensity scores. Next, each case was scored as high or low, using the median final score as the cut-off point for the following test. The immunohistochemical staining patterns of p53 were classified into two subgroups: (a) wild-type pattern, indicated by scattered nuclear staining in tumor cells, and (b) mutant-type pattern, in which the majority of tumor cells (> 60%) showed diffuse strong nuclear positivity or were completely devoid of any staining. Only staining for the expression of cytoplasmic BRAF V600E was considered positive. The MSI status was classified into two subgroups: (a) MSI-high, if any one of the four mismatch repair proteins (MLH1, PMS2, MSH2, and MSH6) was nuclear negative in all tumor cells, but positive in internal controls; and (b) MSI-low, if all four mismatch repair proteins were positive in cancer cells. The p53- and BRAF-staining patterns and MSI status were reported by two single-blinded observers.

Statistical analysis

The clinicopathological findings of the CRC specimens were compared using the chi-square or Fisher's exact test for categorical variables. The non-parametric Mann–Whitney *U* test was used to analyze age, Ki67 labeling index, and TBC datasets because of their non-normal distribution.

Multivariate logistic and Cox regression analyses were used to identify significant independent factors for predicting LNM or recurrence-free survival (RFS). Variables with $P < 0.1$ in the univariate analysis were included in the multivariate analysis model. The RFS of the patients was analyzed using the Kaplan–Meier method and log-rank test. *P* values were obtained using two-tailed statistical analyses, and the significance level was set at 5% ($P < 0.05$). R software (version 4.1.0[1]) was used for all statistical analyses. The R statistical packages "rms," "barplot," "survival," "Hmisc," "MASS," and "pROC" were used to plot the distribution of risk scores and recurrence or distant metastasis, plot calibration, generate receiver operating characteristic curve, build a nomogram, and draw Kaplan–Meier curves. The package "rmda" was used to draw the decision curve analysis (DCA) curves, and "forestplot" was used to draw the forest plot.

Results

Demographic and clinicopathological findings

The baseline clinicopathological characteristics of the participants ($n = 354$) are summarized in **Table 1**. LNM was present in 49 (13.8%) patients (mean age ± standard deviation = 65.2 ± 10.3 years; range = 30–91 years). Recurrence was observed in 38 (10.7%) patients, and the follow-up period was 37.4 ± 16.3 months (range = 14.2–59.9 months).

Evaluation and validation of the lymph node metastasis predictive nomogram

In total, 354 patients were included and randomly allocated to a training cohort ($n = 234$) and validation cohort ($n = 120$) at a ratio of approximately 2–1 based on the data splitting approach. Based on the univariate logistic regression analysis results of the training cohort, seven factors, namely general tumor type, histology grade (**Figure 1A**), LVI (**Figure 1B**), tumor bud stroma (**Figures 1C,D**), tumor bud count, tumor bud cell mitosis (**Figure 1E**), and CDX2 expression (**Figure 1F**), were linked to the LNM status (**Figure 2A**).

General tumor type [pedunculated vs. non-pedunculated; odds ratio (OR) = 0.641; 95% confidence interval (CI) = 0.098–4.185], histological grade (high-grade vs. low-grade; OR = 5.561;

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TABLE 1 Demographics of surgery of 354 patients with pT1 CRC who underwent surgical resection.

Variable		All patients
Age (years)*		65.2 ± 10.3 [30–91]
Sex	Female	131 (37.0%)
	Male	223 (63.0%)
LNM	Absent	305 (86.2%)
	Present	49 (13.8%)
Gross tumor type	Non-pedunculated	161 (45.5%)
	Pedunculated	193 (54.5%)
TP53	Wild-type	27 (7.6%)
	Mutant-type	156 (44.1%)
	-	171 (48.3%)
MSI	MSI-high	11 (3.1%)
	MSI-low	172 (48.6%)
	-	171 (48.3%)
BRAF	Absent	182 (51.4%)
	Present	1 (0.3%)
	-	171 (48.3%)
Ki67 (%)*		78.7 ± 13.2 [5.0–95.0]
Histology grade	Low-grade	339 (95.8%)
	High-grade	15 (4.2%)
Lymph-vascular invasion	Absent	315 (89.0%)
	Present	39 (11.0%)
TB construction	Cluster	201 (56.8%)
	Single	153 (43.2%)
TB location	ITB	118 (33.4%)
	PTB	236 (66.7%)
TB atypia	Non-specific	311 (87.9%)
	Anaplasia-like	43 (12.1%)
TB stroma	Inflammation	100 (28.3%)
	Fibrosis	192 (54.2%)
	Myxoid	62 (17.5%)
TB mitosis	Absent	298 (84.2%)
	Present	56 (15.8%)
TB quantity*		10.7 ± 3.7 [0.0–18.0]
TB CDX2 status	Negative	87 (24.6%)
	Positive	267 (75.4%)
TB EGFR status	Negative	50 (14.1%)
	Positive	304 (85.9%)
Recurrence	Absent	316 (89.3%)
	Present	38 (10.7%)

*Data are mean ± standard deviation. MSI, microsatellite instability; TB, tumor budding.

95% CI = 1.933–16.003), LVI (present vs. absent; OR = 34.194; 95% CI = 9.511–122.930), tumor bud cell stroma type (myxoid vs. inflammatory; OR = 6.746; 95% CI = 1.831–24.851), tumor bud count (high vs. low; OR = 63.429; 95% CI = 14.623–275.130), TB mitosis (present vs. absent; OR = 2.770; 95% CI = 0.643–11.925), and TB CDX2 expression status (negative vs. positive; OR = 15.919; 95% CI = 4.259–59.494) were independent predictors of recurrence in the multivariate analyses (Table 2 and Figure 2B).

The calibration curve of the LNM nomogram was highly consistent with the standard curve, indicating the high reliability of the predictive ability of the nomogram (Figures 2C,D). The DCA curves for the developed LNM nomogram (Figure 2E) and tumor bud count (Figure 2F) in the training and validation cohorts indicated that the DCA of the predictive nomogram had higher net benefits than the tumor bud count, indicating higher clinical application value.

Evaluation and validation of the recurrence-free survival prediction nomogram

Cox univariate and multivariate regression analyses were performed in the training cohort to identify the variables for building the RFS predictive nomogram. RFS was significantly associated with LNM, general tumor type, histology grade, LVI, stroma type, tumor bud count, tumor bud cell mitosis, and CDX2 expression status (Figure 3A). In the multivariate Cox proportional hazards model, LNM, histology grade, LVI, TBC, stroma type, and TB mitosis were independent predictors of local recurrence (Table 3). These variables were used to build a predictive nomogram for RFS (Figure 3B). Calibration curves based on the six variables are shown in Figures 3C,D. There was a positive agreement between the nomogram-predicted and actual probabilities of 5-year RFS in the training and validation cohorts, respectively. The predictive ability of the RFS nomogram was evaluated by analyzing the area under the ROC (AUROC). The nomograms displayed discriminatory power in predicting the postoperative RFS in the training cohort. The C-indices of the RFS nomogram and tumor bud count were 0.944 (95% CI = 0.934–0.952) and 0.689 (95% CI = 0.642–0.736), respectively (Figures 3E,F).

Based on the nomogram score, patients were stratified into low- (score $\leq$ 160) and high-risk (score $>$ 160) for recurrence and mortality, respectively. We used Kaplan–Meier curves and the log-rank test to analyze RFS in patients with pT1 CRC after stratification (low-risk vs. high-risk) using the nomogram ($p < 0.001$; Figure 4A). In the high-risk group, patients who only underwent radical surgery had a lower RFS ($p = 0.029$; Figure 4B), compared to that of patients who underwent radical surgery and a postoperative chemotherapy.

Discussion

Although the traditional TNM staging system remains essential for risk stratification in patients with CRC, the heterogeneity in survival rates within the same stages indicates the need for additional prognostic biomarkers. Furthermore, the

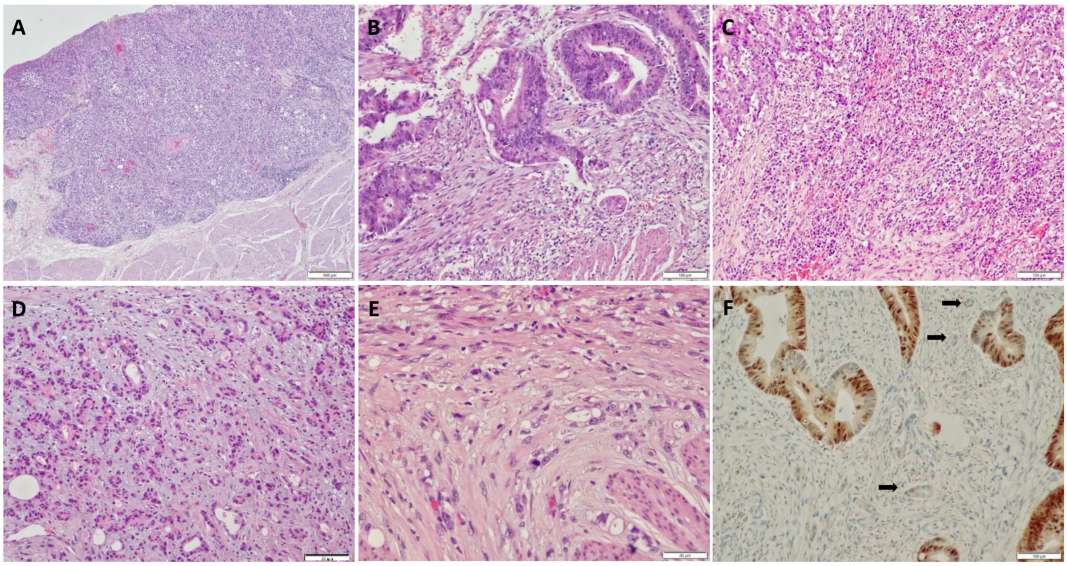

FIGURE 1
Histological and immunohistochemical features of pT1 colorectal cancer (CRC). **(A)** High histology grade; **(B)** lymph-vascular invasion observed in a biopsy specimen; **(C)** inflammatory stroma surrounding tumor budding (TB); **(D)** myxoid stroma surrounding TB; **(E)** mitosis present in TB; **(F)** CDX2 expression in tumor cells, while loss of expression in TB.

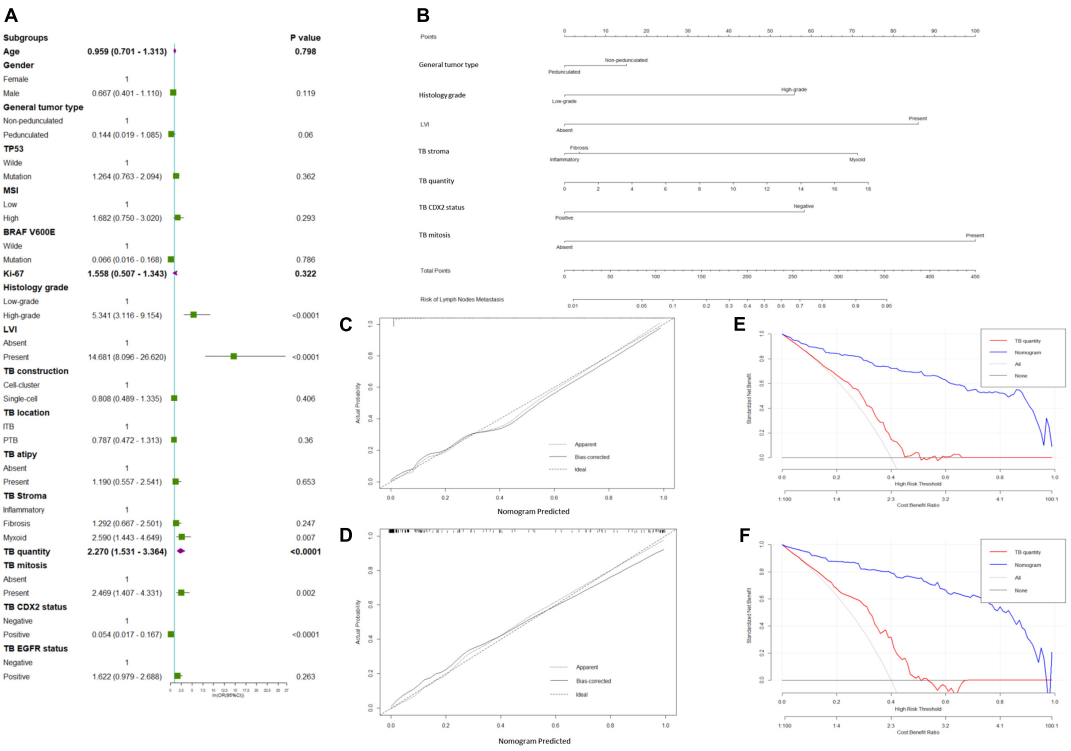

FIGURE 2
Predicted model of lymph node metastasis (LNM). **(A)** Forest plots to decipher the risk factors associated with LNM identified in the univariate logistic regression analysis; **(B)** newly developed nomogram for predicting LNM in patients with pT1 CRC. The calibration curve for predicting LNM of pT1 CRCs in the **(C)** training and **(D)** validation cohorts. Decision curve analysis of the nomogram and TB quantity alone for predicting LNM in patients with pT1 CRC in the **(E)** training cohort and **(F)** validation cohort.

TABLE 2 Multivariate logistic regression analysis of lymph node metastasis.

	Training cohort (n = 234)		Validation cohort (n = 120)	
	OR (95% CI)	***P-value***	**OR (95% CI)**	***P-value***
Gross tumor type				
Non-pedunculated	1.000		1.000	
Pedunculated	0.641 (0.098–4.185)	0.477	0.826 (0.124–4.079)	0.772
Histology grade				
Low-grade	1.000		1.000	
High-grade	5.561 (1.933–16.003)	0.002	4.403 (1.046–18.520)	0.043
LVI				
Absent	1.000		1.000	
Present	34.194 (9.511–122.930)	0.004	11.156 (2.186–56.912)	0.003
TB stroma				
Inflammation	1.000		1.000	
Fibrosis	1.667 (1.278–9.451)	0.527	1.206 (1.1412–6.216)	0.087
Myxoid	6.746 (1.831–24.851)	0.032	4.303 (1.945–15.933)	0.022
TB mitosis				
Absent	1.000		1.000	
Present	2.770 (0.643–11.925)	0.171	1.013 (0.926–2.618)	0.568
TB quantity	63.429 (14.623–275.130)	0.001	28.952 (4.010–208.990)	0.008
TB CDX2 status				
Negative	15.919 (4.259–59.494)	0.021	17.350 (7.689–25.778)	0.003
Positive	1.000		1.000	

CI, confidence interval; OR, odds ratio; MSI, microsatellite instability; LVI, lymphovascular invasion; TB, tumor budding.

invasive front morphology may be more representative of the biological behavior of the tumor than the primary tumor core morphology (15). Our study demonstrated that the morphology and immunohistochemical features of TB in cases of early stage CRC were predictive of tumor progression and local recurrence. Among the various features, non-pedunculated gross type, high histological grade, LVI, myxoid-type tumor bud stroma, high tumor bud count, TB mitosis, and loss of CDX2 expression were independent predictors of LNM, whereas LNM, histology grade, LVI, TBC, stroma type, and TB mitosis were independent predictors of local recurrence in patients with pT1 CRC.

TB is a morphological characteristic that reflects the high aggressiveness of tumors at the invasion margin. A previous study revealed that high-level TB correlated with mutated KRAS or MSS/pMMR (16). Furthermore, patients with CRC who have a KRAS mutation and MSS/pMMR tumor were part of a group with the poorest prognosis (17). It is defined as a morphologic surrogate of epithelial-mesenchymal transition (EMT), a mechanism through which tumor cells acquire motility and invasiveness (18). EMT facilitates the detachment of cancer cells from the tumor mass and their subsequent infiltration in the extracellular matrix as single cells or small clusters (1, 9). TB has a strong risk stratification utility, so much so that the ITBCC system can reliably predict the prognosis of patients with CRC based on tumor bud count alone (19). However, not all patients with a high tumor bud count will have a poor prognosis; thus, further investigation of the characteristics of TB could improve its value for risk stratification. Our study is the first to improve the current ITBCC system by exploring various features of TB to predict LNM in patients with pT1 CRC, rather than limiting the input parameters to the tumor bud count. Moreover, the nomograms that we developed showed stronger discriminative ability than tumor bud count in predicting LNM or local recurrence. Thus, our easy-to-use predictive nomograms could be useful tools to quantify the probability of RFS.

A high histological grade has also been shown to be associated with aggressive tumor biology and to be of prognostic significance; it has been associated with various informative tumor parameters in human malignancies (20–25). Our results agree with the findings of previous studies on non-CRCs and indicate that a high histological grade is of prognostic value for pT1 CRC. Additionally, we focused on the presence of atypia of tumor bud cells, but it was not of statistical significance; it was mainly present in the tumor center. Furthermore, tumor bud cell mitosis was observed in 15.8% of the cases in the present study. Mitosis results from rapid cell proliferation and is correlated with tumor proliferation and invasiveness; it is associated with

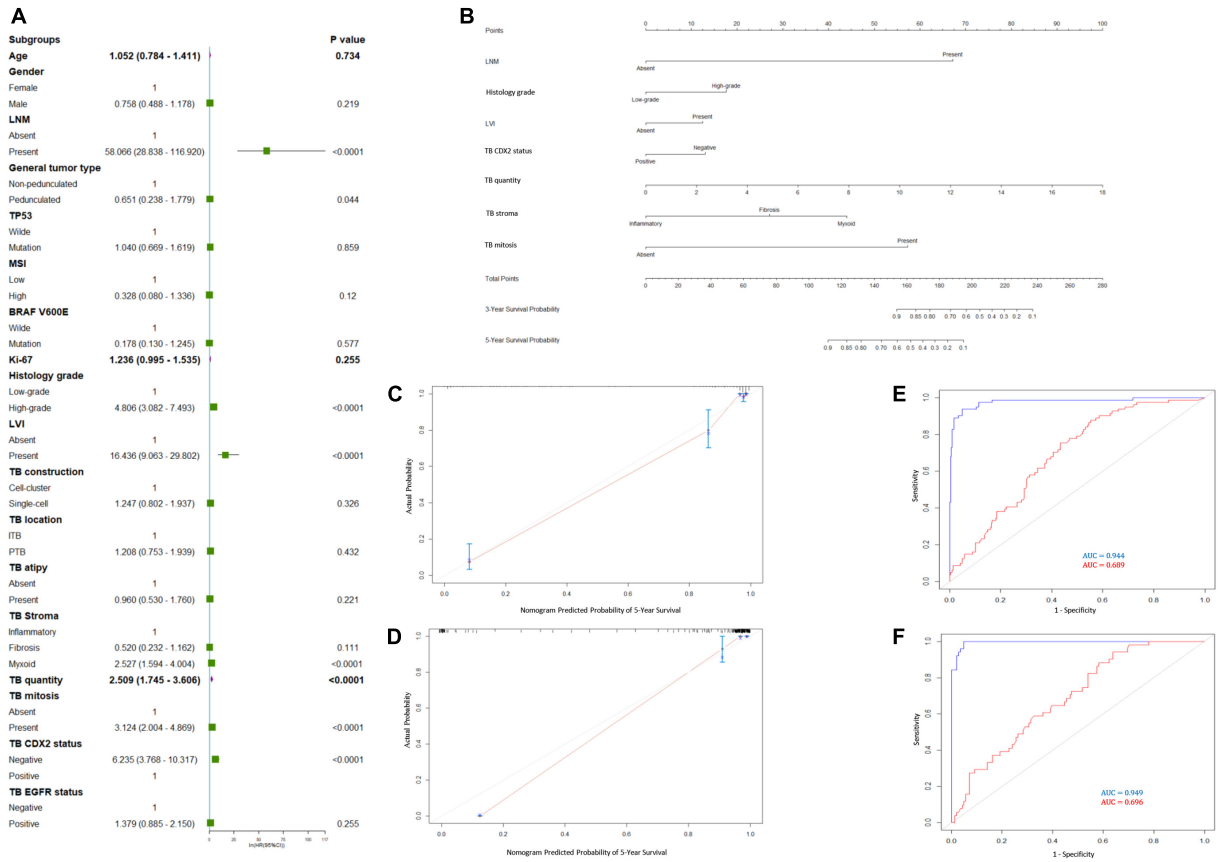

FIGURE 3
Prediction model for recurrence-free survival (RFS). **(A)** Forest plots to decipher the risk factors associated with RFS identified in univariate Cox regression analysis; **(B)** RFS predictive nomogram. The calibration curve of postoperative RFS in patients with pT1 CRC in the **(C)** training cohort and **(D)** validation cohort. Predictive accuracy of RFS-nomogram in the **(E)** training cohort and **(F)** validation cohort.

adverse clinical outcomes in other cancer types (26), although its prognostic value in CRC has not yet been defined. A previous study revealed that a decrease in mitosis is associated with high-level TB (27), which may represent the potential decrease in the mitosis of tumor bud cells because of fibroblastic cells around TB during the EMT process in an effort to slow down tumor invasion. Thus, mitosis in TB may reflect tumor biology and may provide valuable prognostic information.

Unlike the tumor core, the environment at the tumor front is not static; inflammatory, fibrotic, and myxoid stroma are histologic features representing snapshots of the dynamic process of extracellular matrix remodeling. The immature or myxoid stroma desmoplastic reaction has been recognized as an independent prognostic predictor in CRC (28). This feature, however, has not been routinely adopted in pathology reports for clinical care. Furthermore, myxoid stroma is associated with the absence of tumor-infiltrating lymphocytes in CRC (29), which enhance tumor immune escape. Some studies have demonstrated that the immature myxoid stroma is associated with a high degree of tumor budding (30). The myxoid stroma surrounding the tumor buds that appear at the tumor front is regarded as an immature stroma with a high potential to disseminate and metastasize (31). Consistent with the findings of a previous study (32), in the current study, myxoid stroma was significantly associated with the presence of LNM and local recurrence in patients with pT1 CRC.

In addition to the histological characteristics of TB, we also investigated the immunophenotype of TB. We observed a loss of CDX2 expression in 6.6% of tumor buds, which differed from the tumor core. Notably, CDX2 inhibits EMT and metastasis of CRC by regulating Snail and β-catenin expression (33). CDX2, an intestine-specific transcription factor, has been strongly implicated in the development of the intestinal mucosa (34). Emerging evidence suggests the crucial role of CDX2 as a tumor suppressor during colorectal carcinogenesis. CDX2 expression is inversely associated with tumor grade in CRC (35, 36). Consistent with the findings of a previous study, the downregulation of CDX2 expression was associated with LNM in patients with pT1 CRC. The lack of CDX2 expression in tumor buds may indicate that they are in a

TABLE 3 Multivariate COX regression analysis of recurrence.

	Training cohort (n = 234)		Validation cohort (n = 120)	
	HR (95% CI)	***P-value***	**HR (95% CI)**	***P-value***
LNM				
Absent	1.000		1.000	
Present	32.292 (14.401–72.407)	< 0.0001	16.331 (6.007–58.260)	< 0.0001
Gross tumor type				
Non-pedunculated	1.000		1.000	
Pedunculated	0.733 (0.171–2.153)	0.337	0.891 (0.432–3.014)	0.547
Histology grade				
Low-grade	1.000		1.000	
High-grade	1.622 (1.002–2.627)	0.049	1.548 (0.771–3.107)	0.218
LVI				
Absent	1.000		1.000	
Present	2.686 (1.162–6.208)	0.021	2.958 (1.160–7.541)	0.023
TB stroma				
Inflammation	1.000		1.000	
Fibrosis	1.256 (0.449–1.632)	0.551	1.192 (0.312–1.880)	0.715
Myxoid	1.719 (0.264–1.955)	0.001	1.280 (0.950–2.819)	0.151
TB mitosis				
Absent	1.000		1.000	
Present	1.022 (0.540–1.933)	< 0.0001	1.567 (0.615–2.673)	0.094
TB quantity	1.703 (1.055–2.750)	0.029	1.563 (0.838–2.914)	0.020
TB CDX2 status				
Negative	0.935 (0.520–1.679)	0.216	1.789 (0.711–4.502)	0.799
Positive	1.000		1.000	

Statistical analyses were conducted using log-rank tests and a Cox proportional hazards model. CI, confidence interval; LVI, lymphovascular invasion; RFS, recurrence-free survival; TB, tumor budding.

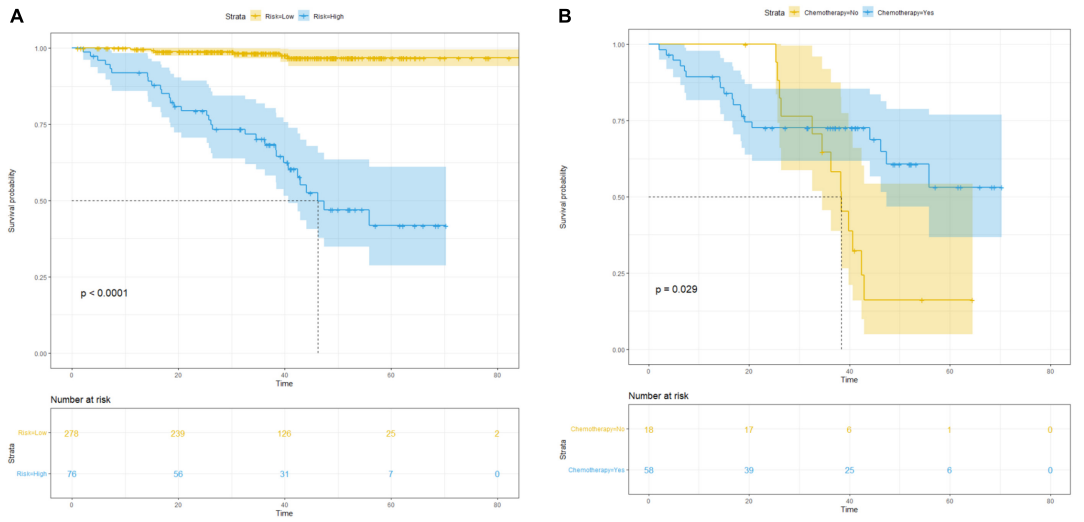

FIGURE 4
Survival curves for subgroup analysis in patients with different risk of postsurgical recurrence stratified by nomogram score. **(A)** Kaplan–Meier survival curves for RFS according to the risk status in all patients; **(B)** RFS according to different therapy in high-risk cohort.

state of EMT; thus, it could predict poor prognosis in patients with CRC. Moreover, because these features are associated with LNM, they can be applied to endoscopic biopsy specimens to better predict tumor progression behavior. To the best of our knowledge, these findings have not been previously reported. Additional studies with larger cohorts are required to validate the prognostic implications of the histological and immunohistochemical features of TB that may help predict the prognosis or occurrence of LNM.

Our novel nomogram can effectively stratify the recurrence risk in pT1 CRC patients, and the KM survival curve shows that the RFS of high-risk patients is much shorter than that of the low-risk. Furthermore, our data revealed that adjuvant chemotherapy is necessary in the high-risk group. Therefore, adjuvant chemotherapy is recommended for high-risk patients even if they do not have LNM. Our method could be applied to determine risk stratification strategies for patients with pT1 CRC; for example, to identify low-risk patients to avoid unnecessary additional treatment, while identifying high-risk patients to enable timely and effective treatment. Low-risk patients could extend their follow-up periods, improving their quality of life and reducing postoperative complications and financial costs. However, this study had some limitations. First, the statistical power was limited because this was a single-center retrospective study. Second, owing to the retrospective study design, potential selection biases could not be ruled out. Finally, although the study focused on identifying the most significant predictors of LNM and local recurrence, it is unclear whether these findings can be generalized.

In conclusion, tumor bud count and other features of TB are associated with LNM and poor prognosis in patients with pT1CRC. Although tumor bud count, stroma type, and the CDX2 expression status in tumor buds were identified as risk factors for LNM, only tumor bud count was significantly correlated with local recurrence in patients with pT1 CRC. Thus, these features of TB should be incorporated into the routine evaluation of CRC, as they may provide valuable information to guide clinical therapy. Additional studies in a multi-institutional setting are needed to confirm these findings.

Data availability statement

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

LC and JT performed the material preparation and data collection and analysis. LC wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Co-delivery of sorafenib and metformin from amphiphilic polypeptide-based micelles for colon cancer treatment

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Colorectal cancer (CRC) is a common clinical disease with a poor prognosis and a high recurrence rate. Chemotherapy is important to inhibit the post-surgical recurrence of CRC patients. But many limitations restrict the further application of chemotherapy. In this study, sorafenib (Sor) and metformin (Met) co-loaded poly(ethylene glycol)-block-poly(L-glutamic acid-*co*-L-phenylalanine) [mPEG-*b*-P(Glu-*co*-Phe)] micelles were developed. The characterizations, drug release, *in vivo* biodistribution, and pharmacokinetics of the micelles were analyzed. The treatment efficacy of the dual-drug loaded micelles was evaluated in a subcutaneous colon cancer mice model. Sor is a common molecular target agent that can inhibit the mitogen-activated protein kinase (MAPK) pathway to treat solid tumors. Met can also regulate the MAPK pathway and inhibit the expression of the phosphorylated extracellular signal-regulated kinase (p-ERK). Moreover, both Sor and Met play important roles in cell cycle arrest. The integration of these two drugs aims to achieve synergistic effects against colon cancer. The micelles can be targeted to cancer cells and possess longer blood circulation time. The two agents can be released rapidly in the tumor sites. The *in vivo* study showed that the micelles can prevent tumor progression by inhibiting the expressions of p-ERK and cyclin D1. This study indicated that the Sor/Met-loaded micelles are suitable for CRC treatment.

KEYWORDS

colorectal cancer, drug delivery system, chemotherapy, micelles, tumor environment (TME)

Introduction

Colorectal cancer (CRC) threatens people's health seriously worldwide. Despite the advanced development in CRC diagnosis and surgical intervention, tumor recurrence tends to happen in lots of patients (1). Systematic chemotherapy is another method to extend the survival of CRC patients (2). However, the concentration of traditional

chemotherapy agents within tumor sites is always not effective for tumor killing (3). In addition, patients with CRC are always intolerant of the side effects of systematic chemotherapy (4). As a result, achieving better therapeutic effects on CRC is important.

Nanotechnology, an emerging science, has promoted the development of pharmacy (5). The nanosized drug delivery systems can overcome the disadvantages of systematic chemotherapy (6). Nowadays, researchers are focusing on developing polymeric nanoparticles, such as vesicles (7, 8) and micelles (9, 10), for tumor therapy. The nanomaterials-based drug carriers not only protect the encapsulated agents during blood circulation but also increase the accumulation in the tumor site (11). Furthermore, co-drug-loaded nanoparticles to deliver combination therapy for CRC treatment have attracted more and more attention (12). Encapsulating chemotherapeutic agents with synergistic effects can increase the antitumor efficacy against CRC (13).

Sorafenib (Sor) can decrease the phosphorylated extracellular signal-regulated kinase (p-ERK) levels and block the mitogen-activated protein kinase (MAPK) pathway to inhibit tumor progression (14, 15). The MAPK pathway in tumor cell lines is associated with tumor development, including tumor growth, differentiation, and apoptosis (16, 17). ERK is a key component in the MAPK pathway, and tumor cell proliferation depends on p-ERK (14). In addition, Sor also exhibits anti-proliferative activity in tumors by inhibiting cyclin D1 expression (18). Sor is approved for the treatment of hepatoma clinically. Recent studies also showed that CRC patients may be benefited from Sor (19) and Sor could prevent the proliferation and metastasis of CRC cell lines (20). Metformin (Met), a safe hypoglycemic agent, has been proved of tumor inhibition effect (21), and can also inhibit the expression of p-ERK (22, 23) and cyclin D1 (24, 25). Therefore, we hypothesize that the integration of Sor and Met can increase the synergistic effects of CRC treatment. Delivering the two drugs while decreasing the side effects is crucial for tumor therapy.

In this study, poly(ethylene glycol)-block-poly(L-glutamic acid-*co*-L-phenylalanine) [mPEG-*b*-P(Glu-*co*-Phe)] micelles were prepared, followed by the encapsulation of Sor and Met. Herein, the copolymers can be self-assembled, and different components of the copolymer possess different functions to deliver Sor and Met. The PEG shell mainly provides the protective effects for the loading agents. Glutamic acid units assist in electrostatic interaction between the glutamic acid carboxyl group and the Met amino group. Sor is hydrophobic and can be loaded into the nanocarrier by physical embedding. Phenylalanine units increase the hydrophobic/aromatic interaction within the inner core of micelles (6). The characteristics of the dual-drug-loaded micelles were analyzed *in vivo* and *in vitro*. A subcutaneous colon cancer mice model was applied to evaluate the treatment efficacy of the Sor and Met co-loaded micelles. Sor and Met were successfully delivered to the tumor sites. Sor and Met loaded mPEG-*b*-P(Glu-*co*-Phe) micelles (NSM) showed a better synergistic effect against colon cancer compared with free Sor and Met treatment. Figure 1 shows the preparation process of Sor and Met co-loaded micelles and the mechanisms against CRC.

Materials and methods

The materials, synthesis of mPEG-b-P(BLG-*co*-Phe) and mPEG-b-P(Glu-*co*-Phe) copolymers, preparation of mPEG-b-P(Glu-*co*-Phe)/Sor/Met micelles, characterizations of copolymers, NSM stability, *in vitro* drug release, cytotoxicity assays, and cellular uptakes are shown in the Supplementary File.

Animal study

This study was approved by the Jilin University Animal Center (KT202002042). BALB/c mice (male, 8–12 weeks) and Sprague-Dawley rats (male, 180–200 g) were used and bought from Jilin University.

The subcutaneous animal model was established by injecting CT26 cells (0.1 mL, 100×10^4 mL^{-1}) into the right flanks of BALB/c mice. When the tumors were about 300 mm^3, the animals were divided into three groups with six animals in each group, i.e., normal saline (control), free Sor and Met (SM), and NSM at Sor dose of 10 mg kg^{-1} and Met dose of 40 mg kg^{-1}. Then, 100 μL normal saline, SM solution, or NSM solution was applied through the tail vein five times every 3 days.

In vivo biodistribution

Twelve mice in the subcutaneous colon cancer mice model with a tumor volume of about 300 mm^3 were selected and were divided into NSM and SM groups. The mice in the SM group were treated with SM saline solution *via* tail vein injection with Sor dose of 20 mg kg^{-1} and Met dose of 80 mg kg^{-1}. The mice in the other group were treated with NSM solution with the equivalent amounts of Sor and Met to those in NSM solution. The mice were euthanized at 6 or 12 h after injection. The tumor tissues and other major organs were resected. The Sor and Met in different tissues were determined with the HLPC method.

Pharmacokinetic detections

Sprague-Dawley rats were divided into NSM and SM groups ($n = 3$). NSM or SM solutions (2 mL) with equivalent Sor dose of 20 mg kg^{-1} and Met dose of 80 mg kg^{-1} were injected *via* tail vein. Blood samples were collected at different time

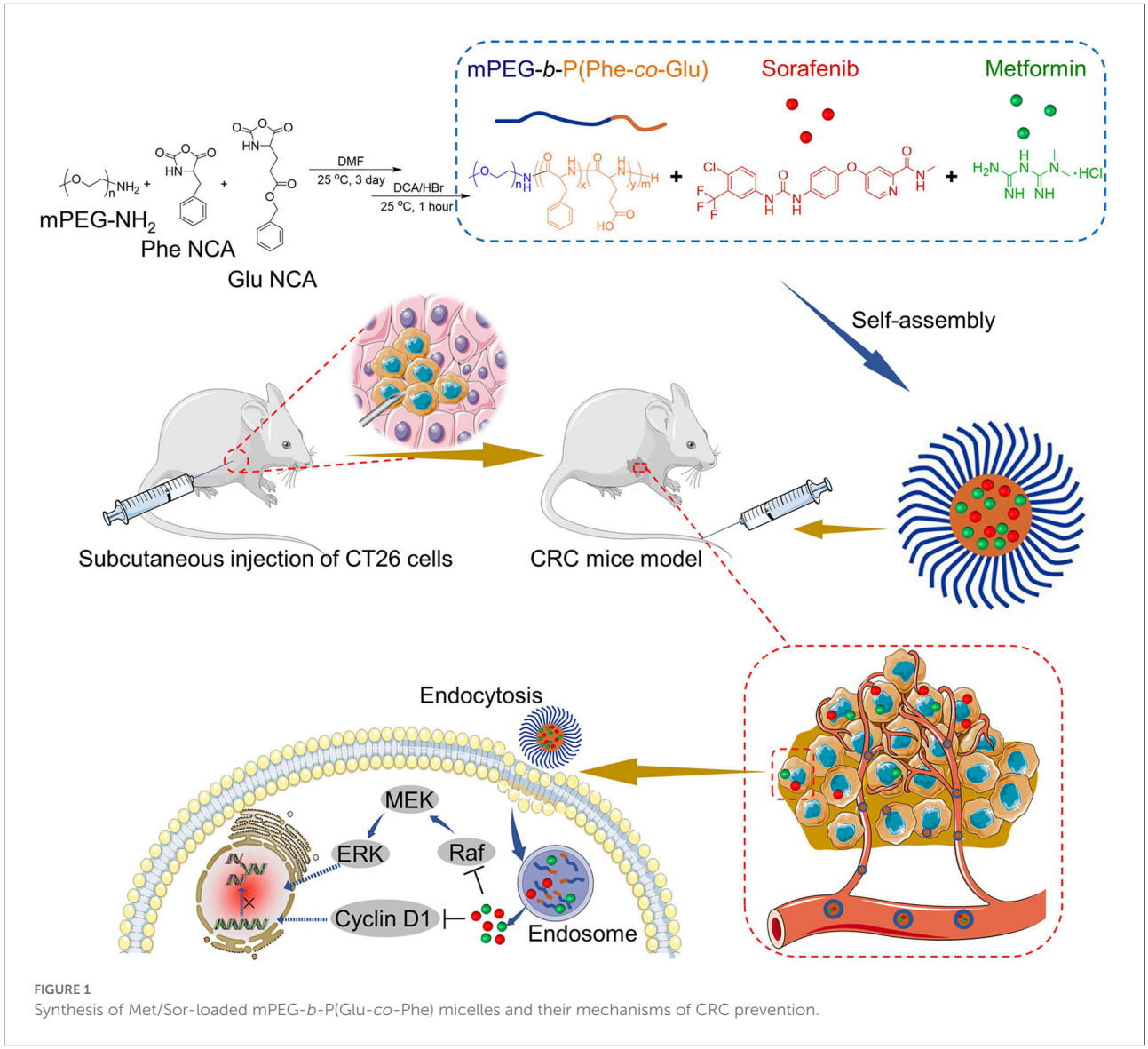

FIGURE 1
Synthesis of Met/Sor-loaded mPEG-*b*-P(Glu-*co*-Phe) micelles and their mechanisms of CRC prevention.

points. The Sor and Met concentrations were analyzed with the HLPC method.

In vivo antitumor efficiency assessment

The largest diameter (L) and smallest diameter (S) of tumors were measured every day, and the tumor volume was calculated with Equation (1).

$$V\ (mm^3) = \frac{L \times S^2}{2} \quad (1)$$

After 14 days post-treatment, all the mice were euthanized. The tumor growth rate (TGR) was calculated by the ratio of the tumor volume at 14 days post-treatment and the tumor volume before treatment. Blood samples were collected and the levels of ALT, AST, CK-MB, BUN, and D-Lac were analyzed by the ELISA method. The tumor weight of each sample was recorded.

Histopathological study

The tumor tissues and other major organs were stained with hematoxylin and eosin (H&E). The immunohistochemical assays were also applied to evaluate ERK, p-ERK, and cyclin D1 levels in tumor tissues. The positive cells were stained brown-yellow, and the relative positive area was analyzed by Image J (National Institutes of Health, Bethesda, Maryland, USA).

TABLE 1 DLC and DLE of Sor and Met of NSM.

Feed ratio (w/w/w) (Polymer/Sor/Met)	DLC Sor (%)	DLE Sor (wt.%)	DLC Met (%)	DLE Met (wt.%)
45:05:15	2.1	77.2	6.6	86.6
40:08:20	4.9	74.8	11.4	85.4
35:15:25	12.3	54.8	18.8	63.6
30:20:30	20.2	46.4	25.6	51.2

Statistical analyses

One-way ANOVA and Student's *t*-test were used. $P < 0.05$ indicated statistically significant, and $P < 0.01$ and $P < 0.001$ indicated highly statistically significant.

Results and discussions

Preparation of NSM and characterizations

Figure 1 shows the NSM preparation and the mechanisms for the CRC therapy.

The FT IR and GPC analyses indicated the successful development of mPEG-*b*-P(Glu-*co*-Phe) copolymers (Supplementary Figure S1). Relevant results are shown in the Supplementary File.

As shown in Figure 1, NSM is prepared in an aqueous solution, utilizing the electrostatic interaction between glutamic acid carboxyl group and the Met amino group. Sor is loaded within the nanocarrier by a simple physical embedding method. The mPEG-*b*-P(Glu-*co*-Phe) copolymers self-assembled in aqueous solutions and entrap Sor and Met within micelles. The electrostatic interactions between the drugs and polymers would benefit the release of drugs. The electrostatic interactions will be damaged in the acidic environment within tumor tissues, thus resulting in drug-releasing (6). As shown in Table 1, when the Sor and Met feeding ratios were 11.2 and 29.4%, satisfactory DLCs and DLEs of Sor and Met could be obtained. A higher drug feeding ratio resulted in slightly increased DLC, while DLE was decreased remarkably. As a result, the DLCs of 4.9 and 11.4% of Sor and Met, respectively, were applied to obtain a rationale DLC and a high DLE. The DLEs of Sor and Met of NSM were 74.8 and 85.4 wt.%, respectively. The polymeric chemotherapy drug delivery systems were acceptable for DLC ranging from 1 to 20% (26).

The micelles' morphology is observed under TEM examination (Figure 2A), demonstrating that NSM is homogeneously spherical with narrow size distribution. The size distributions of NSM were evaluated with DLS in this study. The size distribution results are similar to TEM, in which all the micelles show a pretty narrow distribution (Figure 2A). The average diameter of NSM is 67.3 ± 8.9 nm as observed by DLS analysis. The diameter of nanoparticles of 100 nm is suitable to enhance the permeability and retention (EPR) effect (27, 28). The diameter of NSM is slightly smaller than 100 nm, and the nanoparticles of this size are also suitable for tumor therapy (6).

The stability of drug delivery systems is crucial in drug delivery. As shown in Figures 2B,C, the incubation time lasts for 7 days, but no obvious size changes are observed in both incubation mediums. The NSM shows excellent stability in the neural environment in this study.

In vitro drug release

The release profiles of Sor and Met were studied in PBS solution at pH 7.4, 6.8, and 5.5 at 37°C. The amounts of released drugs were examined with HPLC. The release behavior of Sor was similar to that of Met in which three different release conditions could be observed. As shown in Figures 2D,E, a rapid release happened in the first 24 h, followed by a slower release at 24–48 h and a sustained release at 48–72 h. There are 48.7 ± 3.1% and 57.7 ± 2.6% amounts of Sor and Met released after 72 h of incubation at pH 7.4, respectively (Figures 2D,E). About 50 and 40% amounts of Sor and Met are not released because the electrostatic interaction within the micelles was not seriously weakened at pH 7.4 possibly (29). Besides, hydrophobic phenylalanine units enhanced the stability of micelles, resisting the micelles' dissociation (6). As a result, there were still some drugs not released from the inner core of the micelles. The release profiles of Sor and Met are pH responsive with more Sor and Met released in an acidic environment. There are 69.4 ± 2.7% and 85.8 ± 1.9% amounts of Sor released after 72 h of incubation at pH 6.8 and 5.5, respectively (Figure 2D). The amounts of released Met are 82.3 ± 2.6% and 94.9 ± 2.5% after 72 h incubation at pH 6.8 and 5.5, respectively (Figure 2E). The release of Sor and Met happened simultaneously, but Met was released a little faster than that of Sor at the same pH value. The increased acidity of the tumor microenvironment could facilitate the disruption of electrostatic interaction and promote the instability of micelles, thus facilitating more drug release for tumor therapy (6).

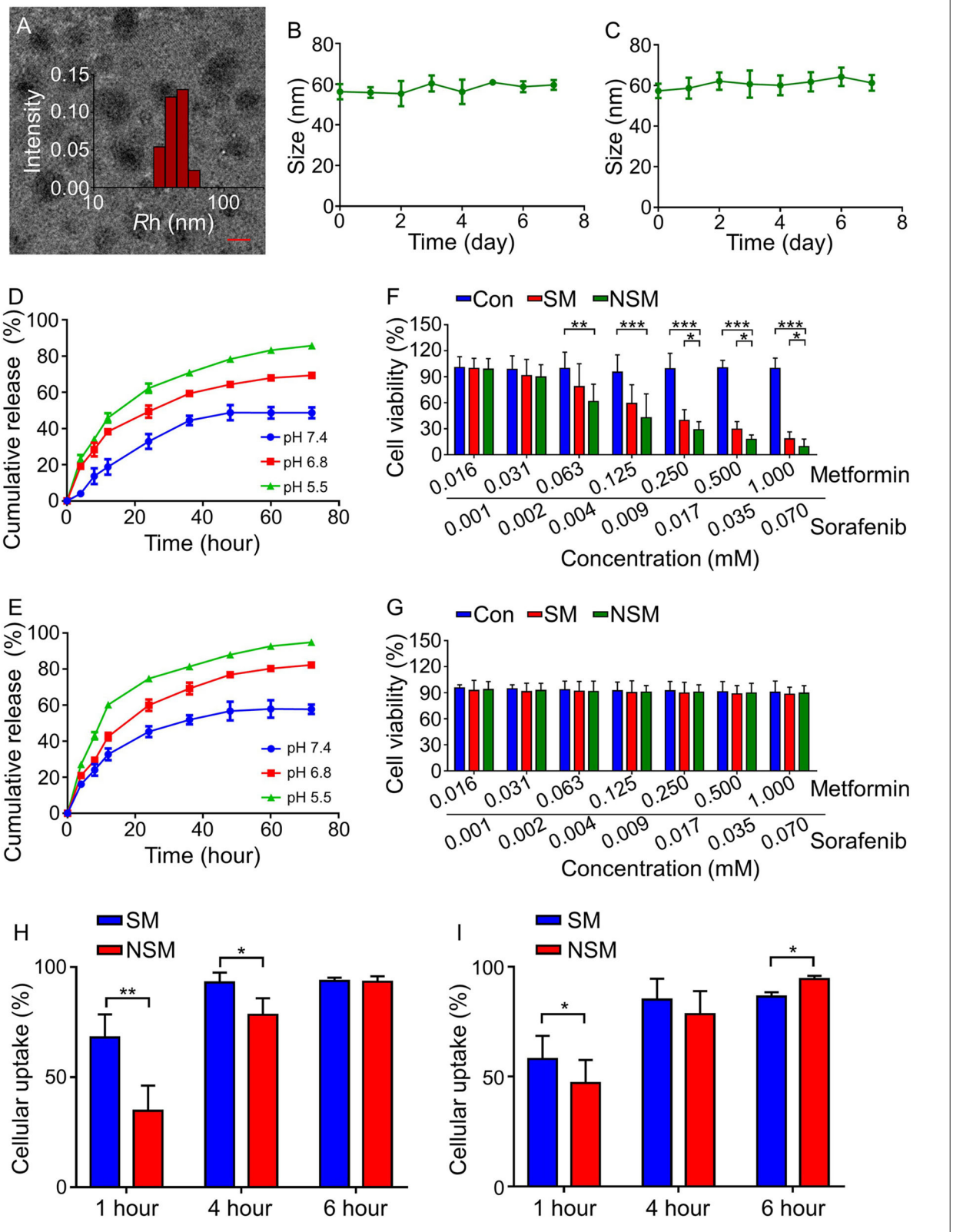

FIGURE 2
Characterizations of mPEG-*b*-P(Glu-*co*-Phe) micelles. **(A)** TEM and DLS analyses. NSM stability in **(B)** PBS and **(C)** BSA solution. Release profiles of **(D)** Sor and **(E)** Met in PBS solution at different pH values. *In vitro* cytotoxicity analyses on **(F)** H22 cells and **(G)** HLL-5 cells at different Met and Sor concentrations in different groups. Cellular uptakes of **(H)** Sor and **(I)** Met of SM and NSM after incubation with H22 cells for 1, 4, and 6 h. Scale bar = 50 nm. *, **, *** represent $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

In vitro cytotoxicity and cellular uptakes

After 48 h, the cell viabilities of CT26 cells are all above 90% (Figure 2F), indicating that the copolymers possess good compatibility and low cytotoxicity. As for the drug-loaded MTT assay, both SM and NSM exhibit dose-dependent cytotoxicity effects toward CT26 cells (Figure 2F). When the concentration of Met is more than 0.063 mM, and Sor is more than 0.004 mM, the cell viability is lower in the NSM group than that in the SM group ($P < 0.05$) and control group ($P < 0.001$), demonstrating that NSM possesses stronger cell proliferation inhibition efficiency than SM. However, NSM did not show severe cytotoxicity to normal human intestinal mucosa endothelial cells HIEC. The viability of HIEC cells was all above 85% and there was no significant difference in cell viability among all groups (Figure 2G). The *in vitro* cytotoxicity was repeated three times.

Efficient cellular uptakes of drugs can increase antitumor activity. The cellular uptakes of Sor (Figure 2H) and Met (Figure 2I) in SM and NSM groups are evaluated with the HPLC. The general cellular uptakes of Sor and Met are higher in the SM group than in the NSM group at 1 h, which may be attributed to the fact that free Met and Sor can be rapidly uptaken by CT26 cells during the first hour. At 4 h, the cellular uptake of Sor is slightly higher in the SM group than that in the NSM group ($^*P < 0.05$). However, there is no significant difference in the cellular uptake of Met between the two groups at 4 h. There is no difference in the Sor cellular uptake at 6 h between SM and NSM groups. The cellular uptake of Met is higher in the NSM group than that in the SM group at 6 h ($^*P < 0.05$). This may be because free SM and NSM may have different cellular uptake methods, and mPEG-b-P(Glu-co-Phe) may increase the ability of Sor and Met to enter the cell. The cellular uptakes of Met and Sor in the NSM group are all above 90% at 6 h. The high cellular uptakes of Sor and Met could benefit the synergistic chemotherapeutic effects against CRC.

Biodistribution studies

The biodistributions of Met and Sor in different tissues were detected with the HPLC method in this study.

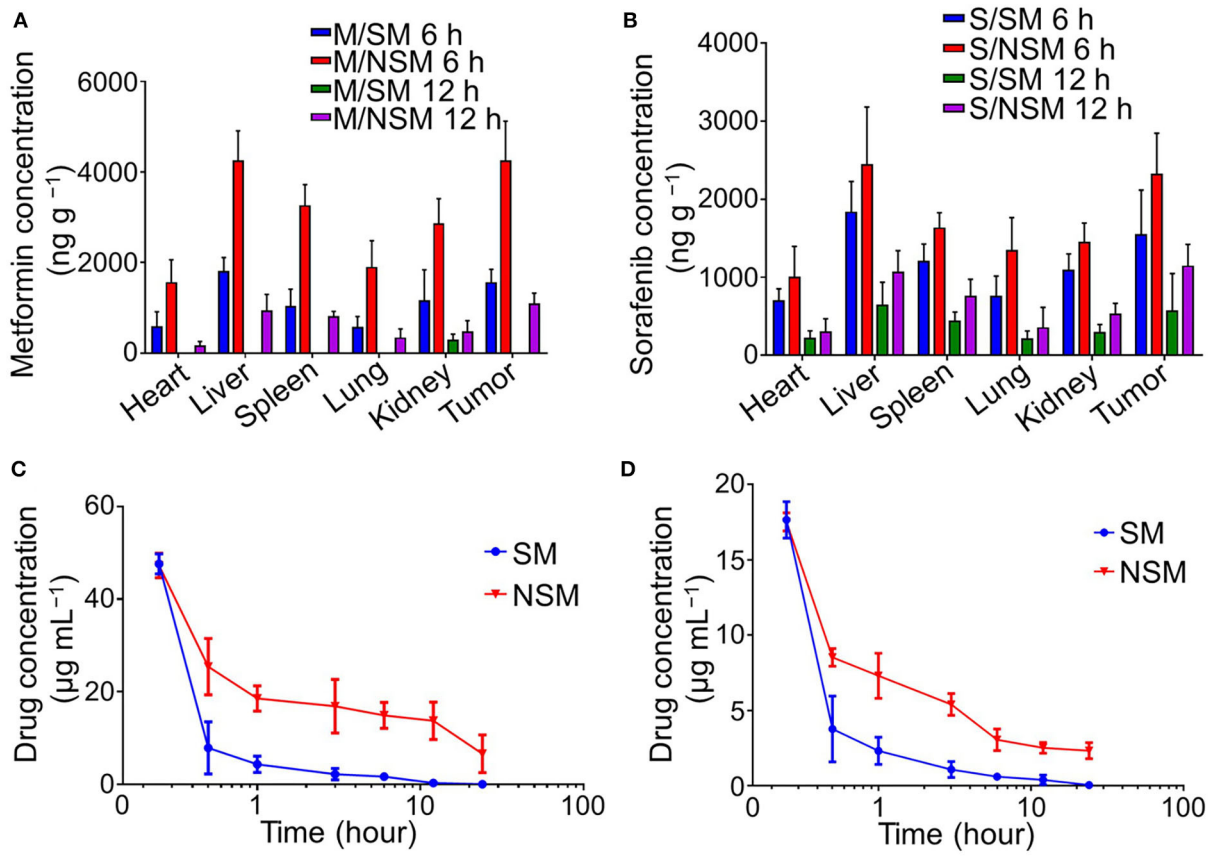

FIGURE 3
Biodistribution and pharmacokinetics studies of Met and Sor. Biodistribution of **(A)** Met and **(B)** Sor in subcutaneous colon cancer mice model. Plasma pharmacokinetics of **(C)** Met and **(D)** Sor in SM and NSM groups.

The amounts of Met and Sor were at higher levels in the tissues at 6 h post-injection than that at 12-h post-injection. There were more Met and Sor accumulated in the NSM groups than in the SM group. Met and Sor are mainly located in liver and tumor tissues in the subcutaneous colon cancer mice model (Figures 3A,B). At 12-h post-injection, Met only accumulates in the kidney and disappears from tumor tissues in the SM group. In the NSM group, amounts of Met in tumor tissues can also be observed. The amounts of Met and Sor are statistically higher in tumor tissues after NSM treatment than after SM injection, indicating that the NSM can target the tumor site. The sustained release of Met and Sor from the micelles within tumors contributed to the accumulation of drugs.

Pharmacokinetic detections

Plasma pharmacokinetics of Met and Sor in SM and NSM are evaluated with HPLC post-intravenous administration (Figures 3C,D). The Met and Sor concentrations in the SM group decrease dramatically in the first 30 min and slowly decrease after that. The burst drug concentrations decrease was not evident in the NSM group, and the drug concentrations decreased much slower than that in the SM group. As a result, the blood circulation time of NSM could be significantly enhanced compared to SM. The Met and Sor clearance in mPEG-*b*-P(Glu-*co*-Phe) micelles decreased due to the increased stability of polymeric micelles and sustained drug delivery possibly.

In vivo anticancer efficiency

The subcutaneous colon cancer mice model was performed to evaluate the anticancer efficiency of NSM. After the sacrifice of mice, the tumors are carefully resected to further assess the *in vivo* antitumor efficiency, and the tumor weights are also recorded. The tumor weight is the least in the NSM group, and the difference is significant between SM and NSM groups ($P < 0.001$) (Figure 4A). Consistent with the tumor weight

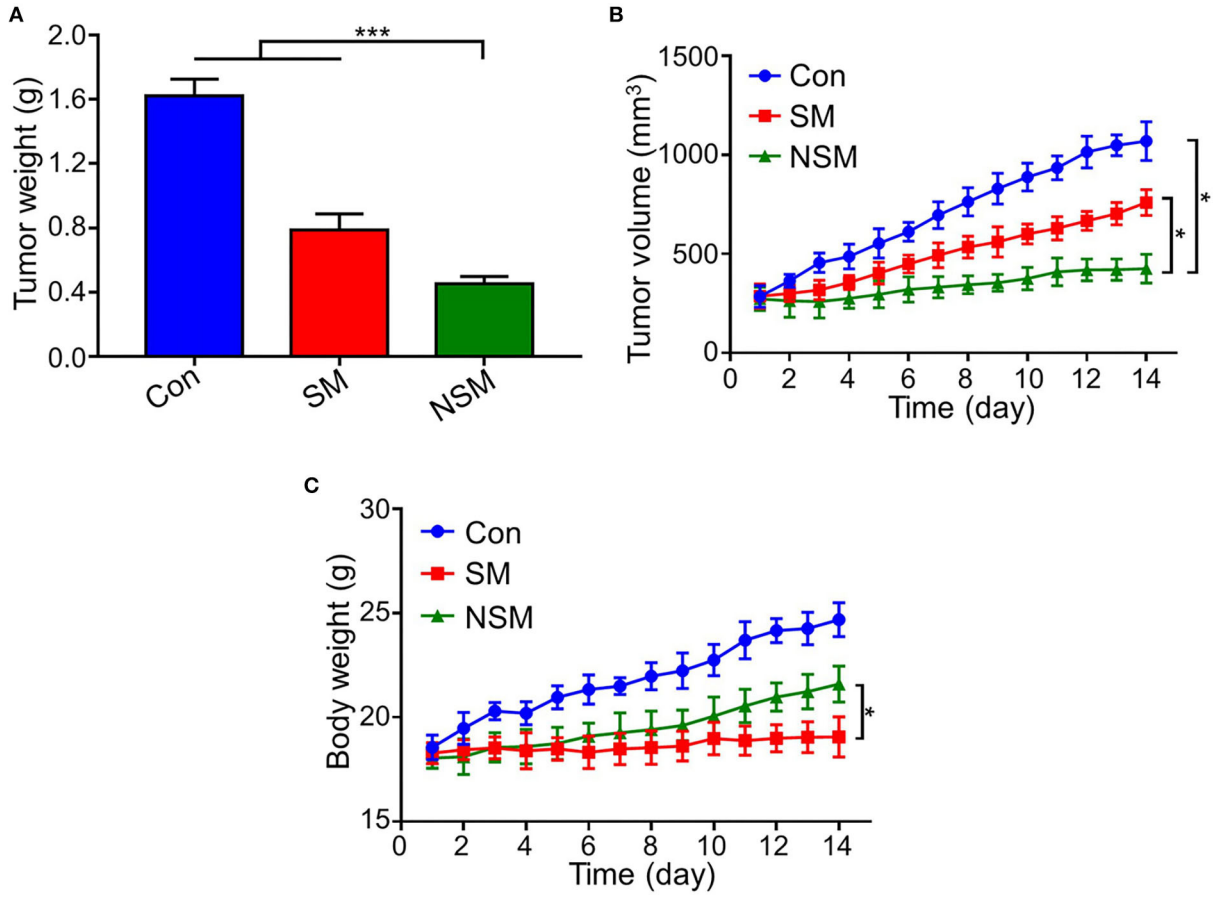

FIGURE 4
Antitumor efficacy of NSM in subcutaneous colon cancer mice model. **(A)** Tumor weight. **(B)** Tumor volume. **(C)** Body weight of mice. * and *** represent $P < 0.05$ and $P < 0.001$, respectively.

results, the tumor volume is also the least in the NSM group (Figure 4B). There is a significant difference between SM and NSM groups in tumor volume. In addition, the TGRs of NSM, SM, and Control groups are 4.13 ± 0.61, 2.42 ± 0.35, and 1.59 ± 0.22, respectively. NSM group presents a higher TGR compared with SM group ($P < 0.05$) and Control group ($P < 0.01$). As shown in Figure 4C, there was no obvious weight loss or increase in body weight in the SM group, which indicates that even the free SM treatment seems to be well-tolerated and causes no weight loss. The mice show an evident increase in body weight in the control group and the NSM group. The body weight of the control group increased gradually and was the highest compared with the other two groups. This may also be explained by the growing tumor and little drug toxicity effect. The difference is significant in body weight between the SM and NSM groups ($P < 0.05$). The general body conditions of animals are good after NSM treatment due to small toxicity. The results demonstrated that NSM possessed higher tumor inhibition efficiency over SM treatment. This is because of the increased accumulation of NSM and the fast release of Met and Sor from the micelles within the tumor tissues possibly.

Biochemical analyses

Biochemical analyses were applied to evaluate the general conditions of major organs. Besides, the toxicity of Met was evaluated by testing D-Lac levels, which were inclined to induce lactic acidosis (30).

Figure 5 shows the biochemical analyses of the subcutaneous colon cancer mice model. There was no statistical difference in ALT, AST, CK-MB, BUN, and D-Lac levels among all the groups, indicating that obvious liver, heart, and kidney injuries are not caused by the NSM and SM treatment. Also, the application of Met in free SM solution or NSM micelles does not increase D-Lac levels or induce lactic acidosis.

Histopathological evaluations

H&E analysis of tumor sections was performed to assess the tumor inhibition efficiency of NSM (Figure 6A). The relative necrosis area of the tumor was analyzed with the Image J software. The necrosis area is small in the control group, indicating rapid proliferation of tumor cells (Figure 6B). However, various necrosis degrees can be found in SM and NSM groups. NSM shows the least necrosis tumor area, and there is a significant difference between SM and NSM groups ($P < 0.05$) (Figure 6B).

The biological values of ALT, AST, CK-MB, BUN, and D-Lac are first tested in this study. The results do not reveal apparent damage in normal organs. H&E analysis of major organs is performed to analyze the security of NSM further. In the control group, the H&E staining of organs shows the normal histological structure (Figure 6C). No obvious pathological changes are

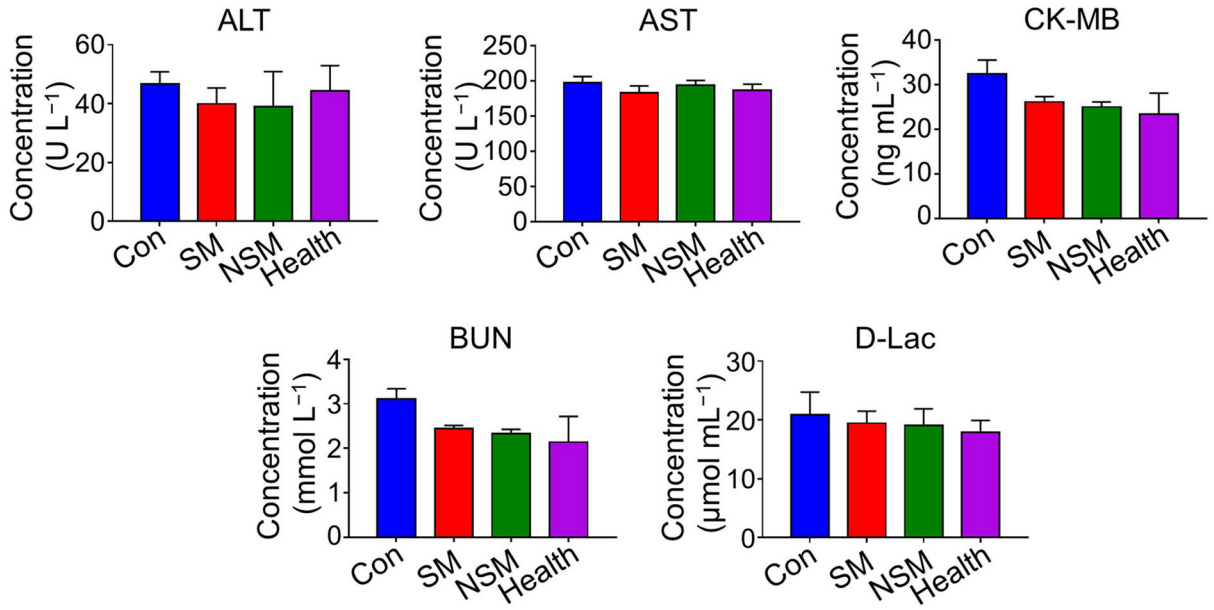

FIGURE 5
Biochemical analyses in subcutaneous colon cancer mice model.

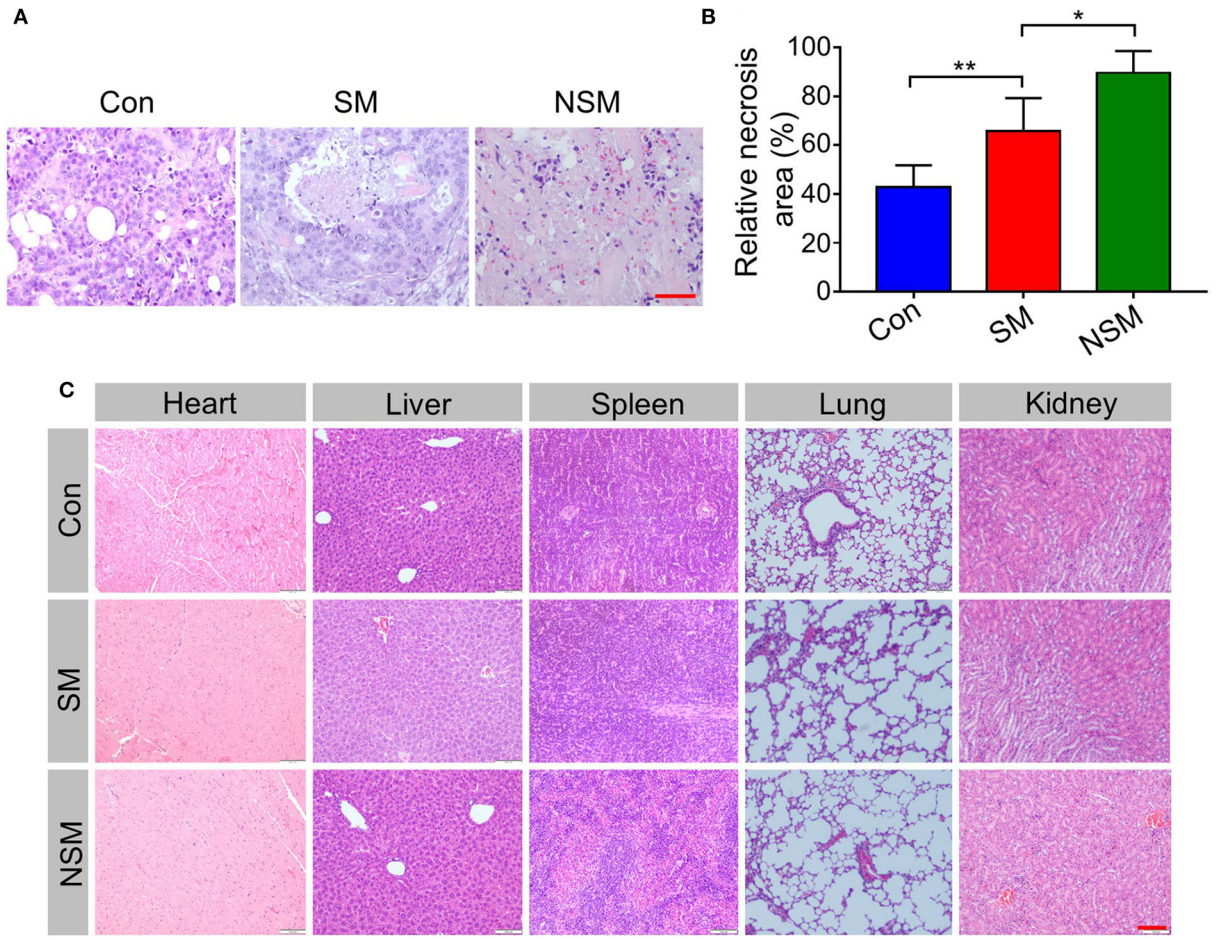

FIGURE 6
Histopathological analysis. **(A)** H&E staining and **(B)** relative necrosis area of tumor tissues. **(C)** H&E analysis of major organs. Scale bars = 100 μm. * and ** represent $P < 0.05$ and $P < 0.01$, respectively.

found in SM and NSM groups, indicating the high security of NSM in CRC treatment.

Immunohistochemical analyses

The ERK, p-ERK, and cyclin D1 levels were analyzed to reveal the anticancer mechanism of NSM.

The MAPK/ERK pathway is one of the key pathways for solid tumor development. The inhibition of the MAPK/ERK pathway could not phosphorylate ERK, reducing the proliferation of tumor cells (31, 32). Immunohistochemical studies first test the expression levels of ERK and p-ERK. All three groups show similar amounts of positive cells for ERK evaluation. However, the most and least amounts of p-ERK are found in the control and NSM groups, respectively (Figure 7A). The immunohistochemical results are confirmed with the semi-quantitative analyses (Figures 7B,C).

Cyclin D1 is one of the most important regulators of the cell cycle (33). The upregulation of cyclin D1 could lead to cell cycle disorders and highly promote cell proliferation (33). The expressions of cyclin D1 are tested to determine whether NSM treatment was associated with cell cycle arrest. The immunohistochemical staining and semi-quantitative analyses show that the expressions of cyclin D1 are most inhibited in the NSM group (Figures 7A,D).

The above results demonstrate that NSM mainly performs its tumor inhibition efficiency through downregulating the expressions of p-ERK and cyclin D1, thus inhibiting the MAPK/ERK pathway and influencing the cell cycle. As a result, the proliferation of tumors can be prevented.

Conclusion

In this study, an mPEG-*b*-P(Glu-*co*-Phe) copolymer-based drug delivery system was developed. Sor and Met were

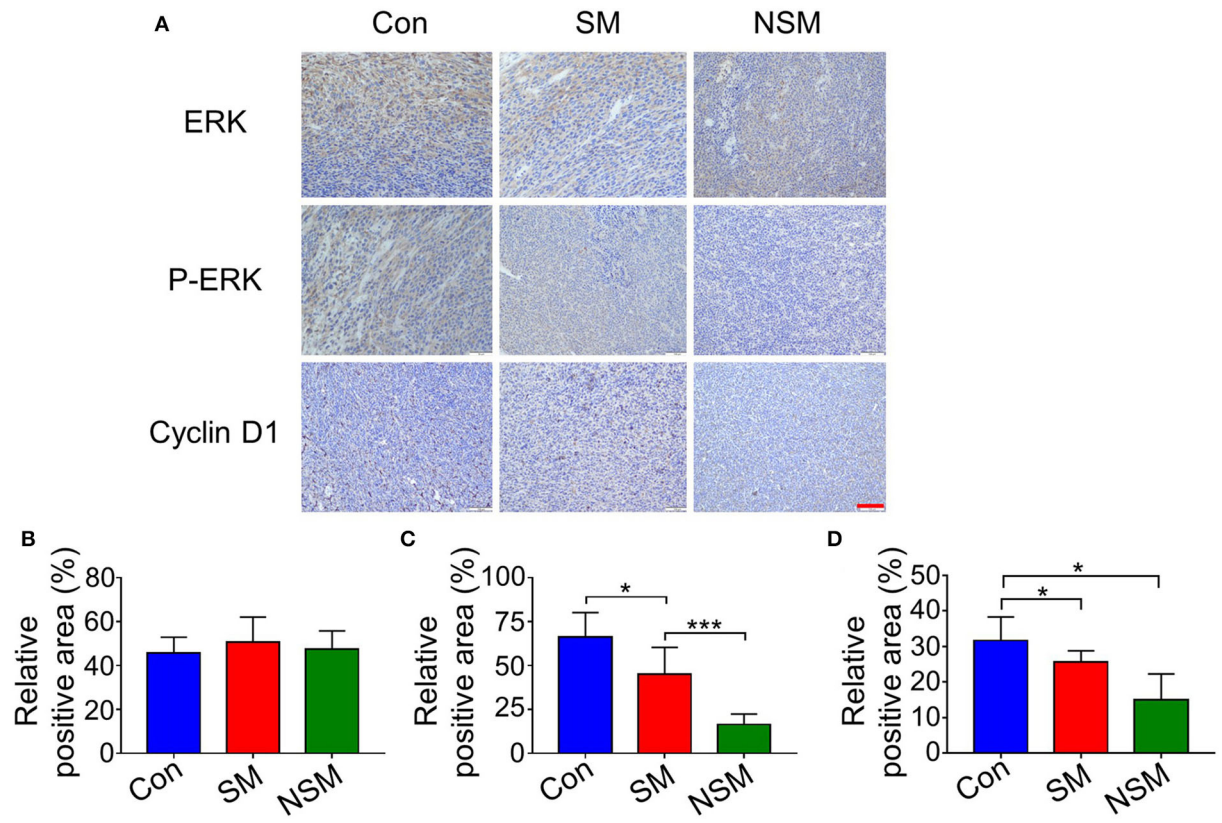

FIGURE 7
Immunohistochemical analyses of tumor tissues. **(A)** Immunohistochemical staining of ERK, p-ERK, and Cyclin-D1 in tumor tissues. Relative positive areas of **(B)** ERK, **(C)** p-ERK, and **(D)** Cyclin-D1. Scale bar = 100 μm. * and *** represent $P < 0.05$ and $P < 0.001$, respectively.

loaded in the mPEG-*b*-P(Glu-co-Phe) micelles to achieve the chemotherapeutic effect. NSM can be targeted to cancer cells and release Sor and Met rapidly within tumors. A subcutaneous colon cancer mice model was developed to assess the anticancer efficacy of NSM. NSM can inhibit tumor proliferation through the synergistic effect of Sor and Met on blocking the MAPK/ERK pathway and arresting the cell cycle of colon cancer cells. All these results demonstrated the superiority of Sor/Met loaded-mPEG-*b*-P(Glu-*co*-Phe) micelles in the treatment of 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 animal study was reviewed and approved by the Experimental Center of Jilin University.

Author contributions

XZ wrote the manuscript. LC, GX, and HH performed the study. HH analyzed the data. TL and HZ revised the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.1009496/full#supplementary-material

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A prognostic signature based on cuprotosis-related long non-coding RNAs predicts the prognosis and sensitivity to chemotherapy in patients with colorectal cancer

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Cuprotosis, a newly proposed mechanism of cell death, can trigger acute oxidative stress that leads to cell death by mediating protein lipidation in the tricarboxylic acid cycle. However, cuprotosis-related long non-coding RNAs (CRLNCs) and their relationship with prognosis and the immunological landscape of colorectal cancer (CRC) are unclear. We have developed a lncRNA signature to predict survival time, immune infiltration, and sensitivity to chemotherapy. CRLNCs were screened using the Cor function of the R software and the differentially expressed lncRNAs were collected with the limma package. Differentially expressed long non-coding RNAs (lncRNAs) associated with prognosis were selected using univariate regression analysis. A prognostic signature was developed using the least absolute shrinkage and selection operator (LASSO) and multivariate regression analysis. Patients with CRC were divided into two groups based on the risk score. The low-risk group had a more favorable prognosis, higher expression of immune checkpoints, and a higher level of immune cell infiltration compared with the high-risk group. Furthermore, there was a close association between the risk score and the clinical stage, tumor mutational burden, cancer stem cell index, and microsatellite instability. We also assessed chemotherapy response in the two risk groups. Our study analyzed the role of CRLNCs in CRC and provided novel targets and strategies for CRC chemotherapy and immunotherapy.

KEYWORDS

colorectal cancer, cuprotosis, prognostic signature, immune infiltration, immunotherapy, chemotherapy sensitivity

Introduction

Colorectal cancer (CRC) is responsible for approximately 10% of cancer cases and related deaths worldwide (1). Only in developed countries does the incidence of CRC show a stable or declining trend, which is primarily due to the widespread use of large-scale screening and colonoscopy, as well as the continuous improvement of people's living and eating habits. It is estimated that there will be 25 million new cases of CRC worldwide by 2035 (2). In addition to surgery, radiotherapy and chemotherapy are still widely applied to reduce recurrence and improve survival. Chemotherapy, which involves the application of chemical compounds to inhibit the growth of tumor cells, is an indispensable part of the treatment process. Currently, platinum-based chemotherapy in combination with 5-fluorouracil is the first-line treatment option in treating patients with CRC (3, 4). However, different patients respond differently to the same chemotherapy regimen, leading to large variations in patient prognoses (5, 6). Exploring new, specific, and effective targets related to chemotherapy sensitivity, as well as recognizing individualized and precise treatment, is therefore critical for CRC therapy.

Copper homeostasis is an ancient phenomenon in living organisms. Copper is an indispensable trace element for the homeostasis of the internal environment (7). Copper contributes to the progression of tumors, such as breast and lung cancer, where it is involved in tumor angiogenesis, epithelial-mesenchymal transition, and cell proliferation and metastasis (8, 9). Therefore, copper-chelating agents have been studied and reported to inhibit tumor growth in some clinical trials (10). Meanwhile, copper can also promote oxidative stress to mediate cell death (11, 12). Copper-specific ionophores can transport copper into cells at specific sites, increasing the copper level in tumor cells, and then mediating the toxicity of copper overload, which results in cell death (10). The role of copper in the treatment of tumors is complex and versatile. Mutations in lncRNAs are believed to mediate several forms of tumor development along with protein-coding genes (13).

LncRNAs can regulate immune and inflammatory responses at the transcriptional and posttranscriptional levels by interacting with proteins, RNA, and DNA (14). At the same time, lncRNAs have a close relationship with the tumor microenvironment (TME) (15). Several lncRNAs, including TUG1, MALAT1, H19, GAS5, LINC00152, UCA1, CUDR, and AA174084, have been identified as predictive biomarkers of CRC. Investigating such lncRNAs as potential targets for CRC therapy is of long-term value. GAS5 is involved in regulating chemotherapy resistance in CRC. The other lncRNAs require further investigation. To determine whether cuprotosis-related long non-coding RNAs (CRLNCs) play a role in CRC, a prognostic signature of the immune infiltration and survival of patients with CRC was developed. A different prognosis was revealed by the Kaplan-Meier analysis. Various methodologies, such as XCELL, TIMER, and ssGSEA, were also used to analyze the immune infiltration in patients with CRC. The analyses of immune checkpoints, clinicopathological data, tumor mutational burden (TMB), cancer stem cells (CSCs), microsatellite instability (MSI), and chemotherapy response were also performed.

Materials and methods

Datasets and samples

The transcriptome, mutation, and clinical data for COAD containing 32 healthy tissues and 375 tumors were downloaded from The Cancer Genome Atlas (TCGA) database. Six fresh frozen CRC and paracancerous paired tissues were obtained from the Second Hospital of Jilin University. The cuprotosis-related genes (CRGs) are shown in **Supplementary Table 1**.

Identification of differentially expressed cuprotosis-related long non-coding RNAs

To identify lncRNAs closely related to CRGs, we performed a screen using the Cor function of the R software, with the filter conditions set to require a correlation coefficient of >0.3 with a false discovery rate of <0.001. Subsequently, differentially expressed CRLNCs between the 32 normal and 375 tumor samples were selected using the limma package ($|\log \text{Foldchange}| > 1$ and false discovery rate < 0.05).

Construction and validation of a prognostic long non-coding RNA signature

The **Supplementary material** provide details about the construction and validation of the prognostic lncRNA signature.

Gene set enrichment analysis and nomogram construction

The gene set enrichment analysis (GSEA) and nomogram are presented in the **Supplementary material**.

Immune landscape, immune checkpoints, and clinical data analyses

Analyses of the immune landscape, immune checkpoints, and clinical data are presented in the **Supplementary material**.

Analyses of tumor mutational burden, cancer stem cells, and microsatellite instability

Tumor mutational burden is an essential marker of immunotherapy response and prognosis. Therefore, we compared genetic mutations in samples from low-risk and high-risk groups. The mutational burdens from all samples were then calculated and compared. A correlation analysis was applied to determine the significant relationships between the risk scores, TMB, and immune infiltration. We also explored the link between CRGs and risk scores. MSI could reflect the effect of immunotherapy. Therefore, the association between MSI and risk score was analyzed. We compared patients' survival times between MSI-H and MSS/MSI-L. We also integrated MSI into the signature for survival analysis.

Drug sensitivity analysis and identification of differential genes

The limma package was used to identify the differentially expressed genes (DEGs) ($|\log \text{Foldchange}| > 1$ and false discovery rate < 0.05). To further search for the hub genes, we used the CytoNCA plugin in Cytoscape software. Based on the scores of Betweenness, Closeness, Degree, Eigenvector, LAC, and Network, we screened the DEGs twice to obtain core genes. Furthermore, Gene Ontology and Kyoto Encyclopedia Genes and Genomes pathway enrichment analyses were used to explore the functional pathways based on the DEGs. Finally, to investigate the differences in response to chemotherapy, we used the pRRophetic package to predict drug sensitivity.

Quantitative real-time PCR

Total RNA was extracted from CRC tissues using Trizol reagent (Invitrogen, Carlsbad, CA, United States). We used a reverse transcription kit (Takara, Tokyo, Japan) to synthesize cDNA. The SYBR Premix Ex Taq^{TM} kit (Takara, Japan) was used to perform the quantitative real-time PCR (RT-qPCR). The expression level of LINC00412, AC016737.1, AC026782.2, AC090204.1, AC129507.1, and AC116914.2 was normalized using glyceraldehyde-3-phosphate dehydrogenase. The data were analyzed using the $2^{-\Delta\Delta Ct}$ method. The primers of the seven genes are listed in **Supplementary Table 2**.

Statistical analyses

All statistical analyses were performed using R version 4.1.1. $P < 0.05$ was considered significant.

Results

Analysis of differentially expressed cuprotosis-related long non-coding RNAs

The study design is presented in **Supplementary Figure 1**. The Cor function was performed to select 880 CRLNCs. The association between CRGs and lncRNAs is shown in **Figure 1A**. We discovered 487 CRLNCs that were differentially expressed, with 445 being upregulated in CRC and 42 being downregulated (**Figures 1B–C**).

Construction and validation of the prognostic signature

As a result of the univariate regression analysis, six CRLNCs were discovered to be linked with the prognosis of patients with CRC (**Figure 2A**). We then selected the genes corresponding to the smallest lambda value for the multivariate Cox regression analysis (**Figures 2B–C**). Finally, LINC00412, AC016737.1, AC026782.2, AC090204.1, AC129507.1, and AC116914.2 were screened to construct the risk signature. The formula of the risk signature is as follows:

$$\begin{aligned} Riskscore = {} & (-1.73912912346949 * expression\ of\ LINC00412) \\ & + (0.6423027570568 * expression\ of\ AC016737.1) \\ & + (0.927870667759444 * expression\ of\ AC026782.2) \\ & + (0.306445754811284 * expression\ of\ AC090204.1) \\ & + (2.09976593806317 * expression\ of\ AC129507.1) \\ & + (-0.802467801481654 * expression\ of\ AC116914.2). \end{aligned}$$

High-risk lncRNAs included AC016737.1, AC026782.2, AC090204.1, and AC129507.1. Low-risk lncRNAs included LINC00412 and AC116914.2 (**Figure 2E**). We found that LINC00412, AC016737.1, AC026782.2, and AC090204.1 were highly expressed in CRC. AC129507.1 was downregulated in CRC (**Figure 2C**). The relationship between CRGs and the lncRNAs is displayed in **Figure 2D**. The low-risk group showed better survival outcomes than the high-risk group (**Figures 2E**, **3A**). The training group and test group confirmed this conclusion (**Figures 3B–E**). The area under the curve (AUC) values demonstrated that our prognostic signature had moderate performance (**Figure 3F**). The AUC values of age, gender, grade, and tumor stage were 0.575, 0.524, 0.558, and 0.586, respectively, indicating that the risk model had the best predictive ability (**Figure 3G**). The Kaplan-Meier survival curve further proved that this risk signature

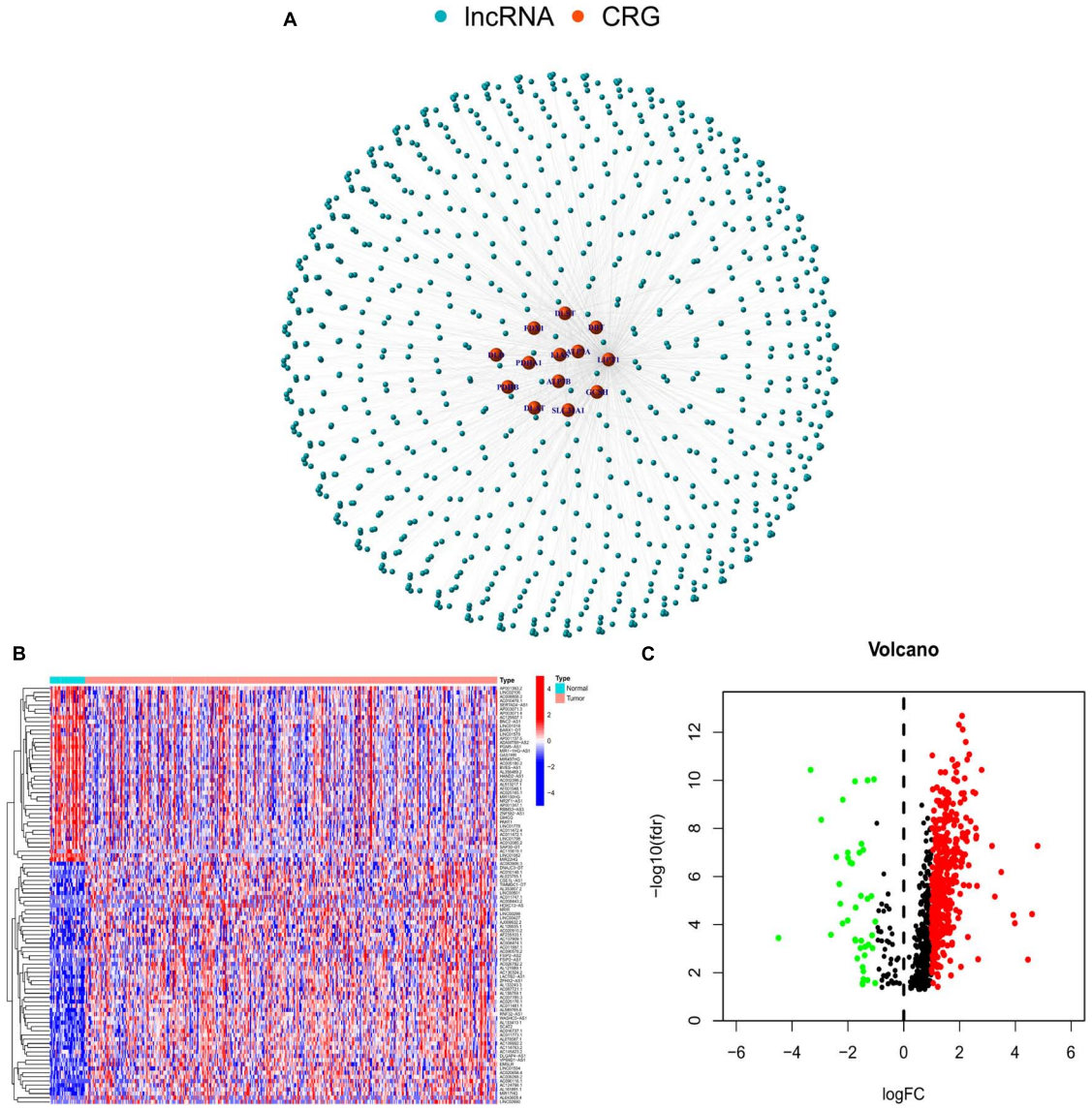

FIGURE 1
(A) Network of long noncoding RNAs and cuprotosis-related genes. **(B)** Differential expression of cuprotosis-related long non-coding RNAs (CRLNCs) between normal and tumor tissues. **(C)** The volcano plot of CRLNCs. Red represents upregulated CRLNCs; green represents downregulated CRLNCs. CRGs, cuprotosis-related genes; CRLNCs, cuprotosis-related long non-coding RNAs.

applied to patients of any age, gender, grade, and TNM stage (Figures 4A–G).

Gene set enrichment analysis and nomogram construction

The results of the GSEA showed that the main functional pathways in the high-risk group were the calcium signaling pathway, GAP junction, extracellular matrix receptor interaction, and complement and coagulation cascades. The main functional pathways in the low-risk group were homologous recombination, neuroactive ligand-receptor interaction, retinoic acid-inducible gene-I-like receptor signaling pathway, and RNA degradation (Figure 4H). The univariate regression analysis showed that age (hazard ratio [HR]: 1.003–1.039; $P < 0.05$), stage (HR: 1.193–1.833; $P < 0.001$), and risk score (HR: 1.054–1.125; $P < 0.001$) were associated with prognosis (Figure 5A). It was demonstrated that age (HR: 1.011–1.049; $P < 0.001$), stage (HR: 1.233–2.075; $P < 0.001$), and risk score (HR: 1.059–1.129; $P < 0.001$) were found to be independent prognostic factors (Figure 5B). A prognostic nomogram was also developed for the prediction

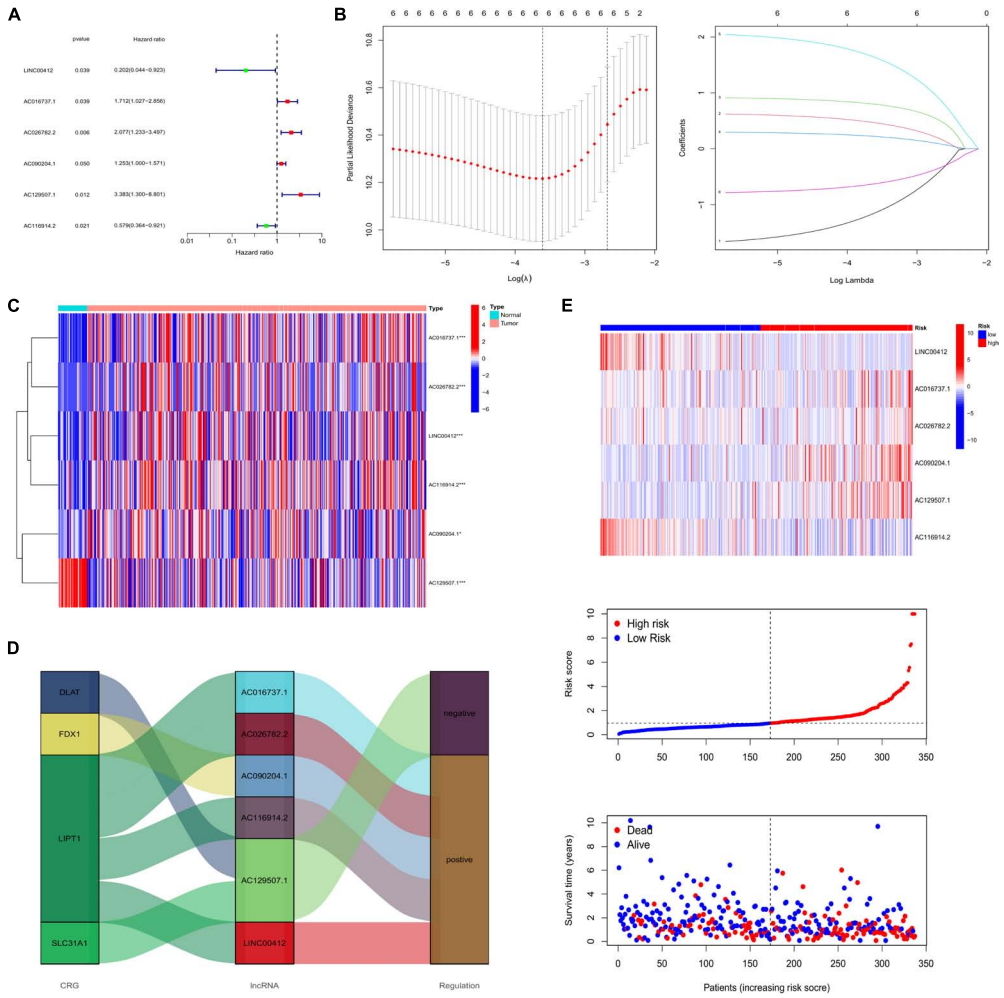

FIGURE 2
(A) Univariate Cox regression analysis. **(B)** The LASSO algorithm further selected the most crucial genes and LASSO coefficient profiles. **(C)** Expression of risk lncRNAs between normal and tumor tissues. **(D)** Sankey diagram showing the relationship between CRGs and lncRNAs. **(E)** Heatmap showing the expression of risk lncRNAs between low- and high-risk groups, and the ranked dot plot showing the risk score distribution in all samples. (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, not significant). LASSO, least absolute shrinkage and selection operator; CRGs, cuprotosis-related genes.

of the survival time (Figure 5C). The calibration curves for the 1-, 3-, and 5-year survival rates confirmed the accuracy of the nomogram (Figure 5D). The decision curve analysis indicated that the nomogram had a better predictive ability for survival time than the stage, age, and risk score (Figure 5E). The AUC values of the stage, age, risk score, and nomogram were 0.590, 0.571, 0.679, and 0.716, respectively (Figure 5F).

Analyses of immune infiltration, immune checkpoints, and clinical data

The low-risk group had more immune cell infiltration (Figure 6B). Additionally, we discovered that some immune cell types had positive correlations with risk scores while others had negative correlations (Figure 6A). The results of ssGSEA indicated that some activities, such as inflammation promotion, were upregulated in the high-risk group (Figures 6C–D). There was a differential expression of 17 immune checkpoints, of which 16 (94.12%) had higher expression levels in the low-risk group (Figure 7A). Meanwhile, higher risk scores were observed in late-stage CRC (Figure 7B).

Characteristics of tumor mutational burden, cancer stem cells, and microsatellite instability

Both low- and high-risk groups had the same top five mutated genes. However, the low-risk group demonstrated

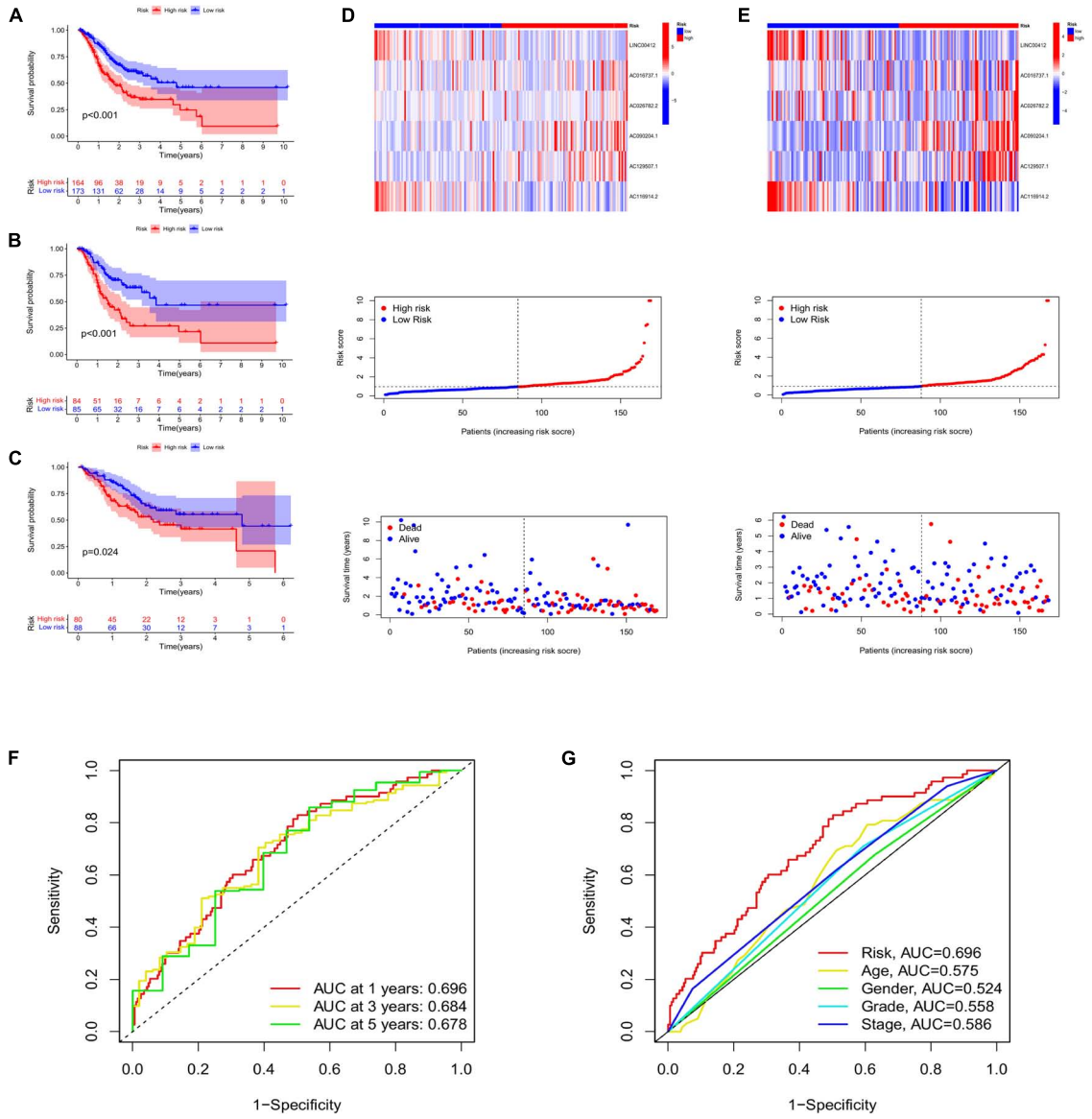

FIGURE 3
(A) Survival curve of low- and high-risk groups in all samples. The survival analysis of low- and high-risk groups in the training group **(B)** and the test group **(C)**. Heatmap displaying the expression of risk lncRNAs between low- and high-risk groups, and the ranked dot plot showing the risk score distribution in the training group **(D)** and the test group **(E)**. **(F)** The AUC values of 1-, 3-, and 5-year survival. **(G)** Comparison of AUC between risk signature and age, gender, grade, and stage. AUC, area under the curve.

a higher mutation probability (Figures 7C–D). TMB was high in the low-risk group (Figure 7F). TMB and risk score were inversely correlated (Figure 7G). Differences in TMB between the two groups may be related to endothelial cells and neutrophils (Figure 7H). Patients with high-risk scores had lower RNA and higher DNA markers in CSCs than patients with low-risk scores (Figures 7I–J). Figure 7E illustrates the relationship between risk scores and CRGs. MSS/MSI-L was strongly associated with higher risk scores (Figures 8A–B). There was no association between survival rates and MSS/MSI-L and MSI-H status. However, the MSI-H + low-risk score had the most favorable prognosis compared with the MSS/MSI-L + high-risk score, the MSS/MSI-L + low-risk score, and the MSI-H + high-risk score groups (Figure 8D).

Analyses of chemotherapeutic drug sensitivity and differential genes

After running the limma package, we selected 140 DEGs (Figure 8E), including 131 overexpressed and 9 underexpressed genes (Figure 8F). Based on the scores of Betweenness,

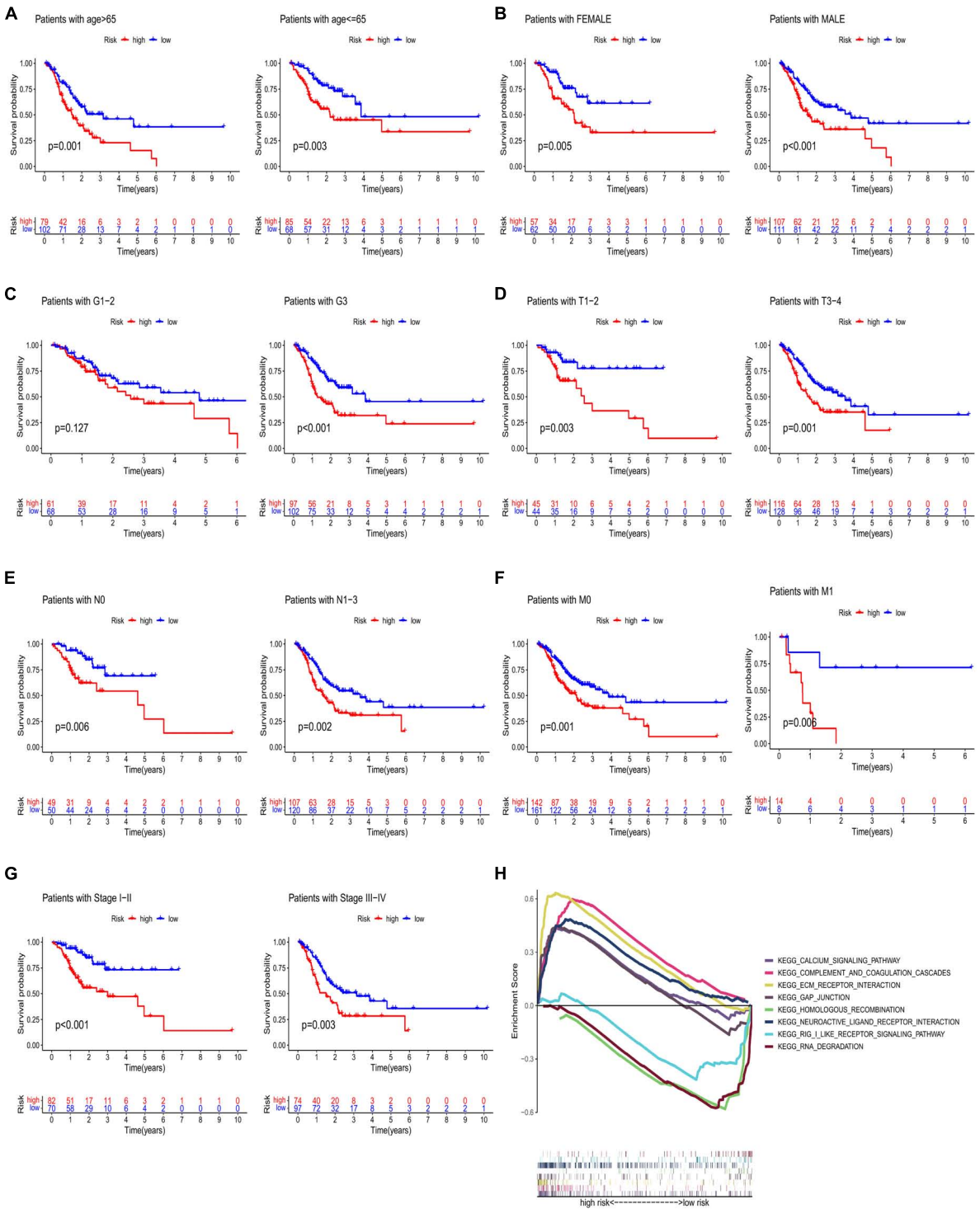

FIGURE 4
Kaplan–Meier analyses of age **(A)**, gender **(B)**, grade **(C)**, T stage **(D)**, N stage **(E)**, M stage **(F)**, and stage **(G)**. **(H)** GSEA of low- and high-risk groups. GSEA, gene set enrichment analysis.

Closeness, Degree, Eigenvector, LAC, and Network, we performed two screenings and obtained eight core genes (Figures 8G–I). The Gene Ontology and Kyoto Encyclopedia Genes and Genomes analyses based on the 140 DEGs showed that the cGMP-PKG signaling pathway, heparin binding, and contractile fiber may be the main biological functions. Finally,

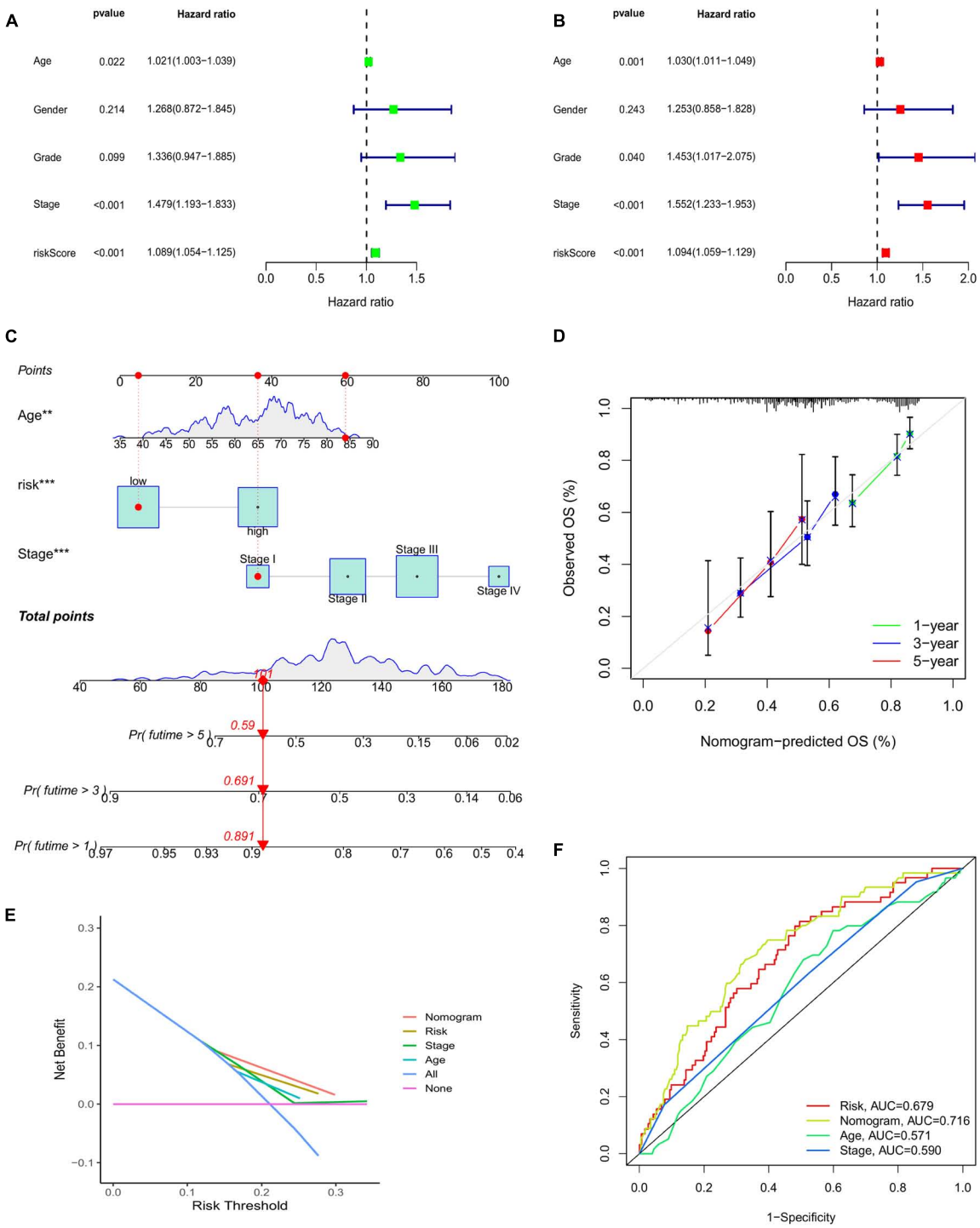

FIGURE 5
(A) Univariate Cox regression analysis. **(B)** Multivariate regression analysis. **(C)** Nomogram to predict the 1-, 3-, and 5-year survival probability for patients with CRC. **(D)** The calibration curves of 1-, 3-, and 5-year survival. **(E)** Decision curve analysis comparing stage, age, risk score, and the nomogram. **(F)** AUC values of the stage, age, risk score, and nomogram. CRC, colorectal cancer; AUC, area under the curve.

we screened a total of 37 chemotherapeutic drugs to evaluate the differences in sensitivity between the two groups. Out of these 37 chemotherapeutic drugs, 32 (86.49%) had higher IC_{50} values in the low-risk group than in the high-risk group. This suggests that high-risk patients may be more sensitive to chemotherapy drugs, contributing to a more favorable prognosis (**Figure 9**).

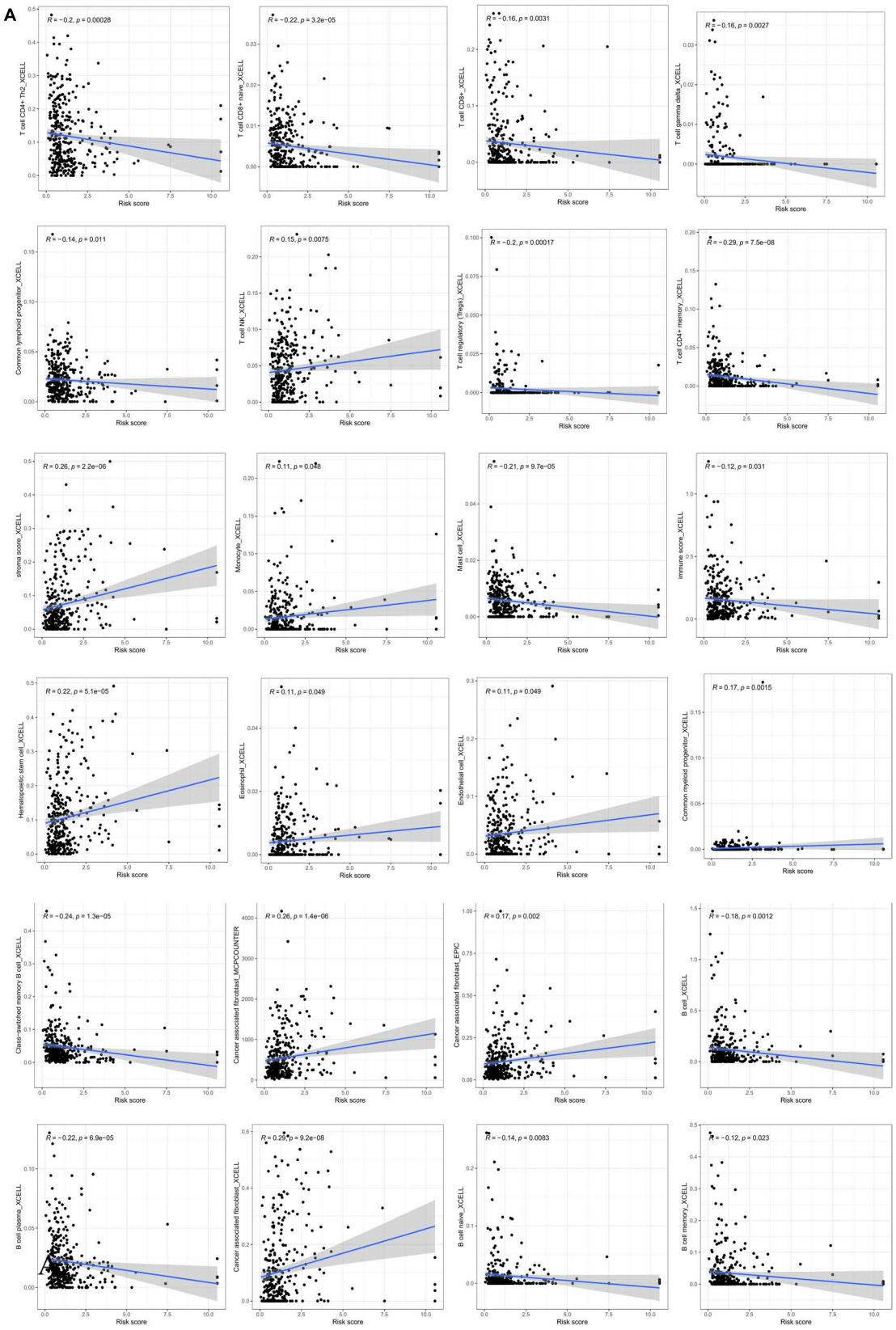

FIGURE 6
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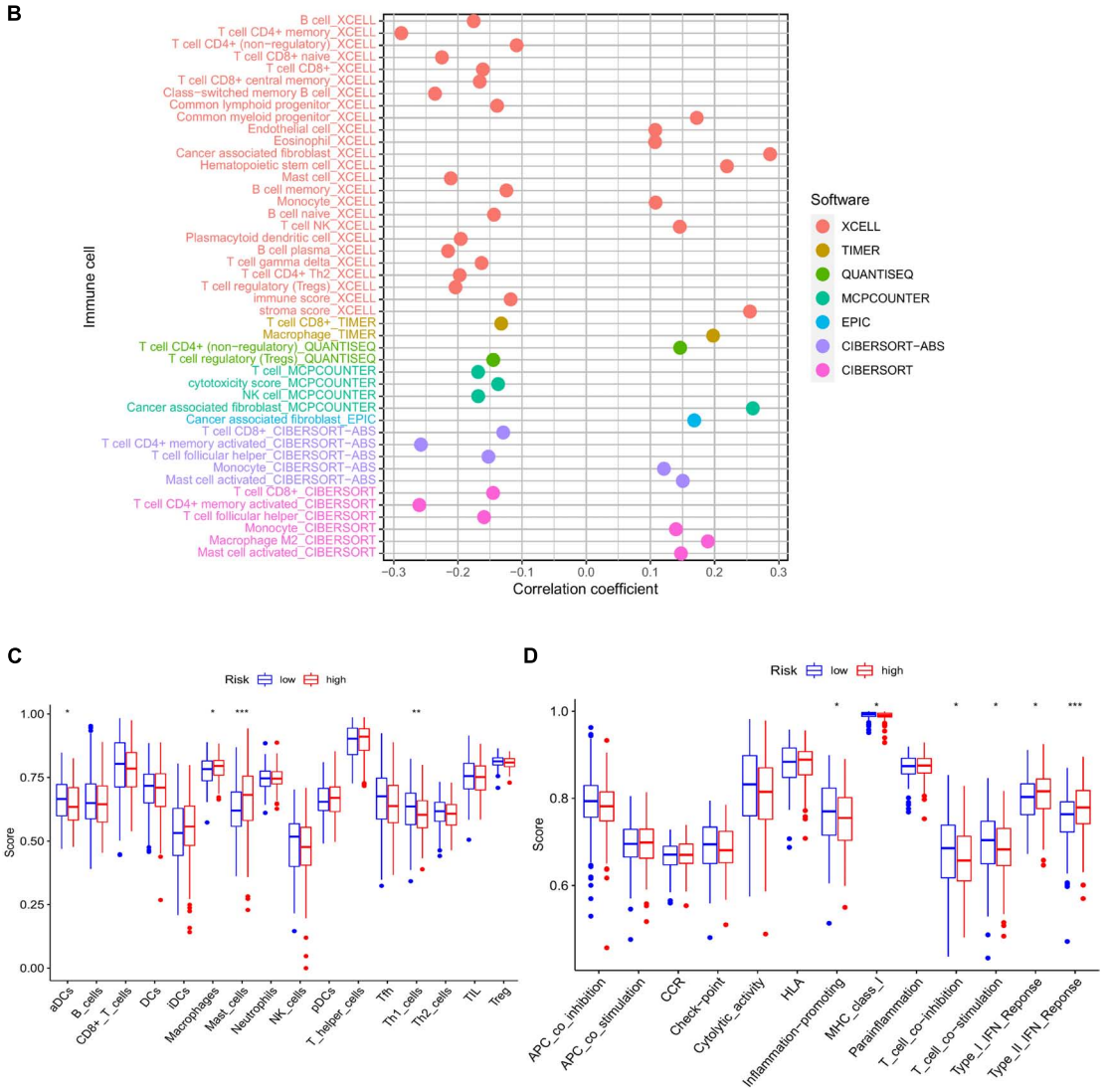

FIGURE 6
(A) Correlation between risk score and immune cells. **(B)** XCELL, TIMER, QUANTISEQ, MCPCOUNTER, EPIC, and CIBERSORT to analyze the immune landscape of patients with CRC. **(C,D)** ssGSEA analysis. CRC, colorectal cancer; GSEA, gene set enrichment analysis.

Discussion

Numerous studies have demonstrated that cell death is related to tumor occurrence and progression (16). The known mechanisms of cell death mainly include ferroptosis, pyroptosis, necroptosis, apoptosis, and autophagy (17–21). Tsvetkov et al. first described cuprotosis, a new mechanism of cell death (12). However, the relationship between cuprotosis and CRC is unclear, especially CRLNCs. In this study, we identified CRLNCs using bioinformatics studies. Six lncRNAs (i.e., LINC00412, AC016737.1, AC026782.2, AC090204.1, AC129507.1, and AC116914.2) were then used to construct a prognostic model for survival time prediction, immune infiltration, and chemotherapy drug sensitivity of CRC. LINC00412 was included in the 10 biomarkers for the construction of the cardia cancer prognostic model and contributed to the modification of the prognostic models by Xin et al. (22). Taniguchi-Ponciano et al. found that LINC00412 was upregulated in all kinds of pituitary tumors (23). Zhang et al. provided evidence that AC016737.1 was associated with the inflammatory response of CRC, and this association was later validated by constructing a prognostic model (24). Chen et al. used hypoxia-related lncRNAs, which included AC016737.1, to construct a model for predicting the outcome of CRC. AC016737.1 was also used in the m6A-modified lncRNA prognostic nomogram by Song et al. and the immune-related lncRNA pair model by Shenglei et al. for CRC prognosis. Zha et al. discovered that AC129507.1 played a role in predicting

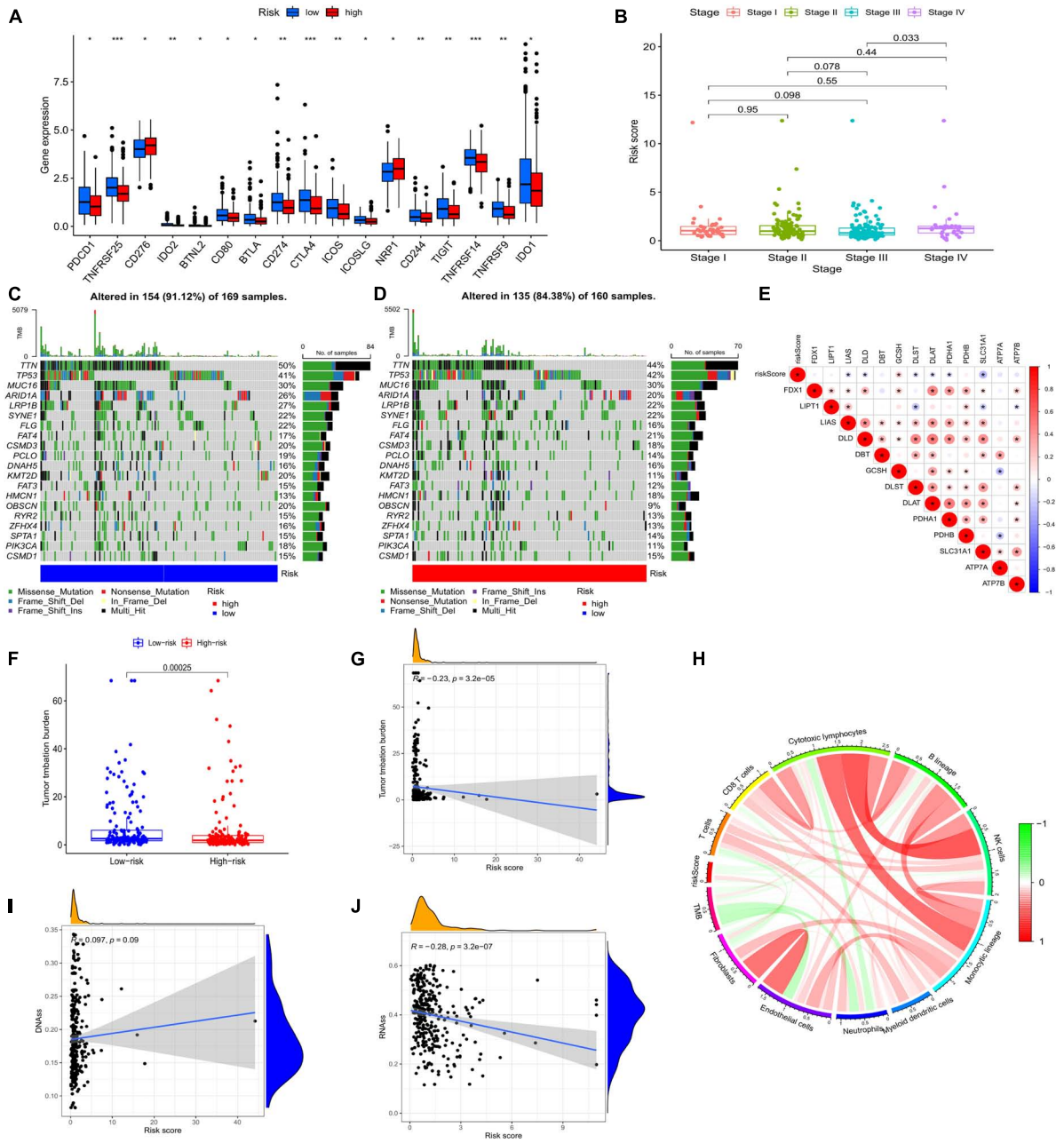

FIGURE 7
(A) Expression of immune checkpoints in the two risk groups. **(B)** Clinical features of the model. Somatic mutations in the low-risk group **(C)** and the high-risk group **(D)**. **(E)** Association between risk score and CRGs. **(F)** TMB of the two risk groups. **(G)** The relationship between TMB and risk score. **(I,J)** Correlation between CSC and risk score. **(H)** Circle picture displaying the relationship between TMB and immune cells. CRGs, cuprotosis-related genes; TMB, tumor mutational burden; CSC, cancer stem cell;

prognosis and multiple tumor-related pathways in CRC (25). AC129507.1 was also used in the exosome-related lncRNA CRC prognostic model by Li et al. as well as in the survival prediction model of gastroesophageal junction adenocarcinoma by Song et al. AC116914.2 was involved in autophagy, m^6A RNA methylation, and hypoxia in head and neck squamous cell carcinoma. Meanwhile, AC116914.2 was associated with survival and immune cell infiltration (26–28). The results of q-RT PCR showed that AC016737.1, AC026782.2, AC090204.1, and AC129507.1 were highly expressed in tumor cells compared with normal cell, while LINC00412 and AC116914.2 were low expressed using Tukey's HSD posttest as the method of multiple comparisons. This is consistent with the fact that AC016737.1, AC026782.2, AC090204.1, and AC129507.1

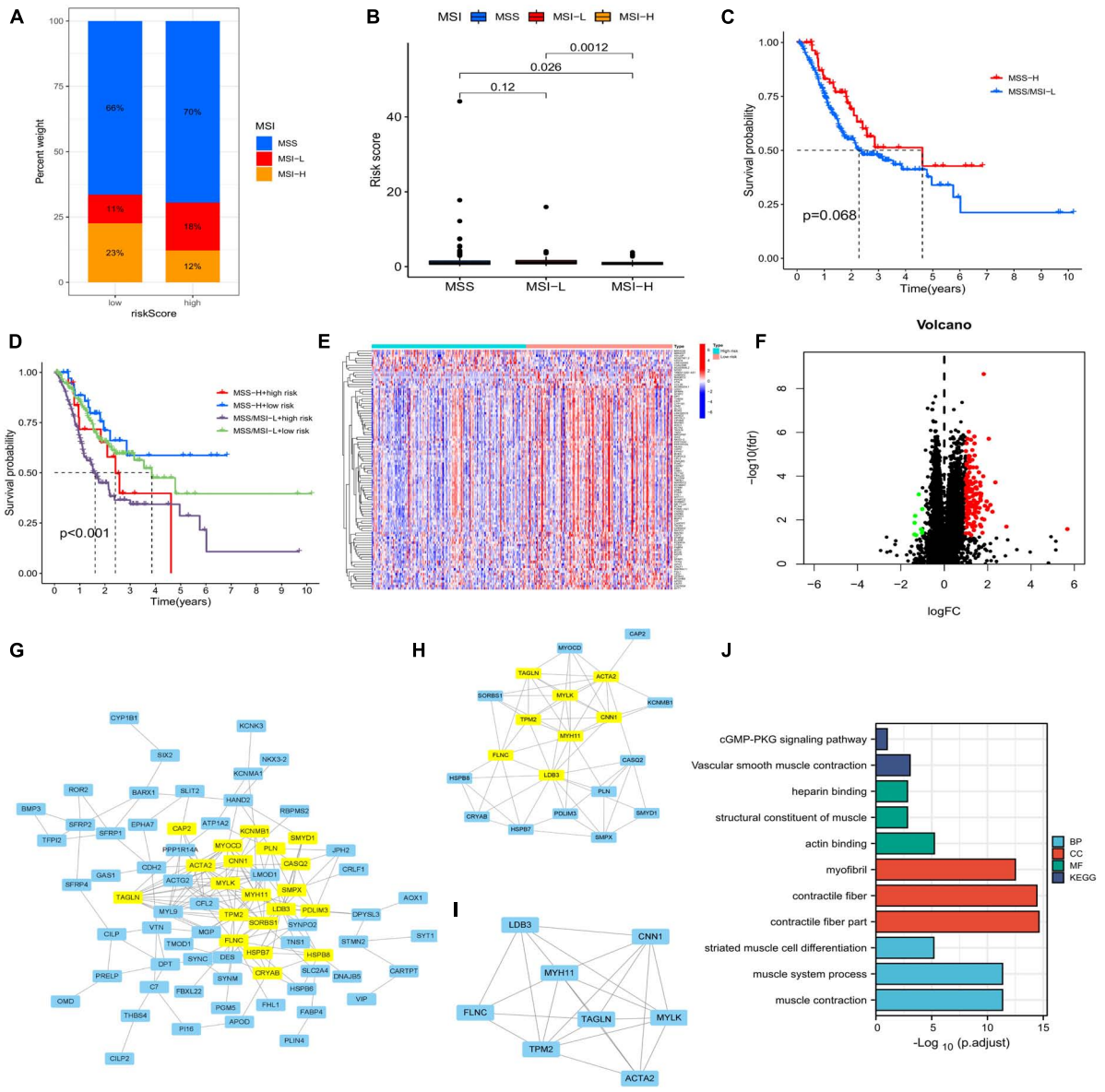

FIGURE 8
(A,B) Relationship between MSI and risk score. **(C)** Correlation between survival time and MSS/MSI-L and MSI-H. **(D)** Survival analysis integrating MSI status and risk signature. **(E)** Differentially expressed genes (DEGs) between the two risk groups. **(F)** Volcano plot of DEGs. **(G–I)** Core DEGs in the two risk groups. **(J)** Gene ontology and Kyoto Encyclopedia of Genes and Genomes analyses of DEGs. MSI, microsatellite instability.

were high-risk genes, and LINC00412 and AC116914.2 were low-risk genes.

The tumor microenvironment is well known to be the site of tumor survival, with multiple components interacting to form a complex and polymorphic environment (29). Immune cell infiltration is one of the key components of TME. Comprehensive analysis of immunological signatures in the TME could facilitate the progress of native and effective immunotherapeutic strategies, as well as the discovery of highly effective biomarkers (30). TME also plays an important role in regulating tumor sensitivity to treatment (31). B cell is the most crucial humoral immune cell, mediating the antitumor response. It is associated with a favorable prognosis and immunotherapy response (32). Notably, the low-risk group had a higher infiltration level of B cell, B cell memory, B cell plasma, and naïve B cell. CD4 + T cells can kill tumors either directly by destroying the tumor cells or indirectly by mediating TME regulation. In addition, CD4 + T cells also can promote gene expression and differentiation of CD8 + T cells (33–35). As a result, we found that some T cell types, including T cell CD4 + memory and T cell gamma delta, were present in higher levels in the low-risk group than in the high-risk

school. The first line of defense in identifying tumors is the ability of CD8 + T cells to recognize MHC class I molecules expressed by tumor cells. CD8 + T cells are the most efficient immune cells against cancer (36). In this study, the infiltration level of CD8 + T cells and CD8 + central memory T cells were higher in the low-risk group than in the high-risk group. Cancer-associated fibroblasts (CAFs) in the TME have been shown to promote the proliferation of multiple tumors by secreting a variety of biological factors to suppress the immune response (37). Various molecules, such as epidermal growth factor and interleukin-6, can be secreted by CAFs to enhance cell proliferation, tumor invasion and metastasis, and epithelial-mesenchymal transition. Notably, a higher infiltration level of CAFs was observed in the high-risk group than in the low-risk group, possibly resulting in the difference in prognosis between the two groups. Meanwhile, we also discovered that the patients with low-risk scores obtained higher immune scores and lower stromal scores than those with high-risk scores, which further gives a reasonable explanation for the difference in prognosis between the two groups.

Immune checkpoint inhibitors have become a promising treatment strategy in almost all kinds of malignant tumors. Several clinical trials involving nivolumab, pembrolizumab, ipilimumab, avelumab, and durvalumab have either been completed or are currently being conducted. In the low-risk group, we found the overexpression of 16 immune

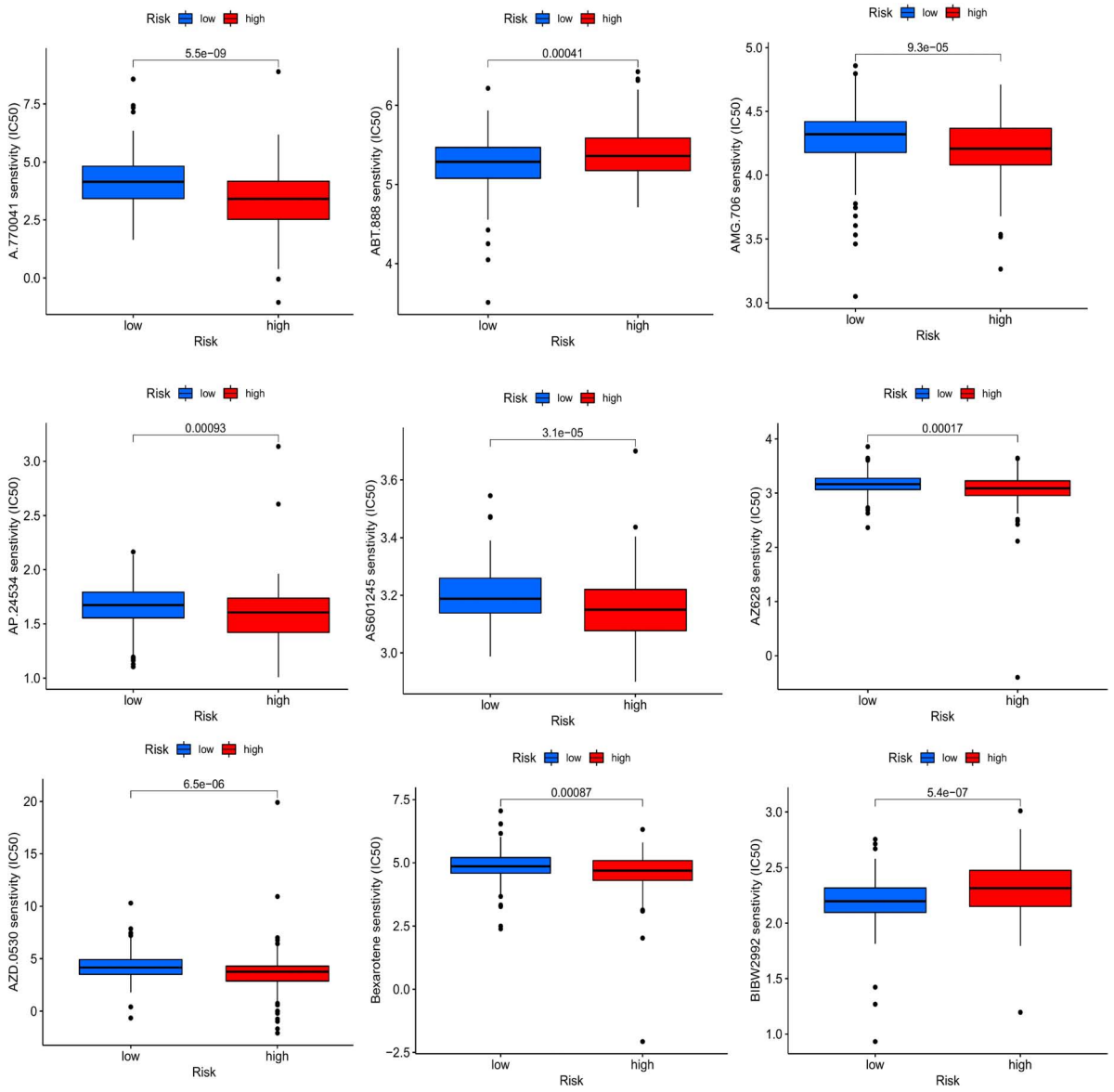

FIGURE 9
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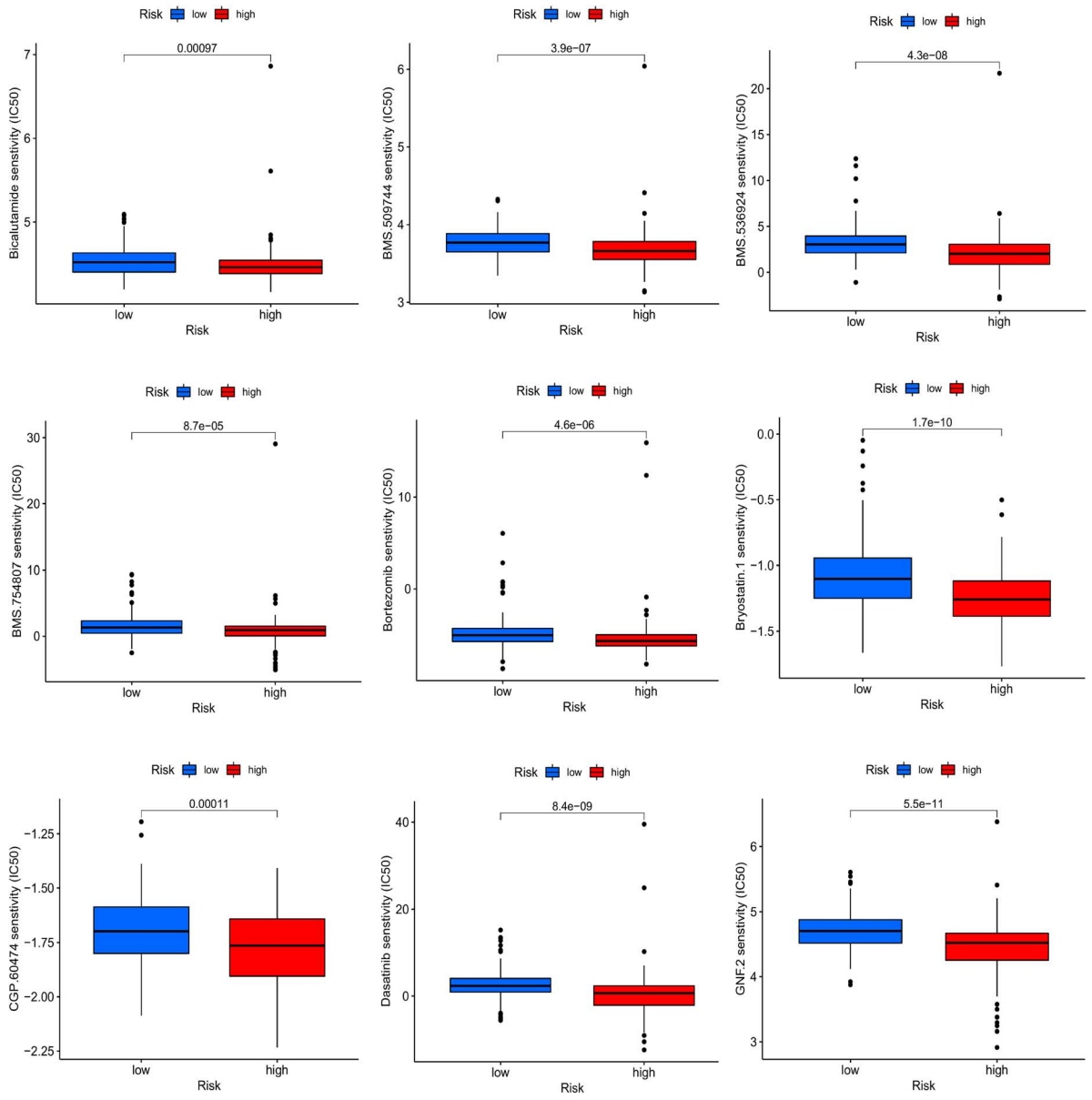

FIGURE 9
Drug sensitivity analysis between high- and low-risk groups.

checkpoints, which could reveal potential immune therapy targets and help develop combination therapies and predictive biomarkers.

Cancer genomics studies have found that most cancers develop with the accumulation of somatic gene mutations (38). In this study, a higher mutation probability was detected in the low-risk group than in the high-risk group. It is widely known that TMB and MSI are predictive biomarkers of immunotherapy response. High TMB and MSI-H appear to be associated with favorable immunotherapy response and prognosis (39, 40). Our findings also confirmed this conclusion and may contribute to revealing potential therapeutic targets.

We also found that patients in the low-risk group were more sensitive to chemotherapy than patients in the high-risk group. Fluorouracil-based adjuvant chemotherapy is recommended for resected stage III and some stage II colon cancers to improve patient survival. Several studies have concentrated on the addition of oxaliplatin to fluorouracil as a novel standardized CRC chemotherapy (41–44). The standard course of adjuvant chemotherapy is 6 months. A major disadvantage of oxaliplatin chemotherapy is cumulative sensory neuropathy. In a clinical trial, 3-month adjuvant chemotherapy in low-risk stage III (not T4 or N2) colon cancer did not compromise treatment

efficacy but reduced drug toxicity (such as neuropathy) (45). Chemotherapy sensitivity is vital for CRC treatment (46). Our prognostic signature can help make chemotherapy more effective or tailor treatment to each individual, which is critical for survival.

Our study also had several limitations. Comprehensive and detailed *in vitro* and *in vivo* experiments are still needed to further validate our conclusion. Also, more clinical samples need to be included.

Conclusion

Based on CRLNCs, a prognostic signature was constructed to predict the survival and chemotherapy sensitivity of patients with CRC. In summary, our study analyzed the role of CRLNCs in CRC and provided new targets and strategies for CRC therapy.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: https://portal.gdc.cancer.gov/repository.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of the Second Hospital of Jilin University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

TL: work concept or design. JZ: data collection. WL and HD: draft the manuscript. GY: make important revisions to the manuscript and approved the final manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.1055785/full#supplementary-material

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Proteomic analysis of the chemosensitizing effect of curcumin on CRC cells treated with 5-FU

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Background: 5-Fluorouracil (5-FU) is one of the most common chemotherapy drugs used to treat colorectal cancer (CRC), which often develops resistance in more than 15% of patients. Curcumin, an active component of Curcuma longa, has been reported to show antitumor activity in CRC and, furthermore, enhance the effect of chemotherapy against colorectal cancer cells. However, the molecular mechanisms underlying the sensitizing effect of curcumin on 5-FU have not been largely elucidated. In this study, we aimed to systematically investigate the role of curcumin as a chemosensitizer for the treatment of CRC, along with the key events responsible for its pharmaceutical effect, which may lead to better clinical outcomes.

Methods: A high-resolution 2DE-based proteomics approach was used to characterize global protein expression patterns in CRC cells treated with 5-FU both in combination with curcumin or without. The differentially expressed proteins were obtained from the 2DE analysis and subsequently identified by MALDI-TOF MS or nano-ESI-MS/MS, some of which were validated by the Western blot. Intracellular reactive oxygen species (ROS) were measured to assess the change in the redox environment resulting from the drug treatment.

Results: A series of proteins with altered abundances were detected and identified by MALDI-TOF or nano-MS/MS. From a total of 512 isolated proteins, 22 proteins were found to be upregulated and 6 proteins were downregulated. Intracellular ROS was significantly elevated after curcumin treatment. Furthermore, mass spectrometry data revealed that some of the proteins appeared to have more oxidized forms upon curcumin treatment, suggesting a direct role for ROS in the chemosensitizing effect of curcumin.

Conclusion: The effect of curcumin in enhancing chemosensitivity to 5-FU is a complex phenomenon made up of several mechanisms, including enhancement of the intracellular level of ROS. Our findings presented here could provide clues for a further study aimed at elucidating the mechanisms underlying the chemosensitizing effect of curcumin.

KEYWORDS

proteomics, curcumin, CRC, 5-FU, ROS

Introduction

Colorectal cancer is a common malignant tumor of the digestive tract with a complicated and multifaceted parthenogenesis (1). It has been well-recognized that both genetic factors and living environment can induce the occurrence of colorectal cancer. Nowadays, the incidence and mortality of colorectal cancer have shown a rapid increase all over the world (2). In China, with the changes in the living environment and dietary habits, the mortality rate of colorectal cancer is also rising rapidly. The incidence rate of colorectal cancer in China has become equal to the world average (3, 4). Currently, methods for the treatment of colorectal cancer include radical surgery, postoperative radiotherapy, and chemotherapy (5–7). Early colorectal cancer can be treated with radical surgery, while in advanced metastatic colorectal cancer, the opportunity for surgery is lost and only chemoradiotherapy and other treatment means can be used though the prognosis is often poor with a 5-year survival rate of only 11%. At present, the clinical treatment of early colorectal cancer is mainly surgical radical resection, followed by 5-fluorouracil (5-FU) combined with other chemotherapy drugs (oxaliplatin, irinotecan, etc.) as postoperative adjuvant chemotherapy, which can further improve the disease-free survival (DFS) and/or overall survival (OS) of patients (8–10). Unfortunately, multiple chemotherapies often lead to drug resistance, which is also a major obstacle affecting the efficacy and prognosis of chemotherapy (11, 12). Curcumin, an active component extracted from Curcuma longa, has been shown to affect the sensitivity of tumor cells to chemotherapeutic drugs, including 5-FU (13). However, the molecular mechanisms underlying the sensitizing effect of curcumin on chemotherapeutic drugs have not been largely explored, which will eventually contribute to the establishment of new treatment strategies to improve drug efficacy, which is of great significance to improving clinical efficacy.

Curcumin and its anti-tumor effects has been subject to extensive exploitation as a third-generation cancer chemopreventive drug for several malignant tumors such as gallbladder cancer, liver cancer, and gastrointestinal cancer (14–16). Although curcumin as an anti-cancer agent has entered the stage of clinical trials, the outcome from some of the clinical trials was not very satisfactory for a variety of reasons (17). Some clinical trials failed because of the low concentration of curcumin adopted in the treatment, while others using high doses of curcumin displayed serious toxic reactions due to its genotoxicity and long-term effects (18). Therefore, it is very urgent for researchers to systematically investigate the detailed mechanisms underlying the pharmaceutical potentials of curcumin as an efficient anti-cancer agent in clinical applications.

Since the concept of the proteome was first proposed in 1994, the field of proteomics has been developing rapidly, providing a high-throughput technological platform for in-depth and systematic research on various life phenomena and their mechanisms, as well as the pathogenesis of various major human diseases, from a dynamic, multidisciplinary, and holistic perspective (19–21). At present, extensive proteomic investigations have been carried out in multiple human tumor tissues or cell lines, including colorectal cancer (CRC), whereas limited proteomic studies have focused on the anti-tumor effect of curcumin on CRC. For example, Lee et al. compared the proteomes of primary and metastatic colorectal cancer cell lines, SW480 and SW620, respectively, which were treated with different chemotherapy agents and natural compounds. The results showed that oxaliplatin, ginsenoside 20(S)-Rg3, and curcumin displayed significant anti-tumor activity, which mainly affected fatty acid synthase and histone H4 (22). In another example using an analog of curcumin with an alkyne moiety that can be conjugated with functional moieties through click chemistry, a list of proteins in HCT116 cells that were bound to curcumin were identified, suggesting that curcumin may target EIF2, eIF4/p70S6K, and mTOR signaling pathways. In addition, mitochondrial dysfunction could be induced by curcumin (23). Although some achievements have been made by several proteomics studies on the anti-cancer potential of curcumin, the underlying multifaceted mechanisms remain unclear and need to be further explored.

In the present study, we adopted 2DE coupled with mass spectrometry to systematically identify the key proteins, as well as the key events, involved in the chemosensitizing effect of curcumin on the CRC cells treated with 5-fluorouracil (5-FU), aiming to further decipher the underlying molecular mechanisms that may eventually lead to better clinical outcomes.

Methods

Cell culture

Human CRC SW480 cells were cultured in DMEM supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum (FBS) at 37°C in 10-cm dishes under a humidified 5% CO_2 atmosphere. Curcumin, as well as 5-fluorouracil, dissolved in DMSO was added into the culture media at different final concentrations.

Cytotoxicity assay

The cytotoxic activities of the two compounds, namely, curcumin and 5-fluorouracil, toward SW480 cells were

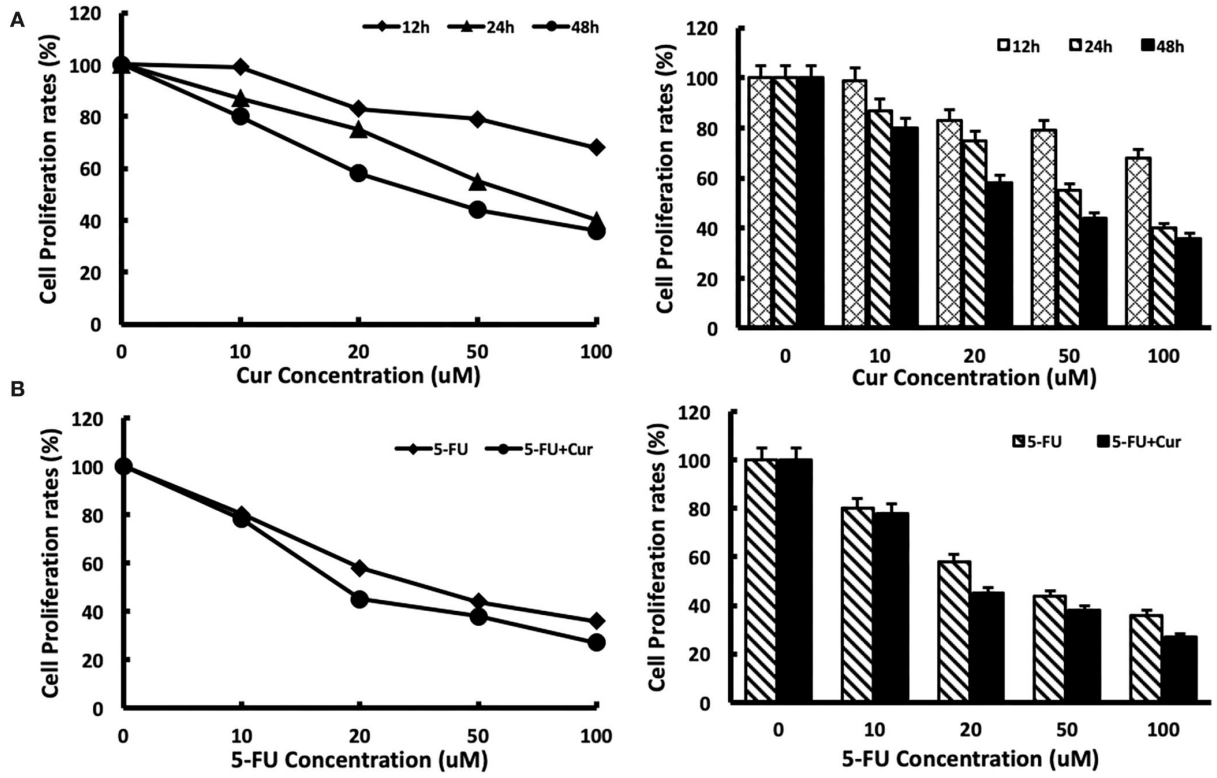

FIGURE 1
Cytotoxicity of 5-FU in SW480 cells is enhanced by curcumin. **(A)** Cytotoxicity of curcumin was determined after exposure of SW480 cells to curcumin with different concentrations (0, 10, 20, 50, and 100 μM) and different times (12 h, 24 h, and 48 h). Cell viability was measured with the MTT analysis. **(B)** Cytotoxicity of 5-FU was determined after exposure of SW480 cells to 5-FU alone and 5-FU in combination with 5 μM curcumin for 48 h. Cell viability was measured with the MTT analysis. The results are provided as mean values with standard deviations from at least three independent experiments.

measured using the MTT assay. Briefly, 1×10^4 cells per well were seeded into 96-well culture plates and cultured for 48 h at 37°C. Then, the culture media were replaced with fresh DMEM containing curcumin and/or 5-fluorouracil and incubated for an additional 48 h. The culture media were then replaced by freshly prepared media containing 0.5 mg/ml MTT. After 4 h of incubation, the resulting insoluble purple formazan was dissolved with 200 μl DMSO. A microplate reader was used to measure the absorbance at 570 nm to calculate the cytotoxicity of the drugs. Triplicate measurements were performed for each concentration of the drugs.

Protein sample preparation

The cells that were used for proteomics analyses were washed with a Tris-buffered 250 mM sucrose solution and collected using a cell scraper. Then, the cells were lysed with a freshly prepared lysis buffer (8 M urea, 4% w/v CHAPS, and 50 mM DTT), which was supplemented with a cocktail of protease inhibitors. The protein samples were obtained from cell lysates by ultracentrifugation at 16,000 × *g* at 4°C for 30 min. The samples were subjected to a DC-RC protein assay and then stored at −80°C until used for 2DE.

2DE

The protein samples that were subjected to 2DE were prepared by dilution of 0.5 mg protein into 300 μl with rehydration solution, followed by loading into IPG strips (24 cm, pH 3–10 nonlinear, Amersham) for 12 h. Isoelectric focusing was carried out for a total of 70,000 V-h. Then, the IPG strips were equilibrated, and the proteins in the IPG strips were separated by the second-dimensional SDS electrophoreses. After 2DE separation, the gels were detached from glass plates and fixed immediately in 10% TCA for 60 min. The proteins in the gels were stained with Coomassie Brilliant Blue G-250. The gel images were scanned with a scanner. The PDQuest software was used to analyze the gel images, by which the total density on each gel was normalized to accurately compare spot quantity between gels.

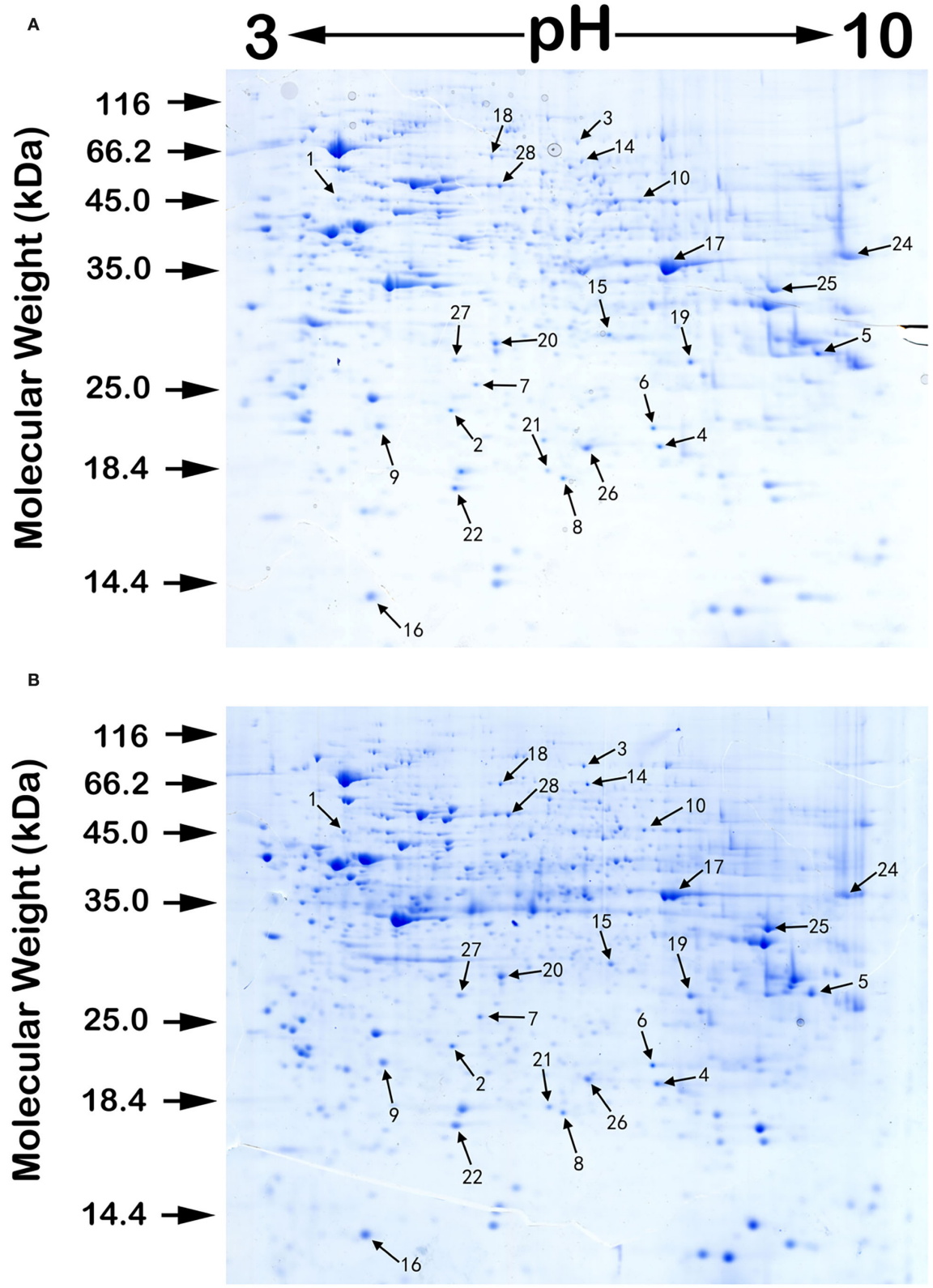

FIGURE 2
A pair of protein 2DE images of SW480 cells treated with 5-FU alone **(A)** and those treated with both 5-FU and curcumin **(B)** with sample loading of 0.5 mg protein each. The isoelectric focusing was carried out on 24 cm IPG strips with a nonlinear pH range of 3–10. Then, the proteins in the IPG strips were separated by the second-dimensional SDS electrophoreses. The gels were stained by Colloidal Coomassie blue G-250. Numbers associated with the spots on the gel images refer to the identified proteins listed in Table 1.

TABLE 1 Summary of differentially expressed proteins (DEP) in SW480 cells in response to 5-FU and curcumin treatment.

Spot number	Accession number	Protein information	Gene name	Observed pI/Mw (kDa)	Sequence coverage%	Theoretical pI/Mw (kDa)
Upregulated proteins in response to curcumin treatment						
1	P27797	Calreticulin	CALR	4.02/47.4	28	4.29/48.14
2	Q04917	14-3-3 protein eta	YWHAH	4.90/25.3	24	4.76/28.2
3	P10809	60 kDa heat shock protein, mitochondrial	HSPD1	5.25/55.4	31	5.7/61.02
4	P49720	Proteasome subunit beta type-3	PSMB3	6.45/22.3		6.13/22.95
5	P15559	NAD(P)H dehydrogenase [quinone] 1	NQO1	8.45/35.5	24	8.91/30.87
6	P30041	Peroxiredoxin-6	PRDX6	5.45/23.0	19	6.0/25.03
7	P25788	Proteasome subunit alpha type-3	PSMA3	5.35/25.5	27	5.19/28.43
8	Q14152	Eukaryotic translation initiation factor 3 subunit A	EIF3A	5.33/24.0	27	6.38/116.57
9	P52565	Rho GDP-dissociation inhibitor 1	ARHGDIA	5.24/22.2		5.01/23.21
10	Q5EBM0	UMP-CMP kinase 2, mitochondrial	CMPK2	6.42/49.8	35	6.57/49.45
14	P17987	T-complex protein 1 subunit alpha	TCP1	5.88/61.5	22	5.8/60.34
15	Q15366	Poly(rC)-binding protein 2	PCBP2	6.41/42.4		6.33/38.58
16	P05386	60S acidic ribosomal protein P1	RPLP1	4.35/12.4		4.21/11.51
17	P06733	Enolase 1	ENO1	6.92/47.7	34	7.01/47.17
18	P07237	Protein disulfide-isomerase	P4HB	4.90/59.3	39	4.76/57.12
19	P32322	Pyrroline-5-carboxylate reductase 1, mitochondrial	PYCR1	7.01/30.7	35	7.18/33.36
20	P09972	Aldolase C, fructose-bisphosphate	ALDOC	6.10/40.9	26	6.14/39.46
21	P15531	Nucleoside diphosphate kinase A	NME1	6.27/20.0	31	5.81/17.15
22	P60660	Myosin light polypeptide 6	MYL6	5.11/18.1	26	4.56/16.93
Downregulated proteins in response to curcumin treatment						
24	P39023	60S ribosomal protein L3	RPL3	10.00/42.8	26	10.19/46.11
25	P22626	Heterogeneous nuclear ribonucleoproteins A2/B1	HNRNPA2B1	9.02/39.3	32	8.97/37.43
26	P33316	Deoxyuridine 5′-triphosphate nucleotidohydrolase, mitochondrial	DUT	5.95/20.5	38	6.15/17.75
27	P12429	Annexin A3	ANXA3	5.89/35.8	24	5.62/36.38
28	P52597	Heterogeneous nuclear ribonucleoprotein F	HNRNPF	5.08/47.0	33	5.37/45.67

In-gel digestion and MS analysis

The protein spots of interest were destained and then subjected to in-gel digestion by TPCK-trypsin for 12 h at 37°C. The tryptic peptides were purified by ZipTip C18 tips before the MS analysis. Most of the tryptic peptide samples were analyzed using a Voyager DE STR MALDI TOF mass spectrometer (Applied Biosystems). A saturated CHCA solution was used as a matrix, which was mixed with peptide samples and then loaded on the sample plate. Besides MALDI TOF MS, nano-ESI-MS/MS was performed on some of the tryptic peptide samples by using a QSTAR mass spectrometer (Applied Biosystems). The tryptic peptide sample was loaded onto a PicoTip emitter and then ionized through an external nanoelectrospray ion source. The ions with multiple charge states were manually selected for MS/MS

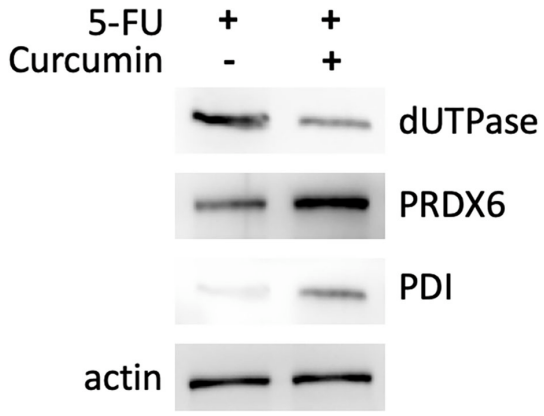

FIGURE 3
Western blotting analysis of three differentially expressed proteins: PRDX6, PDI, and dUTPase. Differential expression of the protein of interest between SW480 cells treated with 5-FU (20 μM) in combination with or without curcumin is normalized to actin level.

analysis to obtain the data for their fragment ions. Both MS and MS/MS data were searched against the human subset in the SwissProt database using the MASCOT software to identify the protein spots.

Western blot

For validation of the results from proteomics analyses, the cell lysates obtained in urea/thiourea lysis buffer (refer to the "Cytotoxicity assay" section) were mixed 1:1 with denaturing loading buffer and subjected to SDS-PAGE 12%. The separated proteins were then transferred onto PVDF membranes. Proteins of interest on the membranes were probed using primary antibodies such as anti-PRDX6 polyclonal antibody (Invitrogen), anti-PDI monoclonal antibody (Santa Cruz Biotechnology), and anti-dUTPase monoclonal antibody (Santa Cruz Biotechnology), followed by incubation with properly diluted secondary antibodies conjugated with horseradish peroxidase. The signals of each protein were then visualized using an ECL reagent.

ROS assay

A DCFH-DA probe was used to detect intracellular ROS levels. The DCFH-DA probe can be captured by cells and enter the cell. After cell metabolism, it is oxidized by intracellular ROS to generate fluorescent products, which can be detected by FCM (flow cytometry). SW480 cells were seeded in six-well plates for 24 h, then curcumin and 5-FU were added to each well at different concentrations (0, 20, 50, and 100 μM), and the culture was continued for 48 h. Triplicate the cells for each concentration of the drug. Then, the cells were collected and washed three times with PBS. The DCFH-DA probe was added to the cell suspension and incubated for 20 min in the dark. The cells were washed three times with PBS, resuspended with PBS, and the fluorescence signal intensity was assayed using FCM (flow cytometry).

Results and discussion

Curcumin can increase the cytotoxicity of 5-FU on SW480 cells

To measure the inhibitory effect of curcumin on the viability of SW480 cells, we used MTT analysis to test the viability of SW480 cells treated with different concentrations (0, 10, 20, 50, and 100 μM) of curcumin for different incubation times (12, 24, and 48 h). The results showed that with the increase in curcumin concentration, the growth inhibition rate of SW480 cells decreased significantly. The inhibitory effect of curcumin was maximal after 48 h of incubation. The IC50 of curcumin was 30 μM at 48 h. No inhibition of the viability of SW480 cells was observed at the concentration of 5 μM. The results are shown in Figure 1A. Then, we tested the inhibitory effect of different concentrations (0, 10, 20, 50, and 100 μM) of 5-FU on the viability of SW480 cells with or without low concentration (5 μM) curcumin for 48 h. The results showed that, compared with those treated with 5-FU alone, the viability of SW480 cells decreased significantly with the increase of 5-FU concentration in a dose-dependent way: in the cells treated with 5-FU alone, when the concentration of 5-FU was 10 μM, the growth inhibition rate was significantly inhibited. When the concentration of 5-FU was 100 μM, the growth inhibition rate was at its maximum, and the IC50 value was about 40 μM. In the cells treated with both 5-FU and 5 μM curcumin, the growth inhibition rate was significantly enhanced compared with those treated with the same concentration of 5-FU, and the IC50 value was reduced to 20 μM. The results are shown in Figure 1B.

Proteomics analysis identified relevant proteins targeted by curcumin treatment

We performed proteomic analysis on the SW480 cells treated with 20 μM 5-FU alone and those treated with 20 μM 5-FU and 5 μM curcumin. Total proteins were extracted from the collected cells and then separated by high-resolution 2DE (Figure 2). The PDQuest software was used to compare the protein spot patterns of the gel images. A total of 28 differentially expressed protein spots between the two experimental groups were detected to be responsible for curcumin treatment.

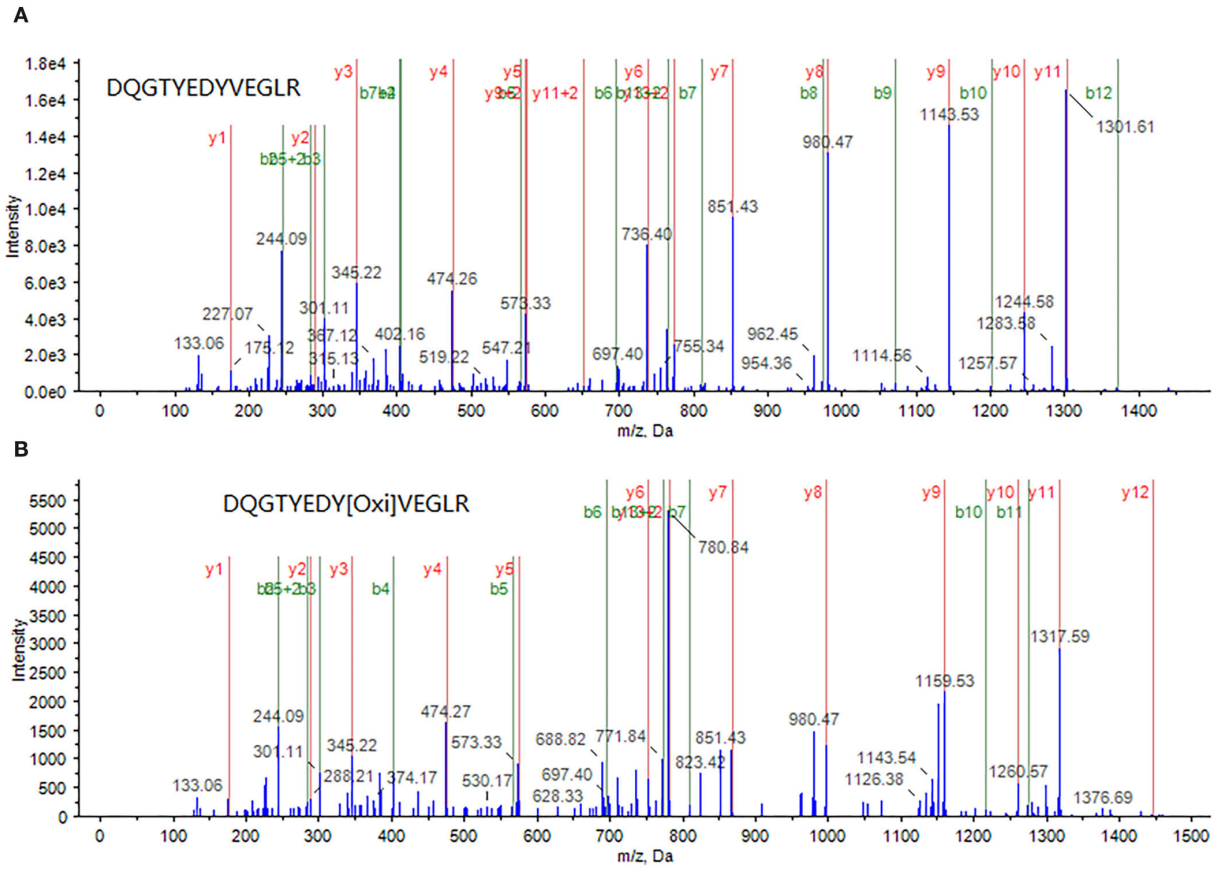

FIGURE 4
A peptide DQGTYEDYVEGLR (82–94) from myosin light polypeptide 6 was identified through MS/MS spectrum of a doubly-charged peak at m/z 772.8 **(A)**, whereas its oxidized form, in which the tyrosine (Y) is oxidized, was identified through MS/MS spectrum of a doubly charged peak at m/z 780.7 **(B)**.

Among these proteins, 22 proteins were upregulated and 6 proteins were downregulated upon treatment with a combination of 5-FU and curcumin. These protein spots were subjected to MALDI-TOF-MS or nano-ESI-MS/MS analyses and subsequently identified by database searching (Table 1). From the identified protein candidates, peroxiredoxin-6 (PRDX6), protein disulfide-isomerase (PDI), and deoxyuridine 5'-triphosphate nucleotidohydrolase (dUTPase) were validated by Western blot analysis, and the expression changes were consistent with the 2DE results as shown in Figure 3.

Through an in-depth examination of the mass spectra of some differentially expressed proteins, evidence of the oxidized form of some peptides was discovered in cells treated with a combination of curcumin and 5-FU, implying an elevated oxidative environment in these cells. For example, in the samples treated with a combination of curcumin and 5-FU, myosin light polypeptide 6 appeared to be oxidized at a tyrosine residue within its peptide DQGTYEDYVEGLR (82–94), as shown in Figure 4.

Intracellular ROS level was significantly increased upon curcumin treatment

In the process of apoptosis mediated by the mitochondrial pathway, with the increase of the degree of mitochondrial membrane potential depolarization, the occurrence of an oxidative stress response will be activated, leading to an increase in ROS level and promotion of the process of apoptosis. Therefore, ROS levels are an important marker of mitochondrial pathway-mediated apoptosis. In this study, ROS levels were measured in SW480 cells treated with different concentrations of 5-FU (0, 20, 50, and 100 μM). For the cells treated with 5-FU alone, the ROS level of SW480 cells was slightly higher than that of the cells untreated, in a dose-dependent way, as shown in Figure 5A(a–d). As indicated in the "Curcumin can increase the cytotoxicity of 5-FU on SW480 cells" section, curcumin could significantly enhance the inhibitory effect of 5-FU on the viability of SW480 cells. Therefore, we also measured the ROS level of SW480 cells treated with different concentrations (0,

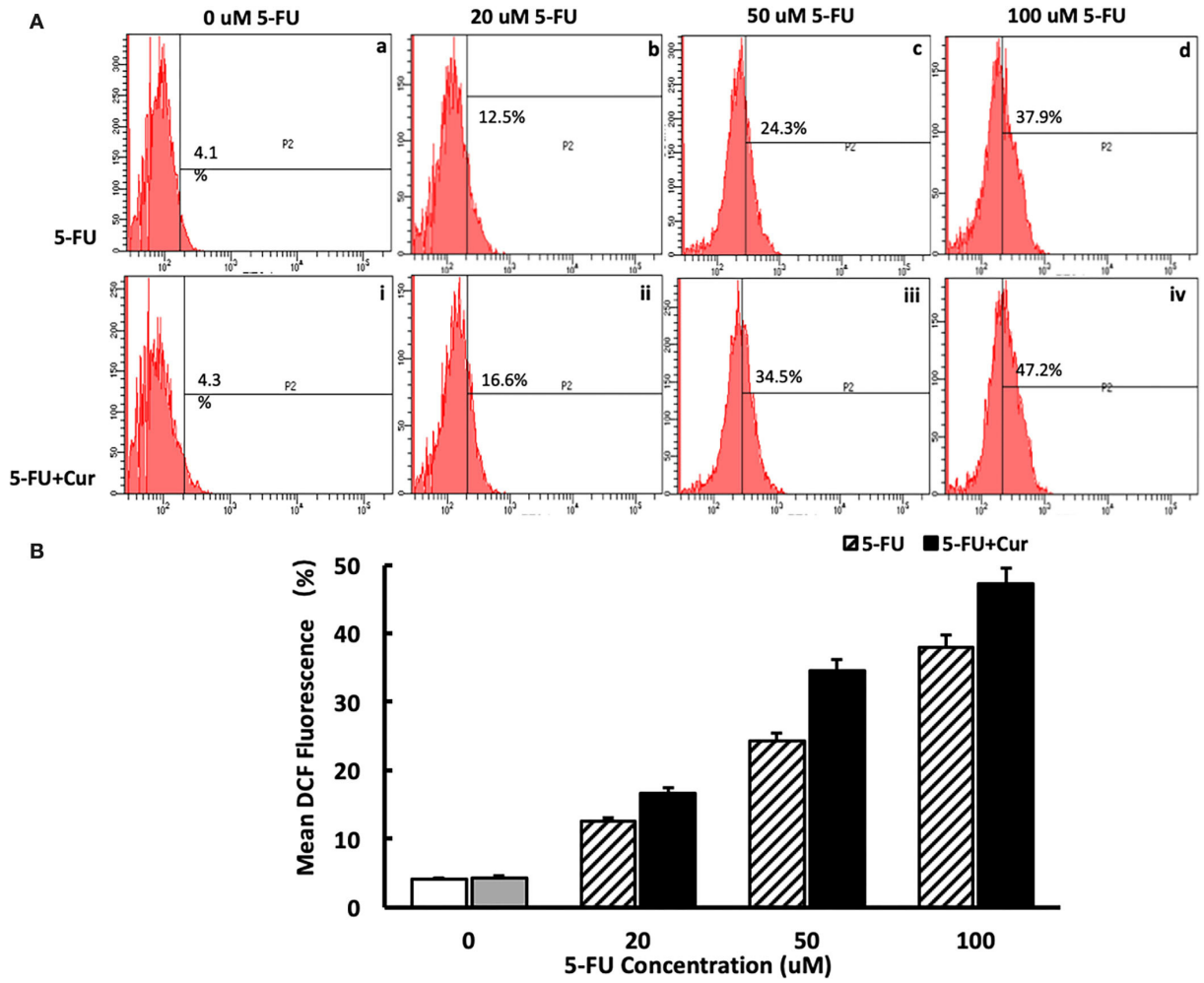

FIGURE 5
Curcumin can increase the ROS level in SW480 cells treated with 5-FU. [**(A)**: a–d] ROS level in SW480 cells treated with 5-FU (0, 20, 50, and 100) alone was slightly higher than that of the cells untreated, in a dose-dependent way. [**(A)**: i–iv, **(B)**] ROS level in SW480 cells treated with 5-FU (0, 20, 50, and 100 μM) and curcumin increased significantly compared to those in the cells treated with 5-FU alone in a dose-dependent manner.

20, 50, and 100 μM) of 5-FU and 5 μM curcumin. The results showed that ROS levels in the cells treated with both 5-FU and curcumin increased significantly compared to those in the cells treated with 5-FU alone in a dose-dependent manner, as shown in Figures 5A(i–iv),B.

Curcumin is a natural phenolic compound extracted from turmeric. It has been proven that curcumin has multiple biological activities, such as antioxidant, hypotensive, anti-inflammatory, and immune enhancement, especially with high anti-tumor activity. Existing studies have found that curcumin has a significant inhibitory effect on colorectal cancer, thyroid cancer, and liver cancer. Chemotherapy plays a vital role in the comprehensive treatment of tumors, especially in patients with advanced tumors. Curcumin can promote the chemosensitivity of a variety of cancers by multiple mechanisms, including enhancement of the production of intracellular ROS (24–26).

Oxidative stress, which is caused by harmful stimulation, intracellular reactive oxygen species (ROS) level, broken oxidative balance, and excessive ROS, can affect mitochondrial function, inhibit the cell cycle, and, through the mitochondria, cause endoplasmic reticulum stress, the death of receptor regulation pathways, cause DNA damage, and induce cell apoptosis (27). Previous studies have found that curcumin can cause an increase in ROS levels and oxidative stress in CRC cells, thereby inducing cell apoptosis (28). Studies have found that curcumin can significantly increase the ROS level in SGC7901 gastric cancer cells, upregulate the protein expression of Bax and P53, downregulate the protein expression of Bcl-2, and activate the apoptosis mediated by the JNK regulatory pathway (29). The increase in ROS induced by curcumin can also cause ER stress and induce cell apoptosis. Studies have found that curcumin can affect the upregulation of ER stress regulatory protein CHOP

and glucose regulatory response protein GRP78 expression in SUNE1 cells of nasopharyngeal carcinoma, activate ER stress, and thus induce cell apoptosis (30).

Conclusion

The underlying mechanisms of the chemosensitizating activity of curcumin are complicated and multifaceted; the intracellular reactive oxygen species are recognized to play a key role, as revealed by the identification of some oxidized protein targets upon treatment by curcumin in the present study. However, the detailed mechanisms within which these oxidative modifications play a role in the chemosensitazing effect of curcumin have not been well established and need further in-depth investigations *in vitro* and *in vivo*. Overall, our findings in the present study could provide a new direction for further elucidating the sensitization mechanism of curcumin.

Data availability statement

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

Author contributions

NL and CH: conceptualization. JY and NL: methodology, writing, review, and editing. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Biomarkers in the development of individualized treatment regimens for colorectal cancer

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Introduction: Colorectal cancer (CRC) is the third most common and second most deadly malignancy in the world with an estimated 1. 9 million cases and 0.9 million deaths in 2020. The 5-year overall survival for stage I disease is 92% compared to a dismal 11% in stage IV disease. At initial presentation, up to 35% of patients have metastatic colorectal cancer (mCRC), and 20–50% of stage II and III patients eventually progress to mCRC. These statistics imply both that there is a proportion of early stage patients who are not receiving adequate treatment and that we are not adequately treating mCRC patients.

Body: Targeted therapies directed at CRC biomarkers are now commonly used in select mCRC patients. In addition to acting as direct targets, these biomarkers also could help stratify which patients receive adjuvant therapies and what types. This review discusses the role of RAS, microsatellite instability, HER2, consensus molecular subtypes and ctDNA/CTC in targeted therapy and adjuvant chemotherapy.

Discussion: Given the relatively high recurrence rate in early stage CRC patients as well as the continued poor survival in mCRC patients, additional work needs to be done beyond surgical management to limit recurrence and improve survival. Biomarkers offer both a potential target and a predictive method of stratifying patients to determine those who could benefit from adjuvant treatment.

KEYWORDS

adjuvant chemotherapy, targeted therapies, colorectal cancer, individualized medicine, biomarkers

Introduction

Colorectal cancer (CRC) is the third most common and second most deadly malignancy in the world with an estimated 1.9 million cases and 0.9 million deaths in 2020 (1). With improved screening and enhanced surgical options, the overall survival in patients with CRC has improved over time with a current overall relative survival of 65% at 5 years (2). However, this survival varies greatly as the disease progresses. The 5-year overall survival for stage I disease is 92% compared to a dismal 11% in stage IV disease (3). At initial presentation up to 35% of patients have metastatic colorectal cancer (mCRC) with 20–50% of stage II and III patients eventually progressing to mCRC (4).

Current recommendations suggest that patients with stage III (lymph node-positive) colon cancer undergo surgical resection followed by adjuvant chemotherapy. There continues to be controversy about the survival benefit of chemotherapy in patients with stage II disease (5). The intention of adjuvant chemotherapy is to decrease the chances of recurrence in the setting of curative resection. As stage II disease is node-negative, there is a presumption of local disease without metastases. Current recommendations suggest that stage II patients do not receive adjuvant therapy, however up to 23% will have a recurrence within 5 years indicating we are not currently capturing a population who may indeed have initial early spread and would benefit from additional therapy (6). Therefore, some argue that "high risk" stage II patients should receive adjuvant therapy in hopes of rescuing this population who will eventual relapse. Some high risk factors in stage II disease that have been suggested as warranting adjuvant treatment include T4 tumors, <12 lymph nodes harvested at surgery, presence of bowel obstruction or perforation, poorly differentiated tumors, and the presence of lymphovascular/perineural invasion (7). Of these, only T4 disease has been validated to help identify the subset of stage II patients who benefit from adjuvant chemotherapy (8).

Standard adjuvant treatment regimens for high risk stage II or stage III disease include combination therapies of CAPEOX (capecitabine and oxaliplatin) and FOLFOX (leucovorin, fluorouracil (5-FU), and oxaliplatin). However, only 20% of patients benefit from adjuvant chemotherapy, exposing 80% of patients to unnecessary toxicity (9). In addition to these combination therapies of classic chemotherapy agents, newer targeted agents exist and may confer benefits in specific patient populations. Better biomarkers that stratify patient risk (prognostic) and predict therapeutic responses (predictive) could reduce the exposure of patient populations to unnecessary toxicity and increase the likelihood of eliminating the chance of recurrence in patients after resection. Biomarkers could aid in defining the optimum regimen of adjuvant chemotherapy, the duration of treatment, the utility of additional targeted treatments, and which patient populations should receive it (Table 1).

Microsatellite instability

High microsatellite instability (MSI-H) is the phenotype of a deficient mismatch repair (dMMR) system and is present in about 15% of colorectal cancers. Microsatellites are short tandem repeats of single nucleotide or di-, tri-, or tetra-nucleotides in DNA sequences found throughout the tumor genome and are a marker of a hypermutable phenotype. The mismatch repair (MMR) system functions to rectify errors that may occur during DNA replication. With the inactivation of at least one of the DNA MMR genes (MLH1, MSH2, MSH6, and PMS2) through either mutations or transcriptional silencing, the MMR system is unable to function leading to an accumulation of errors in the DNA (10). This inactivation stems from either germline mutations in the MMR genes themselves or somatic hypermethylation of CpG islands surrounding the promotor region in the genes. Germline mutations in MMR lead to hereditary non-polyposis colorectal cancer (HNPCC or Lynch Syndrome) which causes ∼3% of all CRCs (11). The somatic hypermethylation of CpG islands is known as the CpG island methylator phenotype (CIMP). These CIMP tumors comprise the majority of sporadic MSI-H CRCs (12). These CIMP tumors are in contrast to tumors with chromosomal instability (CIN) which follow the more traditional pathway of initial APC mutation causing a tubular adenoma with subsequent accumulated mutations leading to cancer (13).

MSI-H/dMMR is more common among stage II tumors compared with stage III CRCs and relatively uncommon in stage IV (metastatic) CRCs suggesting MSI-H/dMMR tumors are less likely to metastasize. Indeed, MSI-H/dMMR is independently associated with improved survival compared with tumors with proficient MMR (pMMR) (14). In addition, MSI-H/dMMR tumors also have lower recurrence rates compared with pMMR tumors (15). It has also been shown that MSI-H/dMMR tumors do not respond well to 5-FU-based adjuvant chemotherapy (16). Indeed, cells require a competent MMR system for 5-FU to be effective (17). Current recommendations suggest that patients with stage II colon cancer with MSI-H/dMMR should not receive adjuvant 5-FU-based chemotherapy based on this known favorable prognosis and lack of response to therapy.

Unlike 5-FU, oxaliplatin leads to DNA-cross linking and inhibits DNA synthesis and transcription. This damage is not recognized by the MMR system and dMMR tumors should not be resistant to oxaliplatin. The MOSAIC trial revealed improvement in 5-year DFS and 6-year OS for stage III colon cancers with the addition of oxaliplatin to 5-FU regardless of MMR status (18). Ten year follow up of the MOSAIC trial confirmed the benefit of oxaliplatin as adjuvant therapy in stage II/III colon cancers. More recent work has revealed a potential benefit to the addition of oxaliplatin to fluoropyrimidines in adjuvant chemotherapy for MSI-H stage III colon cancer (19).

In addition to standard chemotherapy, additional treatment options exist that may specifically benefit in MSI-H/dMMR patients. As previously discussed, MSI-H/dMMR have a baseline improved clinical course compared to tumors with pMMR. This may be due to their hypermutable phenotype contributing to the production of abnormal peptides that serve as neoantigens, producing specific antitumor immune responses leading to decreased tumor growth and metastasis (20). Sporadic MSI-H CRC have a distinct phenotype that includes right colon predominance, increased prevalence in women and poor differentiation/mucinous histology. MSI-H tumors also exhibit an elevated number of tumor-infiltrating lymphocytes (TILs), supporting this neoantigen hypothesis (21). This baseline local immune control contributes to improved patient survival in

TABLE 1 Emerging and established biomarkers.

Biomarker	Targeted drugs	Resistance	Chemotherapy
Microsatellite instability	Pembrolizumab Nivolumab (PD-1 inhibitors) Ipilimumab (CTLA4 inhibitor)		Stage II dMMR patients have not been shown to benefit from 5-FU adjuvant therapy Oxaliplatin may have a benefit in MMR tumors
RAS	Small molecules targeting G12C variant	Confers anti-EGFR agent resistance	
BRAF	BRAF inhibitors	Negative predictor of response to anti-EGFR therapies	
HER2	Trastuzumab Lapatinib Pertuzumab Trastuzumab deruxtecan	Predict resistance to anti-EGFR therapies	HER2 high patients may benefit from adjuvant chemotherapy
APC	Tankyrase inhibitors		
CEA			CEA high patients may benefit from adjuvant chemotherapy
NTRK	Enterctinib Larotrectinib		

Biomarkers offer targets for directed drug therapy as well as potential markers of resistance. In addition, biomarkers can be used to help guide chemotherapy decisions.

MSI-H CRC and also sensitizes tumors in these patients to immune checkpoint inhibitors targeting either programmed cell death-1 protein (PD-1) or cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). PD-1 is expressed on T cells, and binding of its ligands (PD-L1 and PD-L2) downregulates T cell effector function. In that context, tumors can escape immune detection by upregulating expression of programmed death ligand 1 (PD-L1) (22). Inhibitors of PD-1 block the receptor from interacting with its ligands, promoting tumor cell killing by effector T cells. Inhibitors of PD-1, pembrolizumab (Keytruda) and nivolumab (Opdivo), are FDA-approved for patients with mCRC with dMMR or MSI-H and confer a significant survival benefit when used (23, 24). An additional target, CTL-4, is transiently expressed on activated T cells with its expression inhibiting the production of cytokines and providing a negative feedback signal to T cells prompting T cell cycle arrest. Inhibition of CTLA-4 may lead to reactivation of T cells allowing them to overcome tumor-induced immune tolerance (25). Ipilimumab (Yervoy) is an anti-CTLA-4 antibody used in metastatic dMMR/MSI-H patients in combination with nivolumab (26). This combination of nivolumab and low-dose ipilimumab produced an objective response rate of 64%, complete response rate of 9%, and disease control rate of 84%, all of which were durable (27). While the results of immune checkpoint blockade in dMMR/MSI-H CRC patients have been encouraging, single agent checkpoint inhibitors are not efficacious in patients with pMMR which makes up the majority of CRC patients (28). In addition, while immune checkpoint inhibitors are approved in mCRC dMMR/MSI-H disease, their utility as adjuvant therapy in localized disease and their efficacy in combination are being explored (29, 30). The use of dMMR/MSI-H as a biomarker in determining the need for adjuvant therapy, the type of adjuvant chemotherapy and the addition of an immune checkpoint inhibitor could better optimize the alignment of treatment groups and therapies.

MAPK pathway (Ras-Raf-MEK-ERK)

Gain or loss of function mutations in proteins in the mitogen-activated protein kinase (MAPK) pathway lead to dysregulated proliferative cell signaling ultimately driving tumorigenesis. The first protein to be activated in the pathway is RAS, a commonly mutated protein in CRC (31). In the normal cell, activation of RAS begins with an extracellular ligand binding to a receptor-linked tyrosine kinase like epidermal growth factor receptor (EGFR). This binding activates the tyrosine kinase in the cytoplasmic domain of the receptor causing phosphorylation of EGFR and interaction with RAS. This triggers RAS, a GTPase, to exchange a GDP molecule for GTP, activating the pathway and initiating a kinase cascade leading to the activation of Raf, MAPK/ERK (MEK1 or 2) and ultimately MAPK (32).

RAS (KRAS, NRAS, and HRAS) is the most frequently mutated gene family in cancers with the most common oncogenic mutant of the RAS family being KRAS G12C. KRAS mutations are present in 30–50% of CRC with NRAS mutated in 3–5% and HRAS mutated in <1% (33, 34). KRAS mutations account for up 45% of mCRC and ∼15–37% of early stage tumors (35, 36). Historically, RAS was considered "undruggable" due to its picomolar affinity for GTP/GDP, the absence of identified allosteric regulatory sites, and the necessity of wild type RAS in normal biologic functions. However, small

molecules that specifically inhibit the G12C variant have been identified, making RAS a potential therapeutic target (37).

Monoclonal antibodies targeting EGFR, including cetuximab and panitumumab, are routinely used in mCRC. These monoclonal antibodies compete with the endogenous EGFR ligand and after binding, block phosphorylation, leading to internalization and degradation of the receptor. Cetuximab has been approved as a first-line treatment in mCRC patients with wild-type KRAS in combination with chemotherapy (38). Unfortunately, the addition of cetuximab to FOLFOX failed to improve disease-free or overall survival in post-resection stage III colon cancer patients (39). There is emerging evidence of the effectiveness of combining EGFR and KRAS G12C inhibitors in advanced disease. EGFR signaling has been identified as the primary mechanism of resistance to KRAS G12C inhibitors and this combination may overcome this resistance (40). The combination of anti-EGFR and KRAS G12C inhibitors is effective in cell lines, patient-derived organoids, and xenografts (41).

One downstream effector target of RAS is the RAF family, made up of c-RAF1, BRAF, and ARAF. Of these, BRAF is the most frequently mutated in tumors (42). Outside the constitutive activation of RAS, mutations in codon 600 of the BRAF gene produce RAS-independent activation of the MAPK pathway, leading to increased cell proliferation and survival. Sporadic MSI CRCs often show increased co-occurrence of BRAFV600E mutations compared to CRCs overall (43). These somatic BRAFV600E mutations increase BRAF/MEK/ERK signaling leading to the CIMP which silences MLH1, ultimately resulting in dMMR. The presence of a BRAF mutation indicates a sporadic MSI tumor and virtually excludes the diagnosis of Lynch syndrome (44). Patients with BRAF mutations experience poorer survival compared to patients with wild-type BRAF (45). BRAF mutations are associated with more right-sided primary tumors and with an increased risk of metastasis to the peritoneum and distant lymph nodes (46). BRAF and KRAS mutations are not coincident in tumors, and many KRAS wild type mCRC have BRAF mutations. These mutations identify tumors that are unresponsive to anti-EGFR therapies when combined with chemotherapy (47).

BRAF inhibitors are used extensively in BRAFV600E melanomas with positive treatment results (48). While BRAF inhibitor monotherapy in BRAFV600 melanoma leads to response rates of >50%, only ∼5% of BRAFV600 CRC patients respond (49). Since EGFR mediates resistance to BRAF inhibitors, the differing expression of EGFR in CRC, compared to melanoma, may explain this difference in response rates. In CRC, BRAF inhibition leads to feedback activation of EGFR which increases proliferation even in the presence of BRAFV600 inhibition (50). In contrast, simultaneous blockade of EGFR and BRAF produced synergistic inhibition of tumor growth in murine CRC models through enhanced MAPK suppression (51). Dual treatment with EGFR and BRAF inhibitors in previously-treated patients with BRAF V600E mCRC improved overall survival and progression-free survival compared to standard chemotherapy (52). Moreover, triple therapy inhibiting BRAF, EGFR, and MEK is effective against BRAFV600 tumors (53, 54).

HER2

HER2 (human epidermal growth factor receptor 2) is a transmembrane receptor that acts as an intracellular tyrosine kinase. Homo- or heterodimerization of HER2 with an additional member of the EGFR family (EGFR/HER2/ERBB) leads to the activation of either the RAS-RAF-ERK or PI3K-PTEN-AKT pathway leading to increased cellular proliferation. The amplification of the HER2 gene or overexpression of the HER2 protein has been targeted in solid tumor malignancies other than CRC. While therapies that block HER2 (trastuzumab, lapatinib, and pertuzumab) have gained prominence in treating patients with HER2-overexpressing tumors in these other malignancies, there are no HER-2-directed therapies approved by the FDA to treat CRC.

Preclinical work initially showed that HER2-amplified tumors were responsive to dual HER2-directed therapies, but not individual agents alone. Using this information, a phase 2 trial examining dual HER2 therapy comprising a tyrosine kinase inhibitor and anti-HER2 monoclonal antibody in KRAS wild-type, HER2-positive mCRC patients demonstrated that 30% of patients had objective responses and 44% had stable disease (55). A phase 2 trial of trastuzumab deruxtecan, a HER2-targeted antibody-drug conjugate, in patients who had previously progressed on at least two previous treatment regimens, showed an objective response rate of 45.3% (56). In quadruple WT populations (KRAS, NRAS, BRAF, and PIK3CA WT) treated with anti-EGFR therapies, the HER2 pathway may function as a bypass leading to resistance to anti-EGFR agents (57) (Figure 1). Indeed, HER2 expression predicts unresponsiveness to EGFR-targeted therapies in mCRC (58).

In addition to predicting response to HER2 and EGFR directed therapies, HER2 expression could help identify which patients may have a benefit from adjuvant chemotherapy. One study showed that among HER2 high patients, those who received chemotherapy had better OS and DFS than chemotherapy naïve patients. They showed no difference in outcomes among chemo-treated and chemo-naïve patients in the HER2 low group (59). This implies HER2 expression in CRC can be used as a direct target as well as a biomarker of resistance, and even eventually a guide in chemotherapy.

APC

In most CRCs Wnt/β-catenin signaling is activated by loss-of-function mutations in the adenomatous polyposis coli

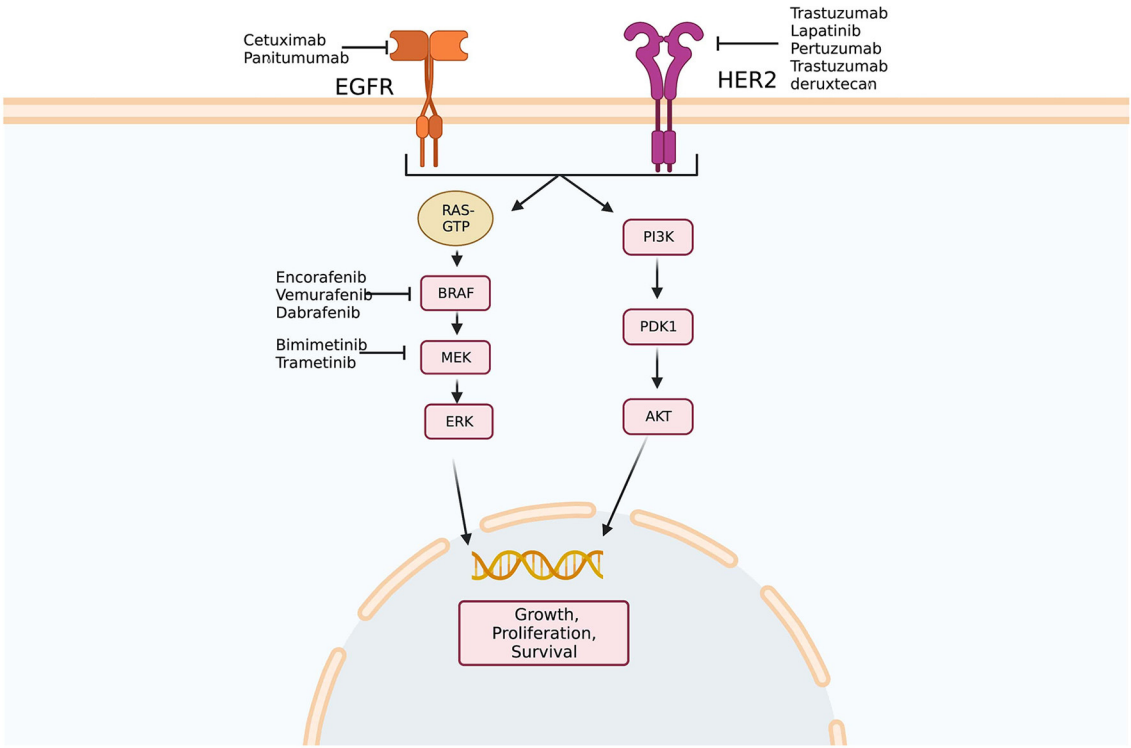

FIGURE 1
Epidermal growth factor signaling pathways. Multiple potential targets for therapy exist along epidermal growth factor receptor (EFGR) and human epidermal growth factor receptor 2 (HER2) pathways. In addition, amplification of HER2 has been implicated in anti-EGFR therapy resistance as activation of the HER2 pathway may bypass blockade of EGFR. Created with BioRender.com. Adapted from Crutcher et al. (37) Overview of predictive and prognostic biomarkers and their importance in developing a clinical pharmacology treatment plan in colorectal cancer patients, Expert Review of Clinical Pharmacology.

(APC) gene. The β-catenin-dependent Wnt signaling pathway is initiated by the binding of secreted cysteine-rich Wnt glycoproteins to LRP5/6 receptors and FZD receptors. The secretion of Wnt ligands depends on acylation by Porcupine (PORCN) (60). Binding of the Wnt ligands to LRP5/6 and FZD receptors on the cell surface induces disheveled (DVL) which leads to suppression of glycogen synthase kinase 3β (GSK3β). Together GSK3β, axin, and casein kinase 1 (CK1a) form a destruction complex which is stabilized by APC and phosphorylates β-catenin, priming it for degradation by the ubiquitin-proteosome pathway. In the presence of Wnt, and suppression of GSK3β, un-phosphorylated β-catenin accumulates in the cytosol, translocates to the nucleus, and interacts with TCF/LEF transcription factors to trigger expression of Wnt targets like c-Myc, cyclin D1, and CDKN1A (61). Inactivating mutations of APC de-stabilize the destruction complex, leading to activation of the Wnt signaling pathway which drives tumorigenesis.

While dysregulation of the Wnt/β-catenin signaling pathway is common in CRCs, this pathway lacks druggable molecular targets. Tankyrases (TNKSs) are members of poly-ADP-ribose polymerases (PARPs) family that poly-ADP-ribosylate and downregulate axins resulting in an overexpression of β-catenin. Tankyrase inhibitors (TNKSi) have been developed as potential therapeutic agents in CRC (62). APC may effect response to tankyrase inhibitors. It has been shown that drug-sensitive CRC cells had truncated forms of APC that lacked all seven β-catenin-binding 20-amino acid repeats (AARs) resulting in cell response to TNKSi. Conversely, drug-resistant CRC cells had longer forms of APCs with two of more 20AARs (63). Identification of APC status could be prognostic in determining potential response to TNKSi.

CEA

Carcinoembryonic antigen (CEA) is a cell adhesion molecule that is elevated in the serum of patients with a variety of cancers, including CRC. CEA levels have been used postoperatively in surveillance and higher preoperative CEA levels have been shown to be an independent predictor of both overall and disease-free survival rates. In addition, patients with node-negative colon cancer but elevated preoperative CEA levels have a poor prognosis similar to those with node-positive

disease (64). These patients may be candidates for adjuvant chemotherapy. As previously discussed stage II colon cancers do not typically receive adjuvant chemotherapy. However those stage II patients with high risk features may benefit from adjuvant therapy but there has been difficulty in defining this group. Studies have shown that CEA levels could potentially be used to risk stratify stage II patients and inform treatment decisions (65).

NTRK

Neurotrophic tyrosine receptor kinase (NTRK) gene fusions are extremely rare in CRC occurring in less than 1% of tumors (66). However, they are more frequently found in patients with dMMR (67). The FDA has approved two tropomyisin receptor kinase (TRK) inhibitors, entrectinib, and larotrectinib, for use in patients with NTRK fusion-bearing cancers in either a worsening metastatic setting or locally advanced unresectable tumors (68). This is an example of tissue agnostic treatments that can be used in any solid tumor, not just CRC.

Consensus molecular subtypes

An additional method of categorizing CRCs that may help guide treatment decisions are the Consensus Molecule Subtypes (CMS). CMS1 or MSI immune tumors account for 14% of CRCs. They have a high rate of mutations, with frequent BRAF mutations, and sizeable immune infiltration. The majority of MSI tumors fall in this category and, as previously discussed, these tumors are responsive to immune checkpoint inhibitors. In addition, the BRAFV600E mutation predisposes resistance to treatment with anti-EGFR agents. CMS2 or canonical tumors make up 37% of tumors and have upregulated Wnt/Myc signaling. These tumors stem from the canonical adenoma-to-carcinoma sequence typified by the initial loss of APC, a following activating mutation in KRAS, and an ultimate loss of TP53. CMS3 or metabolic tumors comprise 13% of cases and have frequent KRAS mutations and dysregulation of cancer metabolic pathways. As discussed previously, KRAS mutation may indicate a poor response to anti-EGFR therapy. CMS4 or mesenchymal tumors form 23% of cases and are characterized by transforming growth factor beta (TGFβ) pathway activation, enhanced angiogenesis, stromal activation and inflammatory infiltrates (69).

These four molecular subtypes can be broadly divided into "hot" and "cold" CRCs based on immune infiltration. The high immune infiltration of CMS1/MSI-H tumors has been discussed, as well as their responsiveness to treatment with immune checkpoint inhibitors. While CMS4 tumors also have increased immune cell infiltrates, responses to immunotherapy may be altered by TGFβ signaling. In comparison to the anti-tumor immune environment of CMS1 tumors, the CMS4 tumor microenvironment is pro-inflammatory (70). Indeed, TGFβ may be immunosuppressive and drive immune evasion in CRC (71). Alternatively, CMS2 and CMS3 tumors are "cold" tumors reflecting low immune cell infiltrates. CMS2 and CMS3 tumors may respond to alternative immunogenic stimuli, like vaccines or co-stimulatory compounds, but do not respond to immune checkpoint inhibitors. CMS2 and CMS3 tumors also respond to anti-VEGF agents (72). CMS classification has the potential to provide prognostic information, since CMS2 and CMS3 tumors have a better prognosis than CMS1 and CMS4. One study examining CMS status among stage II CRC found adjuvant chemotherapy had no benefit in CMS1 subtype tumors, and a significant decrease in DFS for CMS4 tumors (73). In contrast, stage II and III patients with either the CMS2 or CMS3 have benefit from adjuvant therapy (74). While not currently used in clinical practice CMS subtypes may eventually help guide targeted and chemotherapy decisions.

Circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA)

The concept of a "liquid biopsy" for solid tumors has recently emerged, reflecting sampling convenience and its ability to capture the varying molecular markers of a solid tumor. Liquid biopsies have multiple advantages over tissue biopsies, such as assessing molecularly divergent metastatic lesions, capturing the heterogeneity of a tumor, and evaluating potential resistance mutations in real time as treatment progresses. Circulating tumor cells (CTCs) are individual, or clusters of, cancer cells circulating in the bloodstream resulting from passive shedding or intravasation from the primary lesion or metastases (75). The amount of detectable CTCs detected is associated with treatment outcomes and overall survival (76). In contrast to CTCs, cell-free circulating tumor DNA (ctDNA) comprises somatic and epigenetic DNA alterations from tumor cells released into bloodstream following apoptosis or necrosis. ctDNA is more abundant within the bloodstream than CTCs but both can be detected and interrogated for actionable treatment targets and emergent resistant sub-clones, therefore assisting in treatment decisions before and after initiation of therapy.

There is an established relationship between ctDNA and tumor burden, with ctDNA positivity increasing with CRC stage (77). In this sense, ctDNA could identify high risk early stage patients. In addition, as discussed earlier, there are several biomarkers that can predict prognosis or treatment response in CRC such as MSI-H/dMMR (susceptibility to immune checkpoint inhibitors) as well as KRAS/BRAF (anti-EGFR resistance). A study interrogating the emergence of mutated KRAS alleles in ctDNA during anti-EGFR therapy revealed that these alleles decline when treatment is suspended, demonstrating that liquid biopsies can be used to track treatment resistance (78). The ability to accurately capture these

markers prior to the initiation treatment could help tailor therapeutic planning. Furthermore, the ability to track these markers during treatment could both ensure treatment response and monitor for developing resistance.

Currently, there is controversy as to what proportion of stage II CRC patients should receive adjuvant therapy. While some high risk characteristics have been suggested, these are not validated and there is no consensus (79). In stage II CRC, post-operative patients who were positive for ctDNA were at extremely high risk for recurrence when not treated with adjuvant chemotherapy (80, 81). A study surveying ctDNA status in patients after curative-intent surgery revealed that 100% of patients with ctDNA detected after treatment completion ultimately recurred (82). In patients with resectable colorectal liver metastases, patients with ctDNA detected after surgery had a significantly poorer relapse-free survival and overall survival. In addition, all patients with persistently detectable ctDNA after adjuvant chemotherapy recurred (83). A study in stage I-III patients revealed that in the majority, ctDNA identified relapse after definitive treatment. The same study also showed that ctDNA status was independently associated with relapse after adjusting for other clinicopathologic risk factors (84). ctDNA could potentially be used as an adjunct to the traditional TNM staging and other potential prognostic markers in determining which patients receive adjuvant therapy.

Summary

Despite improvements in screening and surgical interventions, CRC has remained the second most common cause of cancer-related death in the United States. While it has an overall favorable relative survival 5 year survival of 65%, inadequacies in treatments are revealed when stage by stage prognosis is examined (2). The 5-year overall survival for stage I disease (small, no lymph node spread) is 92% compared to 11% in stage IV (metastatic) (3). Approximately 35% of patients have metastatic disease at initial presentation with 20–50% of stage II and stage III patients eventually progressing to metastatic disease (4). These survival statistics illuminate multiple areas for improvement in the treatment of CRC. The high recurrence rates among patients who present with localized disease indicates missed opportunities for curative treatment in some patient populations. Currently, adjuvant therapy is consistently given to patients with stage III disease (positive lymph nodes) with some controversy in stage II disease. Again, the high recurrence rates among this population suggest there could be additional benefit from adjuvant treatment.

Further, much like innovations in targeted therapy, strides have been made in novel sampling techniques. ctDNA in the blood of CRC patients reflects the entire tumor genome. Increasing levels of ctDNA have been shown to be correlated with worse survival showing ctDNA could potentially be included in staging algorithms (85). In addition to sampling at diagnosis in order to stage and determine molecular markers, ctDNA levels and mutation expression can be followed to monitor for recurrence or emerging treatment resistance. While CMS subtypes currently are not recommended for use in clinical practice, this may change as a greater understanding of their biology emerges.

Author contributions

Review of relevant papers and manuscript preparation: MC and SW. All authors reviewed the results and approved the final version of the manuscript.

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Conflict of interest

SW is the Chair of the Scientific Advisory Board and member of the Board of Directors of Targeted Diagnostics & Therapeutics, Inc. which provided research funding that, in part, supported this work and has a license to commercialize inventions related to this work.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Psymberin, a marine-derived natural product, induces cancer cell growth arrest and protein translation inhibition

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Colorectal cancer (CRC) is the third most prevalent form of cancer in the United States and results in over 50,000 deaths per year. Treatments for metastatic CRC are limited, and therefore there is an unmet clinical need for more effective therapies. In our prior work, we coupled high-throughput chemical screens with patient-derived models of cancer to identify new potential therapeutic targets for CRC. However, this pipeline is limited by (1) the use of cell lines that do not appropriately recapitulate the tumor microenvironment, and (2) the use of patient-derived xenografts (PDXs), which are time-consuming and costly for validation of drug efficacy. To overcome these limitations, we have turned to patient-derived organoids. Organoids are increasingly being accepted as a "standard" preclinical model that recapitulates tumor microenvironment cross-talk in a rapid, cost-effective platform. In the present work, we employed a library of natural products, intermediates, and drug-like compounds for which full synthesis has been demonstrated. Using this compound library, we performed a high-throughput screen on multiple low-passage cancer cell lines to identify potential treatments. The top candidate, psymberin, was further validated, with a focus on CRC cell lines and organoids. Mechanistic and genomics analyses pinpointed protein translation inhibition as a mechanism of action of psymberin. These findings suggest the potential of psymberin as a novel therapy for the treatment of CRC.

KEYWORDS

patient-derived organoids, patient-derived models of cancer, precision medicine, psymberin, high-throughput screening, protein translation

Introduction

Colorectal cancer (CRC) is the third most commonly occurring form of cancer in the United States and is the cause of over 50,000 deaths per year (1). At initial diagnosis, approximately 20% of patients will have distant metastasis, and another 25–30% of patients with stage II/III disease will develop metastasis (2). Currently, the use of chemotherapy in the metastatic setting can palliate symptoms and improve survival, but do not result in cures for patients. If left untreated, patients with colorectal metastasis can expect an overall survival of approximately 9 months, but with combination therapy, survival can be improved to greater than 24 months (3, 4). The last two drugs that have been approved by the U.S. Food and Drug Administration for the treatment of refractory CRC were regorafenib (5) in 2014 and lonsurf (6) in 2015. Despite these improvements, there remains a lack of new drugs for the treatment of advanced CRC. Unfortunately, the failure rate for new cancer drugs is more than 80% in Phase II and 50% in Phase III trials (7). As a result, despite our advances, CRC still remains an incurable and debilitating disease, and there is an unmet clinical need to develop new therapeutics for CRC.

In our previous work, we developed a precision medicine pipeline to facilitate the identification and validation of new therapies in CRC and other solid tumors (8–11). These studies highlighted the utility of a precision medicine pipeline to identify, test, and characterize novel therapeutics using patient-matched low passage cell lines and patient-derived xenografts (PDXs). Cell lines provide a rapid and low-cost resource to test thousands of compounds and perform genomics studies, while matched PDXs provide robust *in vivo* models to validate top candidate therapies. Despite the utility of this platform, it is limited by (1) the reliance on the generation of cell lines, which can take months to establish and characterize and do not faithfully recapitulate the tumor microenvironment and (2) the establishment of PDXs, which are costly and often time-consuming to produce and maintain.

Given the limitations of our current pipeline, we have turned to patient-derived organoids. Patient-derived organoids are increasingly being accepted as a "standard" preclinical model that is both more representative of *in vivo* tumor physiology than cell lines and a low-cost rapid alternative to PDXs (12–14). We have therefore adapted our precision medicine pipeline to incorporate the use of patient-derived organoids (**Figure 1A**). Using this new pipeline, we first performed a high-throughput drug screen using a compound library on a panel of early-passage cell lines from multiple solid tumors to identify potential therapeutic agents. From this screen, we identified psymberin as one of the top small molecules with potent growth inhibition activity.

Psymberin, also known as irciniastatin A, belongs to a group of biologically active natural products called polyketides. Psymberin was independently discovered in 2004 by the research groups of Crews and Pettit from the sponges *Psammocinia sp*. and *Ircinia ramose*, respectively, and it has been a marine natural product of immense interest since its isolation (15–18). Psymberin has been tested against 60 cancer cell lines and displayed potent cytotoxicity against melanoma, breast, and colon cancer cell lines ($LC_{50} < 2.5 \times 10^{-9}$ M). While these studies pinpoint psymberin as an effective anti-cancer agent, its molecular mechanisms are not extensively understood.

Identification of psymberin as a top hit in our screens prompted us to further characterize the activity and mechanisms of action for psymberin. RNA-Seq on cells treated with psymberin identified negative enrichment of protein translation as a potential mechanism of action. The impact on translation was verified using a fluorescence-based assay of translation inhibition. The rapid inhibition of protein translation within hours was concomitant with the activation of p38, a stress response pathway, and cell cycle arrest. Together, our results pinpoint psymberin as a potent protein translation inhibitor with anti-cancer properties in CRC.

Materials and methods

Establishment and maintenance of low-passage cell lines and organoids

Colorectal cancer patient tissue samples were collected under a Duke Institutional Review Board approved protocol (Pro00089222), obtained from the National Cancer Institute's Cooperative Human Tissue Network or obtained through Duke University BioRepository and Precision Pathology Center. CRC cell lines were established from patient tissue samples as previously described (8). Briefly, patient samples were processed and injected into SCID beige mice to grow as PDXs. After the tumors grew to ∼0.5 cm^3, tumors were then harvested, homogenized, and grown in tissue-culture treated dishes, with subsequent clonal selection as indicated (8). Low-passage osteosarcoma lines were generated as described previously (9). All cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin.

To establish organoids from existing cell lines, 2×10^6 cells from each line were subcutaneously injected into SCID beige mice. After the tumors grew to ∼0.5 cm^3, mice were euthanized following Duke Institutional Animal Care and Use Committee (IACUC)-approved protocols, and the tumor was then harvested. Tumors were mechanically digested in C-tubes with 10 mL of DMEM using a gentleMACS Dissociator (Miltenyi Biotec) and running the m_impTumor_01.01 protocol twice. Cells and tissue fragments were filtered through 70 μm filters and centrifuged at 500 *g* for 5 min. The supernatant was aspirated. 1.25×10^5 cells were plated in 50 μL domes composed of 30% cell suspension in media and 70% Matrigel (Corning).

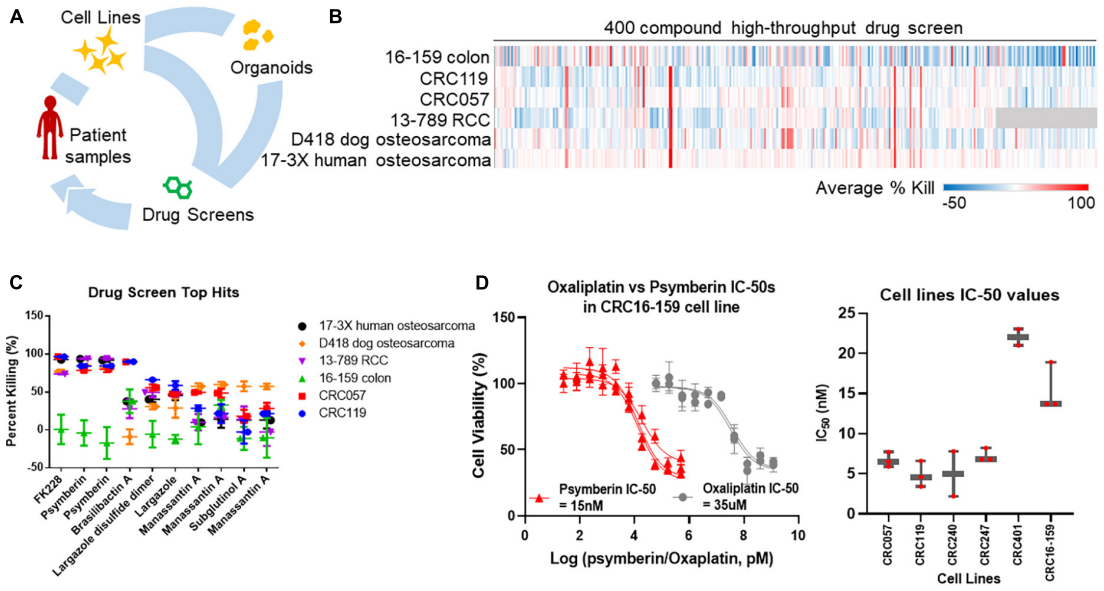

FIGURE 1
Identification of psymberin as a potential anti-cancer therapeutic agent for colorectal cancer (CRC). **(A)** Tumor samples or low-passage cell lines from patients are used to establish organoid cultures *ex vivo*. These organoids are then used in drug screens. **(B)** A high-throughput screen with 400 compounds in six cell lines. Blue indicates a negative average percent killing (net growth) and red indicates a high average percent killing. Compounds that were not tested for a cell line are shown in gray. **(C)** The top ten hits from the high throughput screen in the same six cell lines. Some compounds were synthesized in multiple batches and were therefore tested more than once. **(D)** Dose-response comparison between oxaliplatin and psymberin in the same CRC cell line (left) and comparison of psymberin IC-50 values across different CRC cell lines (right).

CRC organoids were maintained in CRC media, which consisted of DMEM F12 media supplemented with 10 mM HEPES, 1X Glutamax, 100 μ/mL Penicillin/Streptomycin, 500 nM A83-01, 1X B27 without vitamin A, 50 ng/mL EGF, 10 nM Gastrin-1, 1.25 mM N-Acetylcysteine, 10 mM Nicotinamide, 100 ng/mL Noggin, 100 μg/mL Primocin, 10 nM Prostaglandin E2, 100 ng/mL R-Spondin 1, and 3 μ M SB20210.

All cell lines and organoids were maintained at 37°C in a humidified incubator at 5% CO_2.

High-throughput drug screen

Cells from six early passage cell lines (16–159 colon, CRC119, CRC057, 13–789 RCC, D418 canine osteosarcoma, and 17-3X human osteosarcoma) were provided to the Duke Functional Genomics Core Facility for testing with a compound library of 400 natural products, intermediates, and drug-like compounds. A subset of compounds was synthesized in multiple batches and were therefore tested more than once to ensure reproducibility across different batches. The high-throughput screen was performed as previously described (8). Briefly, 384-well plates were stamped with each of the compounds from the library at a final concentration of 1 μM. Cells from each of the lines were plated in these pre-stamped plates at a density of 1 × 10^3 cells/well. Cell viability was assessed using the Cell Titer-Glo luminescent Cell Viability Assay kit (Promega, Madison, WI, USA) after 72 h. Percent killing was calculated as follows: 100*[1–(average Cell Titer-Glo$_{drug}$/average Cell Titer-Glo$_{DMSO}$)].

Cell line drug sensitivity assays

Does response curves for psymberin and oxaliplatin were performed in the following six cell lines: CRC057, CRC119, CRC240, CRC247, CRC401, and CRC16-159. Stock solutions at 10 μM for psymberin and oxaliplatin were made in DMSO and phosphate buffered saline (PBS), respectively. Once cells were 70% confluent, they were plated into 96-well plates at a concentration of 4 × 10^3 cells per well and incubated for 24 h. Cells were then treated with a series of 10 different concentrations in media (DMEM + 10% FBS + 1% penicillin/streptomycin) of psymberin or oxaliplatin starting from 1 μM and 300 μM, respectively, with a serial dilution factor of three. Five replicates were performed for each drug concentration. After incubation in the presence of the drug for 2 days, cell viability was evaluated using the Cell Titer-Glo luminescent Cell Viability Assay kit (Promega, Madison, WI, USA). All drug sensitivity assays were performed in triplicate. Half maximum inhibitory concentration (IC_{50}) values were calculated using a non-linear curve fit with the log(inhibitor) vs. response (3 parameters) function in GraphPad Prism (La Jolla, CA, USA).

Organoid drug sensitivity assays

Organoids were grown in 24 well plates at 37°C for approximately 3 days, after which they were re-plated in 96-well plates for drug sensitivity assays. To do this, media was aspirated from the wells and 1 mL of PBS was added to each well to detach the Matrigel domes from the bottom of the wells. After collecting the Matrigel, wells were washed again with 500 μL of PBS to collect any remaining Matrigel. The Matrigel and PBS was centrifuged for 7 min at 400 × *g*. Supernatants were removed and 500 μL of Trypsin was added to each tube and incubated for 2 min to dissolve the Matrigel. Trypsin was neutralized by the addition of DMEM with 10% FBS and the whole contents were centrifuged for another 3 min at 400 × *g*. Pellets were collected and cells were counted after resuspension in CRC media. Cells were then mixed with Matrigel in a 1:1 ratio and 5 μL of mixture containing 2×10^3 cells was added to the center of each well in a 96-well plate. The 96 well plates were incubated for 10–15 min to allow the Matrigel to solidify before adding 50 μL of CRC media to each well and incubating at 37°C for 72 h. Organoids were treated with a series of six different concentrations of psymberin starting from 1 μM with a dilution factor of five. After incubation in the presence of the drug for 2 days, cell viability was quantified *via* the Cell Titer-Glo luminescent Cell Viability Assay (Promega, Madison, WI, USA). All drug sensitivity assays were performed in triplicate. IC_{50} values were calculated using a non-linear curve fit with the log(inhibitor) vs. response (3 parameters) function in GraphPad Prism (La Jolla, CA, USA).

Protein synthesis assays

Nascent protein synthesis was quantified in the CRC119 cell line using Click-iT® HPG Alexa Fluor® 488 Protein Synthesis Assay Kit (Thermo Fisher Scientific). CRC119 cells were plated into 96-well plates at the concentration of 4×10^3 cells/well in drug-free medium and allowed to recover overnight before treating them with either 1% DMSO, 3X psymberin IC_{50}, or 50 μM cycloheximide. Drug-containing medium was removed at 1 and 6 h intervals, and medium containing 50 μM l-homopropargylglycine (Click-iT® HPG) was added in the dark. After incubation for 30 min, medium containing Click-iT® HPG was removed, and cells were washed once with PBS. Cells were fixed in 3.7% formaldehyde and permeabilized with 0.5% Triton® X-100 in PBS. HPG incorporation was detected using the Click-iT® reaction cocktail prepared according to the vendor's guidelines. Plates were incubated for 30 min at room temperature followed by washing wells with ClickiT® reaction rinse buffer and PBS. Plates were imaged using an Incucyte® S3 live cell imaging system. Luminescence was quantified in FIJI/ImageJ.

Protein isolation and western blotting

CRC119 cells were treated with psymberin at 3X their IC_{50} at different time points. Cells were lysed in radio-immunoprecipitation assay buffer supplemented with protease and phosphatase inhibitors (Thermo Fisher Scientific). Protein concentration was assessed by using the BCA Protein Assay (Bio-Rad). A total of 60 μg of total protein from each sample was electrophoretically separated on 4–20% sodium dodecyl sulfate polyacrylamide gels (Bio-Rad, USA) and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). After blocking the membranes with blocking buffer (Bio-Rad), membranes were incubated overnight with primary antibodies for phospho-p38, p38, poly (ADP-ribose) polymerase (PARP), or cleaved PARP. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. Appropriate secondary antibodies were added subsequently. Blots were scanned using a Licor Odyssey imaging system.

Cell cycle analysis

CRC119 and CRC16-159 cells were seeded at 3×10^5 cells per well in 6-well plates and incubated until they reached 60% confluence. Cells were treated with either 3X their IC_{50} of psymberin or 0.1% DMSO. After 24 and 48 h, cells were harvested and washed two times with PBS followed by fixing in 80% ethanol for 30 min. Subsequently, cells were washed twice more with PBS and resuspended in cell staining buffer (0.1% Triton X-100, 0.1 mM EDTA disodium, 50 μg/mL RNAse A, and 50 μg/mL PI in PBS) immediately prior to flow cytometry. Flow cytometry-based cell cycle analysis was performed by the Duke University Flow Cytometry Shared Resource. A Chi-square test was used to estimate statistical reliability of the observations.

RNA-seq

A total of 8×10^4 CRC119 and 16–159 cells were plated in 6-well plates and allowed to incubate overnight. Cells were then treated with 3X their IC_{50} of psymberin and RNA was extracted after 16 h using the RNEasy Mini Kit (Qiagen). RNA-Seq data was processed using the TrimGalore toolkit (19) which employs Cutadapt (20) to trim low-quality bases and Illumina sequencing adapters from the 3′ end of the reads. Only reads that were 20 nt or longer after trimming were kept for further analysis. Reads were mapped to the GRCh37v75 version of the human genome and transcriptome (21) using the STAR RNA-seq alignment tool (22). Reads were kept for subsequent analysis if they mapped to a single genomic location. Gene counts were compiled using the (23) tool ("HTSeq: High-throughput sequence analysis in Python"). Only genes that had

at least 10 reads in any given library were used in subsequent analysis. Normalization and differential expression was carried out using the DESeq2 (24) Bioconductor (25) package with the R statistical programming environment ("The R Project for Statistical Computing") (26). The false discovery rate was calculated to control for multiple hypothesis testing. Gene set enrichment analysis (27) was performed to identify differentially regulated pathways and gene ontology terms for each of the comparisons performed. False discovery rate cutoffs for positive and negative enrichment were <0.25 and <0.15, respectively. Normalized enrichment score cutoffs for positive and negative enrichment were >0 and < -1.8, respectively. Upset plots and heatmaps were constructed in R using the ComplexUpset and ComplexHeatmap packages.

Determination of apoptosis using the IncuCyte® annexin V green reagent

The IncuCyte® Annexin V Green Reagent (Sartorius), which is a highly-selective phosphatidylserine (PS) cyanine fluorescent dyes that enables real-time evaluation and quantification of cell death, was used. CRC 119 and CRC 16–159 cell lines were seeded into 96-well plates at the concentration of 4,000 cell/well in drug free medium and incubated for 24 h to allow for attachment. Cells were treated with either Psymberin or Cisplatin (as a positive control for inducing apoptosis in CRC) at 3X their IC_{50} dose. Annexin V Green Reagent was added to the wells at the same time according to manufacturer's protocol. An automated platform (Incucyte®) was used for imaging plates at the beginning and 24 h after the treatment. PS exposure on the extracellular surface following apoptosis, enables binding of the IncuCyte Annexin V Reagent resulting in a bright and photostable fluorescent signal.

Drug sensitivity assays using MicroOrganoSpheres

CRC404 and CRC420 organoids were grown in 50 μL Matrigel domes in CRC media at 37°C in a humidified incubator at 5% CO_2. Once the organoids were confluent, the media was aspirated from the wells and 1 mL of PBS was added to each well to detach the Matrigel dome from the bottom of the well. The Matrigel was centrifuged at 750 *g* for 5 min. Matrigel was dissolved, organoids were broken down using 1 mL of TrypLE Express (Gibco) and the mixtures were incubated for 5 min. TrypLE was neutralized by adding 5 mL of DMEM F12 media with 10% FBS and 1% penicillin/streptomycin. After centrifuging at 750 *g* for 5 min, the media was aspirated. Organoid cell suspensions were used to make MicroOrganoSpheres as previously described (28).

Stock solutions for psymberin and two analogs (psy-064 and psy-076) were made at 1 mM in DMSO. MicroOrganoSpheres were plated in 96-well plates at a concentration of 100 MicroOrganoSpheres/well with 1X of each component in the RealTime Glo MT Cell Viability Assay kit (RTG; Promega, Madison, WI, USA). MicroOrganoSpheres were treated with each of the three compounds in a nine-point dilution series starting from 1 μM with a dilution factor of three and five replicates per dose. Fluorescence was measured every day for 3 days using a Varioskan Lux plate reader (Thermo Fisher Scientific). IC_{50} values were calculated using a non-linear curve fit with the log(inhibitor) vs. response (3 parameters) function in GraphPad Prism (La Jolla, CA, USA).

Results

A high-throughput natural product screen identifies psymberin as a potential anti-cancer compound

To identify potential anti-cancer therapeutic agents, we performed a high-throughput drug screen on six early passage cell lines using a library of natural products and drug-like compounds. The six cell lines included three CRC lines (16–159, CRC119, and CRC057) (8), one renal cell carcinoma line (13–789 RCC), and two osteosarcoma lines (D418 canine and 17-3X human) (9). Less than 10% of the compounds were effective across the entire panel of cell lines, with the 17-3X human osteosarcoma cell line the most broadly sensitive to the library and the 16–159 CRC cell line the most broadly resistant (see **Figure 1B**). Across the cell lines, compounds with an average percent killing above 50% included nine compounds against 16–159 CRC cells, 10 compounds in CRC119 cells, 6 compounds in CRC057 cells, 4 compounds in 13–789 RCC cells, 1 compound in D418 canine osteosarcoma cells, and 3 compounds in 17-3X human osteosarcoma cells. To identify the most effective drugs, we focused on compounds with the highest average percent killing across the entire panel of cell lines. A subset of the compounds was synthesized in different batches and tested more than once to ensure reproducibility across different batches. In these cases, analysis of the screen data identified some compounds more than once as consistent top hits, which provides further support for these hits (**Figure 1C**). Among these top hits, FK228 and psymberin had an average percent killing $\geq$50% in all but one cell line (see **Figure 1C**). FK228, also known as romidepsin, has already been approved for the treatment of lymphoma (29), suggesting our screening strategy is capable of identifying natural products with efficacy as anti-cancer agents. Since FK228 is already approved as an anti-cancer agent, we focused on psymberin for further validation.

To understand the relative potency of psymberin against CRC, we compared dose response curves for psymberin and

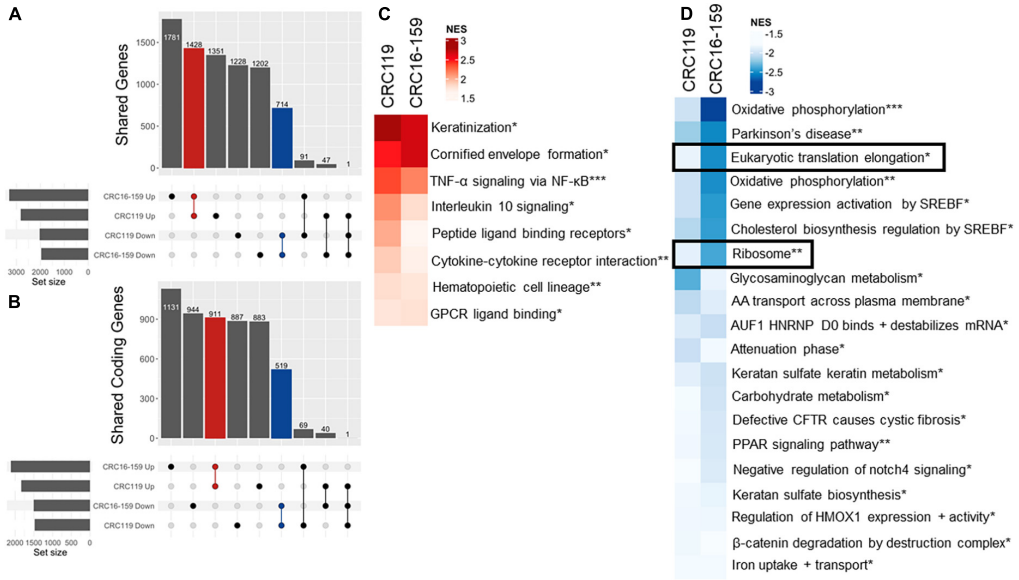

FIGURE 2
RNA-Seq analysis of psymberin treatment in colorectal cancer (CRC) cell lines. **(A)** Upset plot showing overlap of up- and down-regulated genes after psymberin treatment between two CRC cell lines: CRC119 and CRC16-159. Red indicates the number corresponding to upregulated genes consistent between both lines; blue indicates the number of genes consistently downregulated in both lines. **(B)** Same as 3A with coding genes only. **(C)** Most significant positively enriched pathways after psymberin treatment, determined using gene set enrichment analysis. **(D)** Most significant negatively enriched pathways after psymberin treatment, determined using gene set enrichment analysis. *Corresponds to Reactome, **Kegg, and ***Hallmark.

oxaliplatin, a standard-of-care drug for the treatment of CRC. These analyses indicated that psymberin is over 2,000 times more potent than oxaliplatin, with an IC_{50} of approximately 15 nM (**Figure 1D**). The low nanomolar IC_{50} values were consistent across six CRC lines, with IC_{50} values below 25 nM for every line and below 10 nM for four of the six lines (**Figure 1D** and **Supplementary Figure 1**).

Psymberin inhibits protein synthesis

To better understand the mechanism of action for psymberin, we performed RNA-Seq on two low-passage CRC cell lines (CRC119 and CRC16-159) treated with psymberin. At the gene level, we observed substantial overlap in all mRNAs (non-coding and protein-coding mRNAs) (**Figure 2A**) and mRNAs of protein-coding genes (**Figure 2B**) for both CRC119 and CRC16-159 lines. At the pathway level, we observed a consistent positive enrichment in pathways related to differentiation, NF-κB signaling, and pathways relevant to tumor-immune cross-talk (IL-10 signaling, cytokine receptor interaction) (**Figure 2C**) and negative enrichment in multiple pathways, including eukaryotic protein elongation and ribosome pathways (**Figure 2D**).

The observation that psymberin inhibits mRNAs involved in translation and protein synthesis is consistent with previous studies suggesting translation inhibition as a proposed mechanism of action for psymberin (30, 31). To further confirm this, we used a fluorescent reporter of protein synthesis in which the incorporation of a methionine analog into newly synthesized proteins can be quantified by "click" chemistry (Thermo Fisher Scientific). Using this system, we observed rapid inhibition of protein synthesis as early as 1 h after treatment with psymberin, with nearly complete loss of signal by 6 h (**Figure 3A**).

Previous studies have shown a connection between translation inhibition and cellular stress pathways (32, 33), such as p38/MAPK activation. Consistent with these studies, we observed a rapid increase in levels of phospho-p38 upon treatment with psymberin, with the greatest increase at 2 h post treatment (**Figure 3B**). Cell cycle analysis on CRC401, CRC119, and CRC16-159 cells treated with psymberin indicated that psymberin led to significant G1 arrest in the cell line models and G2 arrest in the CRC404 organoid model (**Supplementary Figure 2**). Despite this protein translation inhibition and cell cycle arrest, however, analysis of apoptosis pathway markers by western blotting revealed low levels of cleaved PARP during treatment with psymberin (**Figure 3C**). Similarly, we observed no change in annexin uptake during treatment with psymberin for up to 24 h (**Figure 3D**). This is in contrast to cisplatin, which induced both cleaved caspase and increased annexin uptake. Together, these results suggest that psymberin inhibits protein synthesis, induces phosphorylation of p38, and leads to cell cycle arrest.

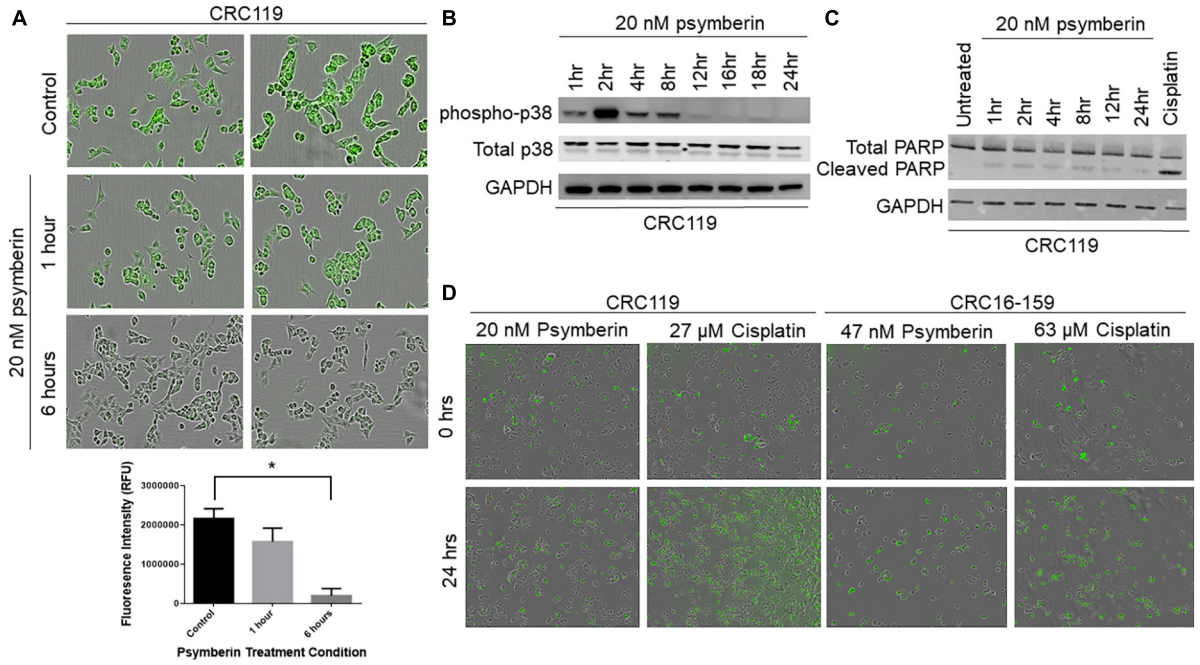

FIGURE 3
Psymberin induces protein synthesis inhibition and p38 activation. **(A)** Protein synthesis assay in CRC119 cells that were treated with 20 nM psymberin for one and 6 h (top). Quantification of fluorescence in protein synthesis assay (bottom) *$p < 0.05$. **(B)** Western blot for phospho-p38 in CRC119 cells treated with 20 nM psymberin at different time points. Total p38 and GAPDH are included as loading controls. **(C)** Western blot for cleaved PARP in CRC119 cells treated with 20 nM psymberin at different time points. GAPDH is included as a loading control. **(D)** Annexin V staining for protein translation in CRC119 and CRC16-159 at 0 and 24 h after treatment with psymberin.

Psymberin inhibits growth of colorectal cancer patient-derived organoids

To further validate the effectiveness of psymberin to induce CRC cell growth inhibition, we performed dose response assays with psymberin across a panel of six CRC patient-derived organoids (Figure 4). With the exception of one organoid line (CRC401; $IC_{50} \sim 70$ nM), the IC_{50} values were all below 20 nM. Morphologically, organoids treated with a low dose of psymberin appear rounded, with refractile spheres throughout the culture. Conversely, organoids treated with higher doses (1 μM) of psymberin are dark and condensed, with few to no viable cells (Figure 4). Comparison of psymberin treatment in CRC240 grown as monolayer cell lines and organoids showed no difference in the IC_{50} values between the two growth conditions.

Psymberin subunits do not inhibit the growth of colorectal cancer patient-derived organoids

In addition to psymberin, we also quantified the cytotoxicity of two truncated psymberin analogs, Psy-064 and Psy-076 (Figure 5A). Both analogs are portions of the original psymberin compound, and Psy-064 itself is a component of Psy-076 (Figure 5A). To validate the effectiveness of the psymberin analogs, dose response assays were performed on CRC MicroOrganoSpheres using psymberin and both of its analogs. Consistent with our previous analyses, psymberin treatment of CRC MicroOrganoSpheres resulted in an $IC_{50} \sim 3.6$ nM and visible inhibition of organoid growth, as noted by the reduction in size and collapse in spherical structure of the MicroOrganoSpheres (Figure 5B); however, the analogs had no negative impact on cell viability either in RealTime Glo fluorescence assays or observed visually (Figure 5B and Supplementary Figure 3). This suggests that the three-dimensional conformation of psymberin and the psymberic acid side chain may be required for its activity.

Discussion

Patient-derived models of cancer, such as early-passage cell lines, PDXs, and organoids, have increasingly been accepted as "standard" preclinical models of cancer (8, 12–14). Each model has its own benefits and drawbacks. For example, early-passage cell lines are the cheapest to maintain of the three models (34). However, cells in monoculture are not representative of cancer growth in the human body and are not able to replicate the

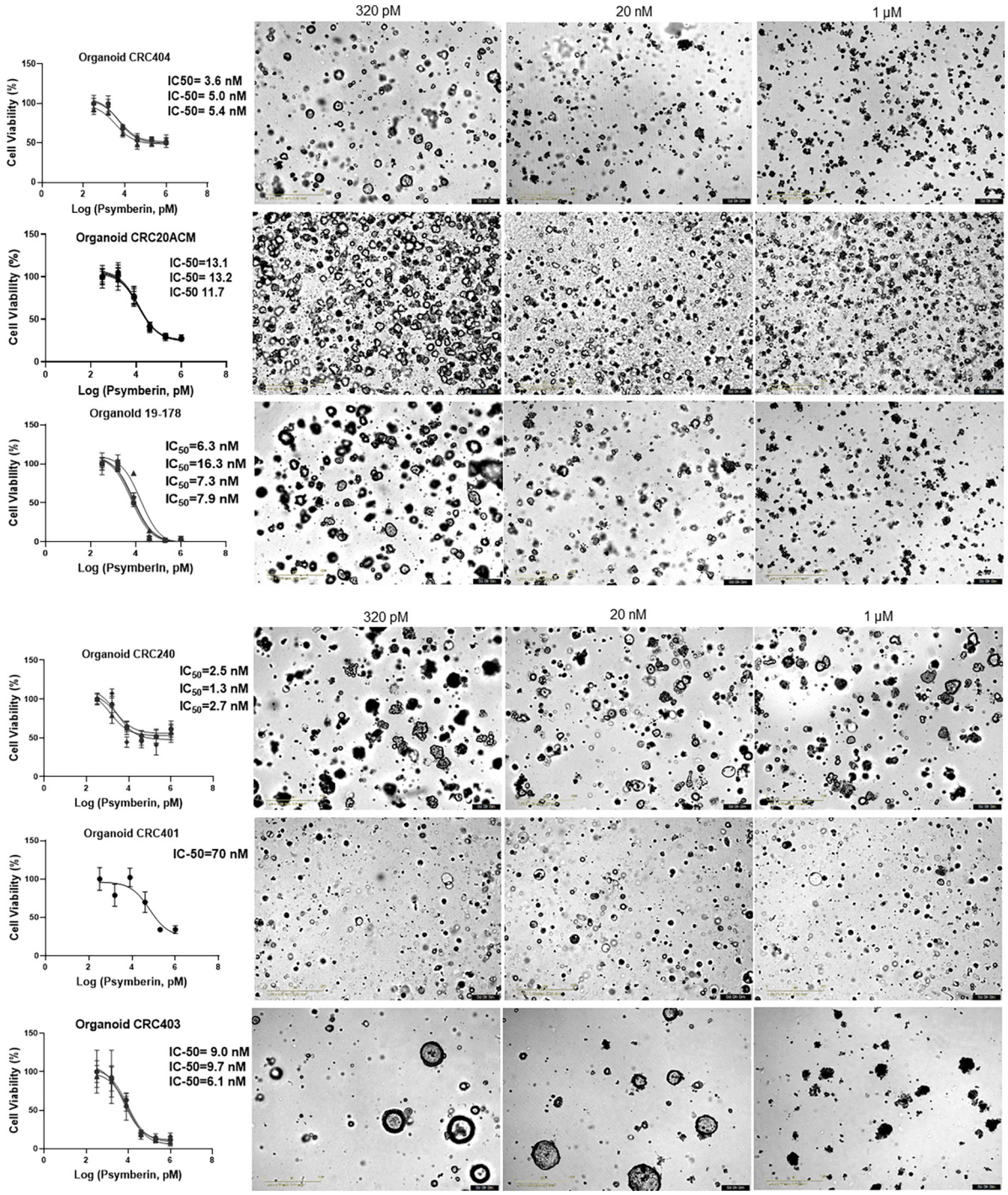

FIGURE 4
Psymberin has IC-50 values in the nanomolar level across multiple colorectal cancer (CRC) patient-derived organoids. Dose response curves are shown for six different CRC patient-derived organoids. Each experimental repeat is depicted in a different curve with different IC-50 values listed on the side of each curve. Images beside each graph show organoids from each line treated with 320 pM **(left)** and 1 μM **(right)** of psymberin.

complexity of the tumor microenvironment (13, 35). Moreover, successful establishment of cell lines from patient tissue is extremely rare, regardless of cancer type (34). On the other hand, PDXs more closely model the tumor microenvironment (14) and tumor heterogeneity (14, 35). One major drawback to PDXs, however, is the high cost and time required to maintain

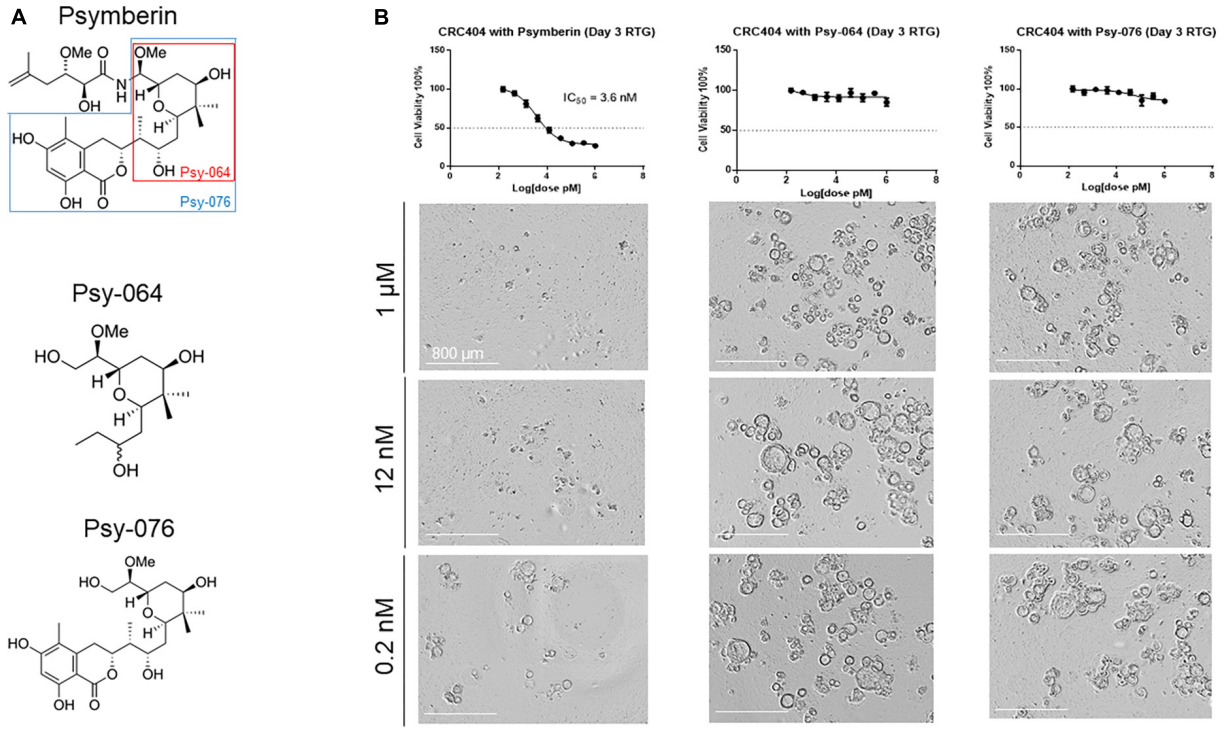

FIGURE 5
The entire structure of psymberin is important for its activity. **(A)** Structural comparison of psymberin and two of its analogs, Psy-064 and Psy-076. **(B)** Dose response curves for psymberin, Psy-064, and Psy-076 in CRC404 organoids. Images below each graph show organoids from each line treated with 1 µM (top) and 0.0002 µM (bottom) of that compound.

these models (34). For these reasons, patient-derived organoids are growing in popularity because they are (1) able to model tumor heterogeneity (12, 13, 36); (2) have a higher uptake rate compared to cell lines (12, 34); (3) can be used to model the tumor microenvironment (36); and (4) are cheaper and faster to grow and maintain than PDXs (34).

We have previously used early passage cell lines and PDXs to develop a precision medicine pipeline to determine patient-specific targets for treatment (10). In this study, we utilize the latest version of our precision medicine pipeline, coupling cell lines and organoids to identify, test, and characterize a natural product library for potential anti-cancer compounds to treat CRC (**Figure 1A**). Using our pipeline, we identified psymberin as a potential anti-cancer agent for CRC (**Figures 1B,C**).

Psymberin, also known as irciniastatin A, was independently isolated by Pettit et al. and Cichewicz, Valeriote, and Crews in 2004 (15, 16). It was later confirmed by Jiang et al. (37) that the compounds isolated by both groups were, in fact, identical, despite being from two different types of sponges: *Psammocinia* sp. and *Ircinia ramosa* (15, 16). Both groups showed that psymberin was active against multiple cancer types, including CRC (15, 16). Moreover, multiple studies, including ours, have shown that psymberin is an extremely potent compound, with IC_{50} values in the low nanomolar range [**Figures 1D**, **4** and **Supplementary Figure 1**; (15, 16, 30, 38)].

The biological properties of psymberin have drawn considerable attention from research groups to develop a complete synthesis of the compound and identify its molecular mechanisms (18, 38, 39). Psymberin has previously been shown to inhibit translation in human leukemia (30) and lung carcinoma cells. This correlates with our data demonstrating that psymberin inhibits translation in CRC (**Figures 2D**, **3A**). The inhibition of translation is connected to cellular stress pathways (32, 33). One such cell stress pathway involves p38/MAPK activation. Both Chinen et al. (30) and we have shown that psymberin induces p38 activation (**Figure 3B**).

Strong activation of p38 through phosphorylation has been associated with apoptosis, senescence, and terminal cell differentiation (40, 41). Our data suggest that by activating p38, psymberin may lead to G1 cell cycle arrest in CRC (**Figures 3C,D** and **Supplementary Figure 2**). While some studies have shown that psymberin induces apoptosis in other cancer types (30, 42), our results are consistent with previous studies linking p38 activation to G1 arrest (43–45).

Overall, psymberin is an extremely effective drug against CRC, both in cell line and organoid form, with IC_{50} values below 10 nM. Our results suggest that psymberin may inhibit protein

translation in CRC and induces the upregulation of p38, leading to cell cycle arrest. Future studies should focus on evaluating toxicity and anti-tumor efficacy in *in vivo* settings.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/bioproject/909790; project accession number: PRJNA909790.

Ethics statement

The studies involving human participants were reviewed and approved by Duke IRB. The patients/participants provided their written informed consent to participate in this study.

Author contributions

JS, JH, and DH: conceptualization, methodology, and supervision. DD, JM, GR, RR, and SV: formal analysis. IS, YZ, SK, and SM: resources. DD, JS, JH, and DH: writing—original draft. DD, JS, JM, GR, RR, SV, IS, YZ, SK, SM, JH, and DH: writing—review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

DH was cofounder of Xilis.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.999004/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Psymberin has IC-50 values in the nanomolar level across multiple colorectal cancer cell lines. Dose response curves are shown for six different colorectal cancer cell lines. Each experimental repeat is depicted in a different curve with different IC-50 values listed on the side of each curve.

SUPPLEMENTARY FIGURE 2
Psymberin treatment leads to G1 cell cycle arrest in CRC cells. **(A)** Cell count for cells in different phases of cell cycle when untreated and treated with psymberin. The P5 label refers to G1, P6 refers to S phase, and P7 refers to G2. **(B)** Percent of cells in different phases of cell cycle in untreated and psymberin-treated cells. **(C)** Cell cycle analysis by flow cytometry in the CRC404 patient-derived organoid. **(D)** Quantification of cell cycle analysis based on the gates shown in panel **(C)**. $*p < 0.05$ by chi-square test.

SUPPLEMENTARY FIGURE 3
The entire structure of psymberin is important for its activity. Dose response curves for psymberin, Psy-064, and Psy-076 in CRC420 organoids. Images below each graph show organoids from each line treated with 1 μM **(top)** and 0.0002 μM **(bottom)** of that compound.

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