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Advances in molecular biology knowledge of rectal cancer and forthcoming role of liquid biopsy

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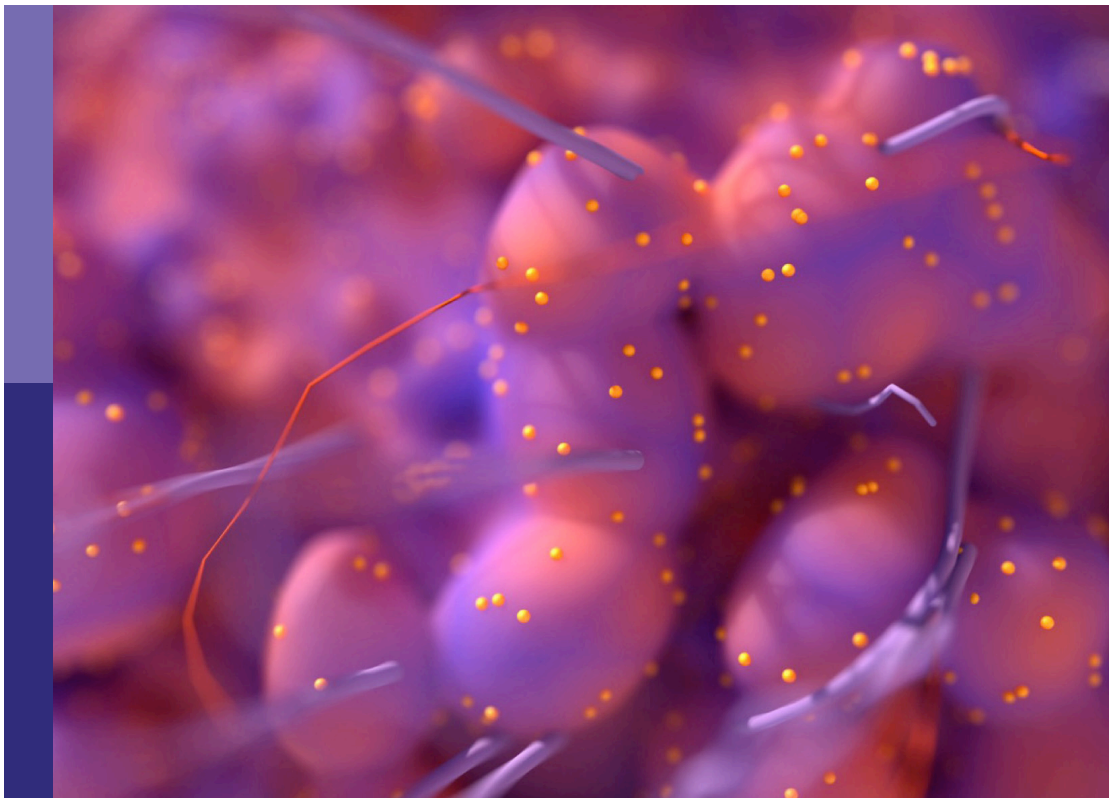

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Advances in molecular biology knowledge of rectal cancer and forthcoming role of liquid biopsy

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Editorial: Advances in molecular biology knowledge of rectal cancer and forthcoming role of liquid biopsy

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Editorial on the Research Topic
Advances in molecular biology knowledge of rectal cancer and forthcoming role of liquid biopsy

Advances in molecular biology may significantly impact our understanding of rectal cancer and inform the development and positioning of novel therapies (1). Total Neoadjuvant Treatment (TNT) for locally advanced rectal cancer is gaining consensus and has evolved, emphasizing the need of a multidisciplinary strategy. TNT has increased complete response rates, disease-free survival, control of distant metastases and has become a new standard of care (2, 3). Also, a raising amount of reports indicates that a non-operative management (NOM), entailing close surveillance of patients with clinical complete response (cCR) after neoadjuvant therapy, could be an appropriate alternative option to rectal surgery (4). Recently, the International Watch & Wait Database (IWWD) reported superiority in terms of quality of life and a small risk of local inoperable disease recurrence without compromising survival in patients with rectal cancer managed nonoperatively after achieving a cCR following neoadjuvant therapy (5).

Selecting predictive molecular markers is thus becoming even more crucial. The tumor microenvironment plays a critical role in colorectal tumor development, progression and immune escape (6). Stromal cells (i.e. adipocytes, vasculature, lymphocytes) interact with cancer cells and may affect therapy response. As reported in the review by Mirza et al., tumor microenvironment evaluation during treatment may inform on new therapies, uncover responses and tumor resistance. The intratumoral immune contexture is a main factor of clinical outcome in both early- and advanced-stage colorectal cancer (7, 8). Specifically, in rectal cancer a local hot immune signature in the tumor before neoadjuvant therapy is correlated with improved response and prolonged disease-free survival (9). Besides, the 100% complete clinical response rate after programmed cell death protein-1 (PD-1) blockade soundly suggests that the *in situ* innate immune response released and enhanced by immune checkpoint inhibitors treatment can fully eradicate cancer cells

precluding recurrences (10). Tumor immune infiltrate has also been described as an independent prognostic marker in a large international cohort of rectal cancer patients with cCR managed nonoperatively and could pave the way for prospective therapeutic trials guided by immunoscore to adapt follow up and/or therapy of NOM patients (11).

Colorectal cancers show genetic variations and clonal evolution, which proffer noteworthy difficulties in selecting appropriate therapies (12). In the traditional approach, the identification and choice of therapy have mainly depended on the employment of invasive tissue biopsies and imaging assays. Currently, core tumor biopsy specimens represent the gold standard biological tissue to identify and analyze predictive biomarkers. However, anatomical feasibility, tumor heterogeneity and cancer progression are major limitations of this single-snapshot approach. Liquid biopsy is increasingly gaining attention as a complementary and potentially alternative non-invasive tool to bypass these limitations.

Liquid biopsy assessment of circulating tumor DNA (ctDNA) is useful for risk stratification and detection of minimal residual disease (MRD) in early colorectal cancer (13). ctDNA can also outline the tumor mutational profile, detect mutations not identified in the tissue biopsies and offer a comprehensive and dynamic evaluation of tumor genetics, classify specific therapeutic targets thus allowing clinicians to monitor disease progression and the efficacy of treatments (13). The introduction of liquid biopsies has endorsed a noteworthy move towards precision medicine in colorectal cancer; the presence of ctDNA in high-risk stage II (T4) and stage III colorectal cancer patients correlates with adverse prognosis both post-surgery and post-adjuvant treatment independent of other conventional clinical-pathological risk factors (13). More recently, Tie and co-authors demonstrated that patients with liver-only metastases undergoing surgical resection had a lower relapse-free survival in the case of ctDNA positivity (14), thus confirming the potential role of serial ctDNA analysis as an immediate marker of therapy activity. As reported by Choi et al., liquid biopsies using extracellular vesicle DNA (evDNA) secreted by tumor cells may be a different source for the detection of cancer driver mutations and a complementary tool for the diagnosis and surveillance of colon cancer patients. Their results showed that evDNA isolated from the plasma of colon cancer patients harboring KRAS G12D and G13D mutations was significantly associated with both CEA level and survival. Unlike fragmented pieces of 'cell-free' DNA (cfDNA) shed from apoptotic or necrotic cells, extracellular vesicles arise from viable tumor cells. Therefore, evDNA might reveal the underlying biology of living cancer cells (15, 16) and reflect cancer driver mutations even in the early stages of cancer development (17).

In rectal cancer liquid biopsy could be important in several steps: at the time of diagnosis, for the evaluation of MRD, treatment response and possible acquired resistance and also to modulate treatment during TNT (18). The up-to-date trimodality approach for locally advanced rectal cancer comprises chemotherapy, radiotherapy and surgery and may cause considerable morbidities; moreover, it might not be mandatory for some patients, and fails to prevent disease relapses in others. The main drawback in the present managing of rectal cancer is the absence of consistent and accurate techniques of predicting responsiveness to neoadjuvant therapies without surgical resection and subsequent pathological evaluations. For instance, among patients candidate to sphinter-sparing surgery who demonstrate adequate clinical responses to induction systemic chemotherapy, omitting radiotherapy and its associated toxicities might be a valuable therapeutic option (19). Compared to standard pathological evaluation criteria, ctDNA or modifications in ctDNA could be useful in directing therapeutic decision in this setting of patients. Additionally, a tool to improve the degree of concordance between clinical and pathological complete response could assist a NOM strategy.

In conclusion, the knowledge of tumor microenvironment and immune changes together with introduction of liquid biopsies could offer a measure of these dynamic interfaces, thus enabling the development of immunotherapies and tailored therapies. The inclusion of liquid biopsies in the design of clinical trials, in addition to other analytical modalities such as conventional tissue biopsies, is a crucial component of this development. The Research Topic gives an overview of liquid biopsy and other new technologies and methods as well as emphasizing the clinical usefulness of liquid biopsy, particularly investigating its implication as an analytic, predictive, or MRD marker.

Author contributions

FN: Writing – original draft, Writing – review & editing. LG: Writing – original draft, Writing – review & editing. CA: Writing – original draft, Writing – review & editing.

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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Patient-derived rectal cancer organoids—applications in basic and translational cancer research

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Colorectal cancer (CRC) is one of the most commonly diagnosed cancers and among the leading causes of death in both men and women. Rectal cancer (RC) is particularly challenging compared with colon cancer as the treatment after diagnosis of RC is more complex on account of its narrow anatomical location in the pelvis adjacent to the urogenital organs. More and more existing studies have begun to refine the research on RC and colon cancer separately. Early diagnosis and multiple treatment strategies optimize outcomes for individual patients. However, the need for more accurate and precise models to facilitate RC research is underscored due to the heterogeneity of clinical response and morbidity interrelated with radical surgery. Organoids generated from biopsies of patients have developed as powerful models to recapitulate many aspects of their primary tissue, consisting of 3-D self-organizing structures, which shed great light on the applications in both biomedical and clinical research. As the preclinical research models for RC are usually confused with colon cancer, research on patient-derived RC organoid models enable personalized analysis of cancer pathobiology, organizational function, and tumor initiation and progression. In this review, we discuss the various applications of patient-derived RC organoids over the past two years in basic cancer biology and clinical translation, including sequencing analysis, drug screening, precision therapy practice, tumor microenvironment studies, and genetic engineering opportunities.

KEYWORDS

rectal cancer, organoids, patient-derived, precision medicine, treatment prediction, tumor microenvironment, cancer modeling

Introduction

Cancer is a leading cause of noncommunicable disease deaths (9.9 million people annually) and has sparked a health crisis worldwide (1). Colorectal cancer (CRC) is the third most common type of cancer and second in terminology of cancer mortality. CRC includes colon cancer (CC) and rectal cancer (RC); 40% of CRC is represented by RC (2). Although early diagnosis and multiple treatment strategies optimize outcomes for individual patients, we still need a more accurate and precise model to facilitate cancer research, including predicting response to standard therapies, as large numbers of newly developed strategies fail in clinical trials but go well on cancer models (3).

Traditional immortalized two-dimensional cancer cell lines and patient-derived xenografts (PDX) have long been applied in CRC research (4–11). Several studies from Bardelli's laboratory are focused on 151 established CRC lines (6) along with xenopatient-derived cell lines or xenolines (XLs) (7, 8). The authors later improve this platform to compare drug responses and better carry on genomic analysis. As a result, this system is able to detect CRC dependencies on kinases for which clinically approved drugs are available and to discern CRC cross-sensitivity to Olaparib and Oxaliplatin. However, the translation of the obtained results from laboratory to bedside is often hampered as cell lines fail to recapitulate primary tumor characteristics due to lack of heterogeneity, fewer cancer cells expand from the microenvironment, and experience loss of their primary polarity (12). Furthermore, different types of PDX models, such as murine (9) and zebrafish (10, 11) are developed to study CRC tumor initiation, progression, immune response, and response of novel strategies. The histology and genomic mutational patterns of their primary tissue are maximally retained in PDX (13). Yet, due to high costs and low success rates, their use in cancer research is limited. In addition, animal models have conquered some of these drawbacks, but they do not mirror human physiology, resulting in the high failure rate of new cancer strategies in the clinic (14).

Organoid technology bridges the gap between cell culture and animal models, providing *in vivo*-like conditions. In general, organoids involve most types of cells from primary organs and can recapitulate the key features, main structure, and tissue functions along with gene expression profiles of the organs from which they were derived. To date, organoid technology nowadays has shown great capacity in breaking down mechanisms associated with gastrointestinal cancer and improving patient outcomes as recently reviewed by Lau et al (15). The novel technology based on generating patients' tumors from biopsies as patient-derived organoids (PDOs) constitutes a major breakthrough for the study of translational cancer research, such as studying tumor biology, discovering new biomarkers, drug screenings, and testing targeted personalized therapies. Furthermore, huge efforts have been made in CRC research using the technology of tumor-derived organoids. The success establishment rate could reach around 63% by using small amounts of starting material from tissue biopsy samples (16, 17). These cancer organoids can be accomplished in a few weeks with high efficiency, and most of them have inheritable stability after several generations.

More than one third of CRC is represented by RC (2). Although CC and RC are synonymously called CRC, as they share the same histological classification and characteristics (18), RC is particularly challenging compared with CC since treatment of the RC patient is usually more complex than that of the CC patient due to their differences in anatomical position (19). Some RC patients can avoid surgery if they respond completely to neoadjuvant chemoradiotherapy alone (20, 21), and others responding poorly to CRT require radical surgery (22). Therefore, it is promising to use a correct and specific research model for identifying patients' response to treatment in order to minimize potential harm from overtreatment and enable personalized therapy of RC.

Despite the fact that RC treatment is more complicated by utilizing trimodal therapy consisting of neoadjuvant chemoradiation, surgical resection, and 5-fluorouracil-based chemotherapy (19), few efforts have been made to develop RC-specific research models—not to mention that one preclinical practice for treatment of RC has unexpectedly depended on CC cell lines (23). Cell lines are also established from RCs (SW837, Caco-2, etc) (24–26), but whether they were derived from the correct side of the rectum or from patients in the context of multimodal therapy is hard to prove. As for the PDX platform, the first study to establish a PDX model from RC patients' biopsies before treatment is reported recently, supporting the translational suitability of the PDX platform that predicted the response of corresponding patients, and to identify cetuximab as a strategy to enhance the efficacy of 5-fluorouracil/radiotherapy (27).

As PDOs can be easily converted into PDX models and represent one of the models that comes closest to the primary patient's tumor, it currently shows potential in high-throughput drug screening and personalized therapy testing. Whereas extensive research is available for CRC organoids, most studies have not distinguished CC organoids from RC. For instance, we can find 214 full text results from the past two years through searching "(Patient-derived organoid) AND (colorectal cancer)" in PubMed and 132 full text results by applying the MESH "Patient derived organoid AND Colorectal Cancer" with the same year range, whereas only 26 full text results were found with the same year range by using "(Patient-derived organoid) AND (rectal cancer)" and 14 full text results were found by applying the MESH "Patient derived organoid AND rectal Cancer." Furthermore, we find that some CRC-associated research did not mention whether it used samples from CC or RC patients (Table 1), and some clinically derived CRC organoids or biobanks exclude tissue from RC patients and mainly focus on CC specimens (30, 38) with RC PDOs remaining

an underexplored field. This may be because patients with RC are often irradiated prior to surgery or treatment, which can affect the efficacy of derived organoids to assess the effectiveness of subsequent treatment strategies. However, with the continuous improvement of technology, we are looking forward to seeing that research in the field of CRC can be more refined in the future, and research models specifically used for RC will continue to be applied. Recently, specific research or protocols on the organoid model of RC is gradually garnering attention from scientists in the last two years, and translational applications are springing up (16, 39–41) (Table 2). The translational application of the RC PDO model is also mentioned in some recent literature and systematic reviews (44, 46, 50, 52, 53). There is a need for standard procedures and methods to improve the reproducibility and stability during laboratory practice.

In this review, we aim to review the latest in RC organoid development and its cutting-edge applications for basic or translational cancer research (Figure 1), including mutational analysis, drug screening, personalized medicine therapy, tumor heterogeneity, and microenvironment study as well as cancer modeling.

Analysis of the mutational landscape using high-throughput sequencing technologies

Organoids over a period of continuous culture represent one of the *in vitro* models that comes closest to the primary tumor tissue, and they retain the function, histology, and genomic mutational patterns of the primary tumor, thereby preserving the heterogeneity of the origin tissue. By undergoing RC PDO sequencing, even low-frequency subclonal mutations can be detected and identified, and this gives scientists a hand in studying cancer pathobiology.

In the field of RC PDOs, two published articles in *Cell Stem Cell* and *Nature Medicine* (16, 39) illustrate the successful practice of RC organoids derived from patients, representing a major breakthrough in the precision medicine field. One group generated a biobank of 80 locally advanced RC PDOs from biopsy samples. Whole-exome sequencing was constructed in 18 culture lines, and the results displayed the mutational profile level of the corresponding tumor tissue. A 94.4% overlay between known cancer driver mutations and the TCGA data set was found in PDO samples. In 12 of 18 cases, they compared the genome-wide gene copy number variations of RC PDO cultures with that of primary tumors. The results displayed that the DNA copy number losses and gains of the original tissue were retained in PDOs. At the same time, WNT signaling was reported to represent the most related mutated genes pathway (88.9%), including APC (72.2%), FBXW7, TCF7L2, ARID1A, LRP5, and SOX9 mutation (16). Another group investigated the mutational fingerprint and larger landscape of molecular alterations in RC PDOs by undergoing the Food and Drug Administration–cleared tumor-profiling panel, MSK Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT). The data displayed that 92% (range: 0.66–1.00) of the oncogenic mutation was retained in the paired PDO cultures

TABLE 1 Excluded records of the CRC cancer organoid studies, rectum organoid research and others.

Category	Reference or NCT number	Excluded reasons
CRC cancer organoid studies	(28)	CRC research with organoids generated from naive WT mice normal intestine without from rectal tumor.
	(29)	CRC research with organoids generated from naive WT mice intestine without from mice rectal tumor.
	(30)	Built biobank excluded tissue from RC patients.
	(31)	CRC Immuno-genomic research used samples collected both from CC and RC patients without distinguishing them.
	(32)	CRC research used PDO model only derived from right-sided colon tumors without from rectal tumor.
	NCT05304741, NCT04996355, NCT05384184, NCT04755907, NCT05183425, NCT04220242, NCT02732860, NCT04587128, NCT04279509, NCT05412706	These clinical CRC studies did not mention whether RC patients were involved in their studies.
	NCT05038358, NCT04896684	Clinical studies related to colon cancer patient-derived organoid.
Rectum organoid research	(33)	Normal Rectum research used adult normal mucosa organoids.
	(34)	Normal Rectum research used mouse Rectum Crypt organoids.
	NCT03874559	Rectal cancer study used malignant colonic organoids model.
Others	(35)	Review did not focus on RC PDOs models.
	(36)	Colonic inflammatory bowel disease research used PDO model without mentioning Rectal cancer and related PDO models.
	(37)	Research used colonic epithelial organoids without mentioning Rectal cancer and related PDO models.

TABLE 2 Summary characteristics of included publications (n = 21) and clinical studies (n = 5).

Reference or NCT number	Study type	Source & type of experimental model	Limitions	Main findings
(42)	Analysis of the mutational landscape using high-throughput sequencing technologies	Excised from mice with RC patient tumor xenograft *Ex vivo*	• Lack of data from post treatment human rectal cancer specimens. • Did not show accordance exists between organoid and biopsy data • Small sample size	Assessed for ST6GAL-1 protein with and without chemoradiation treatment on patient-derived xenograft and organoid models and identified ST6GAL-1 protein as a mediator for resistance to clinical chemoradiation therapy through restraining apoptosis.
(43)	Personalized medicine based on the testing of individual PDOs; Studying the tumor microenvironment with PDOs; Cancer modeling by genetic engineering of organoids	Biopsies from pre-CRT tumor and normal *In vivo* and *ex vivo*	• Lack of data from post treatment human rectal cancer specimens. • Did not mention about the type and number of cells seeded for organoid culture. • Lacked the success rate and cell composition of the established organoid.	Developed RC PDOs and primary stroma cells and identified that interleukin-1α (IL-1α) after irradiation polarizes cancer-associated fibroblasts toward the inflammatory phenotype together with triggering oxidative DNA damage; Displayed the impact factor in chemoradiotherapy resistance and disease progression.
(44)	Reviewing biomarkers and models used in RC	–	–	Reviewed published findings associated with biomarkers discovery and pre-clinical models (included RC PDOs) in RC.
(45)	Personalized medicine based on the testing of individual PDOs	Surgically or endoscopically resected tumor tissues of patients undergoing neoadjuvant therapy *Ex vivo*	• Organoid culture lacked microenvironmental regulation of tumor response. • Did not mention about the type and number of cells seeded for organoid culture. • Lacked the success rate and cell composition of the established organoid.	Analyzed radiosensitivity of PDOs and provided a readout predictive of neoadjuvant therapy for selecting patients who need pre-treatment.
(46)	Reviewing PDOs models for precision medicine	–	–	Evaluated the potential of PDO models (included RC PDOs and distinguished RC research) in predictive translational research.
(47)	Conducting clinical trial for translational research from bench to bedside; Personalized medicine based on the testing of individual PDOs	–	–	Started ACO/ARO/AIO-21 phase I trial to test the IL-1 receptor antagonist (IL-1 RA) anakinra combining with CRT therapy for RC based on previous achievement (43), which set up a great example for translational application from bench to bedside.
(38)	Personalized medicine based on the testing of individual PDOs	0.5×0.5×0.5 cm for surgically resected specimens and 1.5×0.2×0.2 cm for ultrasound-guided core-needle biopsy tissue *Ex vivo*	• Results need further validation in the prospective, randomized controlled study. • Organoids culture was in the absence of tumor microenvironment. • Lacked the purity and cell composition report of the established organoid.	The sensitivity, specificity, and accuracy of the RC PDOs for predicting chemotherapy regimens response were 63.33%, 94.12%, and 79.69%.

(Continued)

TABLE 2 Continued

Reference or NCT number	Study type	Source & type of experimental model	Limitions	Main findings
(48)	Analysis of the mutational landscape using high-throughput sequencing technologies; Personalized medicine based on the testing of individual PDOs	Biopsy samples Ex vivo	• Small sample size • Did not mention about the type and number of cells seeded for organoid culture. • Organoids culture was in the absence of tumor microenvironment. • Did not perform a drug sensitivity test.	Established a prediction model through a machine learning algorithm combining clinical and experimental radio response data; Radiation responses in clinic were positively correlated with the paired cultures.
(41)	Analysis of the mutational landscape using high-throughput sequencing technologies	Colon-endoscopic biopsy from participants accepted preoperative chemoradiotherapy (pCRT) Ex vivo	• Did not show accordance exists between organoid and biopsy data	High expression of VSTM2L reduced γ-H2AX expression in RC PDOs treated with CRT.
(49)	Analysis of the mutational landscape using high-throughput sequencing technologies	Biopsy samples Ex vivo	• Small sample size • Did not mention about the type and number of cells seeded for organoid culture. • Lacked the success rate and cell composition of the established organoid.	Developed RC PDOs to detect genes and pathways that participate in the radio-resistance of LARC by biological and bioinformatic analysis approaches; Identified cathepsin E (CTSE) that was negatively correlated with the radio-resistance in PDOs.
(50)	Reviewing PDOs models for precision medicine	–	–	Described CRT prediction value of organoids (included RC PDOs) for GI cancers.
(51)	Drug screening to develop novel treatment strategies; Personalized medicine based on the testing of individual PDOs	Resected specimens Ex vivo	• Small sample size • Did not mention about the type and number of cells seeded for organoid culture. • Lacked the purity and cell composition report of the established organoid.	Screened PDOs with a customized medium-throughput drug library consist of 33 single agents and three 5-FU-based drug combinations with Leucovorin (FLV), Oxaliplatin (FLOX), and SN-38 (FLIRI).
(52)	Reviewing pre-clinical models used in RC	–	–	Described different pre-clinical model (included PDOs) used in RC research.
(53)	Reviewing biomarkers and models used in RC	–	–	Reviewed published paper associated with potential biomarkers and cell-based models (included RC PDOs) to predict treatment response in RC.
(16)	Analysis of the mutational landscape using high-throughput sequencing technologies; Drug screening to develop novel treatment strategies; Personalized medicine based on the testing of individual PDOs	Tissue biopsies from patients with newly diagnosed LARC who were treatment-naive in a phase III clinical trial NCT02605265 Ex vivo	• Lacked the purity and cell composition report of the established organoid.	Established an organoid biobank with PDOs obtaining similar histological and genetic features of original tumors; identify the role of predicting LARC patient Chemoradiation responses in the clinic.
(54)	Drug screening to develop novel treatment strategies	7 rectal endoscopic biopsy and 1 colon cancer sample from low anterior	• Lacked the success rate and cell composition of the established organoid.	Butyrate could enhance the curative effect of radiotherapy while protecting the normal mucosa; Identified FOXO3A as a factor with non-responsive cases to butyrate in PDOs.

(Continued)

TABLE 2 Continued

Reference or NCT number	Study type	Source & type of experimental model	Limitions	Main findings
		resection *Ex vivo*		
(27)	Personalized medicine based on the testing of individual PDOs	Endoscopic biopsies from 26 Stages 2 and 3 rectal cancer patients prior to receiving 5FU/RT *In vivo* and *ex vivo*	• Small sample size	Identified the ability of cetuximab to enhance RT effectiveness; Used PDOs to improve patient selection based on mutational profile. Success rate:90%
(55)	Analysis of the mutational landscape using high-throughput sequencing technologies	Endoscopic Biopsies from therapy-naïve rectal cancer patients *Ex vivo*	• Did not mention about the type and number of cells seeded for organoid culture. • Lacked the success rate and cell composition of the established organoid.	Compared the gene profiling of organoids derived from a normal rectum and rectal tumors and their responses to calcitriol; Identified rectal tumor organoid-specific genes associated with biosynthetic machinery, including those encoding the RNA polymerase II subunits POLR2H and POLR2J.
(56)	Personalized medicine based on the testing of individual PDOs	Did not mention *Ex vivo*	• Did not mention about the type and number of cells seeded for organoid culture. • Lacked the success rate and cell composition of the established organoid. • Lack details in culture methods • Small sample size	Similarly, two patient-derived organoid models containing relatively low AC expression were found to be comparatively more radiosensitive than three other models containing higher levels of AC.
(39)	Analysis of the mutational landscape using high-throughput sequencing technologies; Drug screening to develop novel treatment strategies; Personalized medicine based on the testing of individual PDOs; Investigation of intratumoral heterogeneity and tumor evolution	Endoscopic biopsies from pre- and post-treatment patient samples *In vivo* and *ex vivo*	• Need studies with larger populations to investigate the prediction value. • Did not mention about the type and number of cells seeded for organoid culture.	RC PDO cultures reserved architecture and molecular features of the original tumors and their in vitro responses to clinical treatment correlated with the outcomes of individual patients' tumors; PDOs from patients with RC under multimodal therapy engraft into the rectal mucosa of mice, which indicating a success in vivo RC PDO model.
(40)	Protocols for RC PDO establishment	Surgery or biopsy *Ex vivo*	• Lack details in culture methods • Lacked the success rate and cell composition of the established organoid.	Developed protocols for establishing RC cancer organoids; performed high-throughput drug sensitivity testing.
NCT03577808	Personalized medicine based on the testing of individual PDOs;	Pre-treatment biopsies *Ex vivo*	–	Validation of Organoids Potential Use as a Companion Diagnostic in Predicting Neoadjuvant Chemoradiation Sensitivity in Locally Advanced Rectal Cancer
NCT05352165	Personalized medicine based on the testing of individual PDOs; Drug screening to develop novel treatment strategies;	Not mentioned	–	A Prospective Multicenter Randomized Controlled Trial of the Clinical Efficacy of Neoadjuvant Therapy Based on Organoids Drug Sensitivity Versus Empirical Neoadjuvant Therapy in the Treatment of Advanced Rectal Cancer.
NCT04371198	Determine the feasibility of establishing patient-derived organoids.	Pre-treatment rectal adenocarcinoma biopsies. *Ex vivo*	–	Accessing the feasibility of the Biospecimen Collection Protocol for establishing Patient-Derived Organoids for Rectal Cancer

(Continued)

TABLE 2 Continued

Reference or NCT number	Study type	Source & type of experimental model	Limitions	Main findings
NCT05401318	Personalized medicine based on the testing of individual PDOs; Drug screening to develop novel treatment strategies;	Fresh tumor samples from colon and rectal cancer patients *Ex vivo*	–	Accessing the prediction value of the PDOs and investigating the effect of Pre-treatment with cytotoxic agents which can induce cellular immunotherapy efficacy against solid tumors in PDOs
NCT04842006	Personalized medicine based on the testing of individual PDOs;	Not mentioned *Ex vivo*	–	Population distribution of PDO treatment response is compared to their corresponding clinical response by response MRI and pathological response.

compared with an 88% concordance (range: 0.62–1.00) reported of organoids from colon and colon metastases (with RCs excluded), and 77% of the clonal oncogenic mutations in the original tumors were also detected as clonal mutations in PDO cultures. The top mutations in the RC PDOs included the alterations of genes *APC*, tumor protein P53 (*TP53*), *KRAS*, and F-Box and WD Repeat Domain Containing 7 (*FBXW7*) (39).

After a previous successful practice, more specific works are springing up. Costales-Carrera A et al. analyze the RNA-seq transcriptomes of six normal rectum or colonoscopy and rectal tumor organoid cultures by establishing a biobank of 50 organoids from therapy-naive RC patients. They confirm that these established RC organoids involve classic genetic alterations of sporadic CRC (APC, TP53, KRAS). Interestingly, some aberrant genes presented expression differences in RC and CC organoids. A cluster of genes regulating protein synthesis (ribosomal biogenesis, translation components, and regulation) was only expressed at a high level in rectal tumor organoids, indicating that CC and RC mainly differ in protein expression rather than the RNA level (55). In a new coclinical trial, Park et al. compares the result of PDTOs after irradiation with the individual patients' response of radiotherapy. They used targeted next-generation sequencing analysis to check the ability of organoids to recapitulate the paired patients' tissue and found that the match rate of KRAS, NRAS, and BRAF mutations in PDTOs could reach to 86.6%, 100%, and 100%, respectively. WNT signaling pathway–associated genes (including *APC* and *FBXW7*) were mutated in 68.4% of established cultures with identified *APC* (68.4%) and *FBXW7* (31.5%). But, there was a problem in that the sample size was small, and they did not mention whether these PDOs were derived from patients who were therapy-naïve or not (48).

To analyze relevance between gene expression and the radiosensitivity of PDOs, Lee et al. checked 27,685 genes and identified 1741 differentially expressed genes from radio-resistant (RR) and radio-sensitive (RS) organoids by RNA-seq. In RR organoids, up-regulation genes that encode the control protein include calcium-dependent interactions (ANXA2, S100A4), immune cell activation (CD55, IL18, RUNX3),

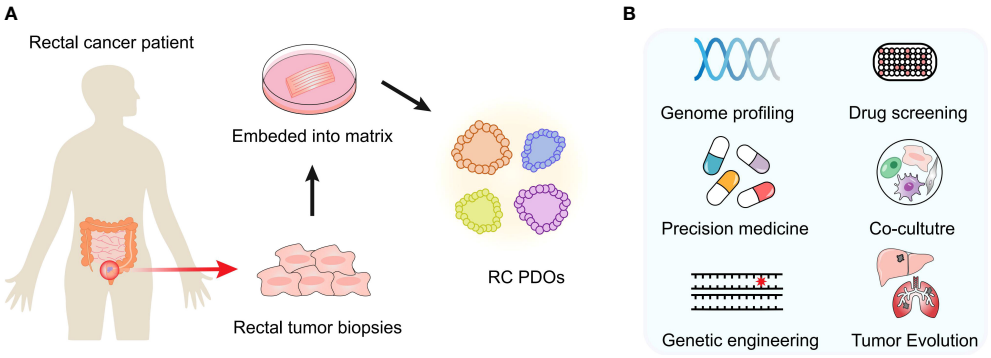

FIGURE 1
Developments and cutting-edge applications of the PDO model. **(A)** After an RC patient tumor's surgery or biopsy, the tumor specimen is collected. PDOs can be generated in the laboratory and expanded to create sufficient material for biobank building and storage. Once established, these models are expanded in order to create sufficient material for storage and biobanking. **(B)** Multiple applications in vitro or ex vivo can be performed, such as genome profiling, drug screening, coculture experiments, etc.

receptor catabolic processes (NPC1, APOE, LGMN), and plasma membrane proteolysis (ADAM9, BACE1, CTSE). They found the enhanced expression of CTSE regulated by DNA methylation status in RR organoids, suggesting that CTSE could be applied as a biomarker for radio-responsiveness (49).

One group combined the results from PDX, RC organoids generated from xenograft mouse models, and RC cell lines and identified ST6GAL-1 protein as a mediator for resistance to clinical chemoradiation therapy through restraining apoptosis. The data might need further studies in a larger sample size system (42).

Another report mentions that the high expression of VSTM2L could be one of the bad factors leading to patient resistance to CRT through diminished γ-H_2AX expression, but they all did not mention about the correlation between PDTO response and patient outcomes (41).

Overall, these studies confirm the feasibility that rectal organoids can be generated and cultured from the clinical patients' specimens and the great potential of PDO libraries as powerful platforms for RC research *in vitro* since the gene expression profiles of PDOs are very close to the organs from which they were derived.

Drug screening to develop novel treatment strategies

Some RC patients can avoid surgery if they respond completely to CRT alone (20, 21), whereas others responding poorly to CRT require radical surgery (22). So it is crucial to undergo drug screening to minimize potential harm from overtreatment and enable personalized therapy.

PDOs now represent the most precise and effective models for drug screening and research. To date, Yao et al. generated 80 organoid cultures derived from patients with locally advanced RC and tested their susceptibility to 5-FU, irinotecan, or radiation. These data support that RC PDOs can function as a biomarker to help identify therapy-sensitive patients and promote precision medicine (16). Another group further leveraged an RC PDO platform to test responses to treatment in the front line, including FOLFOX (5-FU, leucovorin, and oxaliplatin) or radiation. Particularly, the responses of RC PDOs were consistent with the progression-free survival of the patients (39). It is also reported that PDOs derived from RC patients are used as a system to access the effect of combined radiotherapy with the short-chain fatty acids (such as butyrate, propionate, and acetate) by regulating HDAC activity. They further identified FOXO3A as a factor with cases nonresponsive to butyrate in PDOs. These data displayed the advantages of PDOs for testing novel drugs and modulating drug response (54). Kryeziu K et al. tested the drug efficacy of a medium-throughput drug library comprising 33 single agents and three 5-FU-based drug combinations containing Leucovorin (FLV), Oxaliplatin (FLOX), and SN-38 (FLIRI) by screening RC PDOs. They applied an interdisciplinary method to analyze spatiotemporal pharmacogenomic heterogeneity in a recurrent patient with KRAS-mutated liver metastases from RC. Coclinical *ex vivo* analyzes of front-line neoadjuvant combination chemotherapy regimens in three subsequent liver resections simulate actual responses to treatment, including indications of acquired resistance to FLOX. This case sets a great example for clinical application except for the small sample size. It is promising to conduct practice based on more large samples for evaluating the potential of PDOs in precision medicine (51).

These studies provide evidence that the capacity of PDOs to discover novel strategies and undergo drug screening overcome the flaws of 2-D cancer cell lines and upgrade the success of newly developed drugs.

Personalized medicine based on the testing of individual PDOs

RC is an ideal disease type to conduct precision medicine practice due to the different clinical responses of different patients and the subsequent tailored treatment. If the expected radiosensitivity is poor, patients may need more intensive chemotherapy application, and if the pretreatment worked well with the patients, they could avoid radical surgery. Accordingly, stable and reliable prediction tools play an important role in clinical practice for RC patients. It is reported that PDOs preserve high predictive value and the possibility to validate clinical biomarkers.

Ganesh et al. treated organoid culture from RC patients' tumors of different stages with clinical chemotherapy (5-FU alone or with oxaliplatin together) or radiation and analyzed the corresponding clinical response of each patient. They revealed that the PDO predictions were correlated with the results from clinical patients after treatment. They also successfully used tumoroids to establish the endoluminal model as a feasible and reproducible *in vivo* platform of RC and invested its chemosensitivity. They found that the *in vivo* model could also recapitulate the clinical response to therapy such as 5-FU and FOLFOX (39). In a clinical phase III study, Yao et al. reported an LARC organoid biobank that could foresee the clinical response to 5-FU, irinotecan chemotherapy, irradiation, or combined treatment (16). They compared the radiosensitivity of organoids with clinical patients' responses and found that 16 out of 17 patients with irradiation-sensitive organoids displayed positive response upon noadjuvant chemoradiation (NACR) treatment. Among the 64 patients whose organoids were resistant to irradiation, 42 of them had a poor response to NACR. They also exposed RCOs to 10 μM 5-Fu or 10 μM CPT-11. Twenty-seven patients with 5-Fu-sensitive organoids achieved a good response except for five poor response cases, and 38 of the 53 patients with 5-Fu–resistant PDOs had a poor response, and another 15 performed well. As for CPT-11, 32

sensitive PDOs of paired patients achieved a good response except for seven cases, and 27 of 34 patients with resistant organoids had a poor response. They, together, reveal that patients can acquire a good clinical response if their paired tumor organoids were sensitive to at least one of the three preceding treatments, which means that those with 5-Fu– or CPT-11–sensitive PDOs would probably benefit from 5-Fu or CPT-11 treatment alone although their PDOs were resistant to irradiation. Although PDOs generated by Yao et al. lack sources from patients of different stages compared with that from Ganesh et al., the data from these two groups could provide the evidence that the PDOs may enable tailored, personalized strategies with chemoradiation or chemotherapy and reduce overtreatment or toxicities for RC patients.

Later, Janakiraman et al. found that cetuximab has the potential to selectively sensitize the radiation effect with a KRAS mutational condition existing, and this group also detected cetuximab sensitivity gene impactors by establishing novel PDX and related PDO platforms from RC patients under pre-neoadjuvant therapy (27). Another observational coclinical study checked the standard combination of chemotherapy regimens in a PDO model generated from a patient with recurrent KRAS-mutated liver metastases from RC. They identified the SMAC mimetic as a unique therapeutic option in PDOs from the recurrent tumor tissue (51). Park et al. developed a prediction model to analyze clinical and laboratory patients' radiotherapy response data by using a machine learning algorithm. The whole coclinical experiment involved 33 patients with diagnosed mid-to-low RC, and the machine prediction accuracy could achieve over 89% (48). The other study from Ting et al. displayed that the sensitivity, specificity, accuracy, and positive and negative predictive value of the PDO model for predicting chemotherapy drug responses were 63.33%, 94.12%, 79.69%, 90.48%, and 74.42%, which indicated that PDO responses consisted of the sensitivity in the respective patient in the clinic, which indicates that PDTOs can serve as a prediction model to guide the individualized selection of chemotherapy regimens for patients with RC (38). The work from another group identifies acid ceramidase (AC) as a target for improving radiotherapy treatment with the finding that PDOs presenting low AC expression were more radiosensitive than other models possessing higher levels of AC. However, the sample size of this study was small, and they did not demonstrate the original sample collection location of the organoid cultures (56). Another group investigated the radiosensitivity of CRC with PDOs of both colon and rectal tumors and focused on how the radiosensitivity of 13 RC PDOs correlated with clinical treatment outcomes. A significant mutual correlation is seen between the D0 (from the single hit multitarget algorithm, which is a single value to evaluate tissue radiosensitivity) of primary tumor PDO and the clinical response to neoadjuvant therapy (45). In a nutshell, the prediction accuracy could reach a level of 60%–90%, demonstrating the potential of RC PDOs to correctly identify clinical therapy responders and nonresponders and guide personalized therapy selection for RC patients.

In a newly published study, PDOs were very helpful in finding a new resistance mechanism of iCAFs and IL1α in RC patients. These PDOs were generated from biopsies collected prior to CRT from (non-pCRs) patients with poor along with fine prognoses and (pCRs) patients with pathological complete responses, and subsequently, these organoids detected different IL-1ra expression levels but comparable IL-1α expression. This group also created a preclinical RC mouse model (APTKA) through orthotopically transplanting organoids into C57BL/6 mice. Through a series of experiments, they identified the therapy-resistant capacity of iCAFs in RC and proposed the IL-1 pathway as a potential target for matrix repolarization and prevention for cancer-associated fibroblast senescence (43). Based on the previous achievement, this group recently further started an ACO/ARO/AIO-21 phase I trial to test the IL-1 receptor antagonist (IL-1 RA) anakinra combining with CRT therapy for RC, which set up a great example for a translational application from bench to bedside (47).

According to the above research, PDOs show high accuracy in predicting therapy responses and guiding personalized strategies. There is a matter of modifying culture methods to achieve a stable growth rate and improve the prediction accuracy in drug tests and targeted therapy research, which enables the application of RC PDOs in clinical practice and trials.

Investigation of intratumoral heterogeneity and tumor evolution

It is currently reported that PDOs facilitate the study of intratumoral heterogeneity and tumor evolution by analyzing the differences between PDOs from primary tissue and from the metastatic sites in a patient. Organoids derived from different sites in the same CRC patient possess different patterns and distinct responses to diverse therapies (26, 57, 58), whereas there are only rare reports about RC PDOs in this field.

One RC-related study from Ganesh et al. mentions the metastatic potential of an *in vivo* transplanted RC organoid model. They created a patient-derived orthotopic xenograft model with human tumoroids, which could reflect the actual process, including cancer initiation, invasion, and metastasis in the rectum. After 22 and 30 weeks post-transplant, these *in vivo* models turned into invasive adenocarcinoma, which is consistent with the stage I or II characteristics in human RC. They also found lung and liver metastases with infiltrating normal parenchyma by poorly differentiated carcinoma. It is worth mentioning the observation that metastases beginning from the endoluminal rectum shared the same appearance with the metastases found in the individual patients. Their success in the generation of a RC PDO endoluminal model paves the way forward in the field of tumor evolution research (39). This application of the PDO system again proves the potential and sheds light on the RC metastasis assays field.

Studying the tumor microenvironment with PDOs

RC has a poor prognosis for both metastases and a higher risk of local recurrence than CC in CRC. Four different molecular CRC consensus subtypes (CMS1–S4) have recently been delineated according to their transcriptome profiles and cellular heterogeneity. The CMS4 type was identified as having the worst prognosis with a feature of an abundant mesenchymal signature (59). Therefore, the tumor microenvironment (TME) and, particularly, cancer-associated fibroblasts (CAFs) consisting of heterogeneous populations have great importance in CRC research.

In vivo models are familiar tools to study the interaction between cancer cells in the microenvironment, whereas organoids as a model *in vitro* make the interactions visible. With the purpose of overcoming the limitation that organoids do not harbor diverse cell types or complex tissue compositions, scientists developed a cocultivation method incubating human organoids with human CAFs in order to activate fibroblasts and promote the growth of desmoplastic stroma (60). Immune cells can also be successfully activated and possess killing capacity when cocultured with PDOs.

For instance, a cocultivation method is reported by a research group in which circulating tumor reactive T lymphocytes could be coincubated together with human CRC organoids and stimulated into active status (61, 62). By establishing these models, it is promising that we could assess the efficacy of immune therapy under a controlled environment and produce individual T cells for adoptive T cell transferring. Another study treated CRC organoids with chemoradiotherapy and examined the capability of tumor-infiltrating T lymphocytes within the culture (63). They checked the cytotoxic effects as well in the co-culture system containing RC organoids and infiltrating T lymphocytes and found that the killing ability of PDOs from patients with a good therapy response was stronger than in PDOs from nonresponders. In newly published research, Nicolas et al. cocultured the supernatants from low or high IL-1ra expression organoids together with matched human intestinal fibroblasts and found that supernatants with insufficient IL-1ra expression activated a series of pro-inflammatory gene expression profiles (43). Patient RC organoid application in the study of the TME is indeed a remarkable achievement. We expect to see more high-quality research using PDOs as a tool in RC microenvironment field.

Cancer modeling by genetic engineering of organoids

It is reported that cancer genetic mutations can be generated in wild-type organoids through technology such as CRISPR/Cas9 to facilitate research in oncology pathway mutants, tumor origin, and invasion. To date, there are fewer reports about RC organoid application in this field, so we use the research of CRC organoids.

As for CRC metastasis research, it has underscored the need for engineered organoids and emphasized the importance of genetic composition (64, 65).

By using CRISPR/Cas9 technology, oncologists could establish both advanced cancer and early tumor organoids with various mutations. The Medema group generated sessile serrated adenoma (SSA) organoids with the BRAFV600E mutation by engineering normal colon organoids to ensure regular growth. They later put forward that the CRC mesenchymal subtype could originate by activating RAS signaling through TGFβ stimulation together with BRAFV600E alternation (66). Another group made use of the SSAs characteristic of Rspondin gene fusions and first established chromosomal rearrangements with CRISPR/Cas9 to dig the impact of Rspondin fusions (67). This study introduced the idea that CRISPR/Cas9 technology can also be applied to delete, insert, or translocate chromosomes in addition to the use for mutating genes, which led the way to discover new therapy through targeting a specific pathway.

It is worth mentioning that Nicolas et al. activated *Il1a* transcription in irradiation-sensitive APTK organoids (Apc, Trp53, Tgfbr2, and K-rasG12D mutant) by CRISPR/Cas9 (43). Their data supports the conclusion that tumor cell–derived IL-1α polarizes CAFs toward an inflammatory phenotype, which then causes resistance to irradiation. A strong expression of inflammatory genes has been discovered in fibroblasts exposed to the fluid of APTK-sg*Il1a* organoids. APTK-sg*Il1a* organoids show an increased postirradiation stromal response with the features of suppressed $CD8^+$ T cell infiltration along with increased Sirius red staining. However, irradiation did not further improve the level of macrophage and neutrophil infiltration with the IL-1α-dependent increase. Nicolas et al. combined CRISPR/Cas9 technology with RC organoids, enriching the field of study known as RC modeling.

In summary, CRISPR/Cas9 technology in combination with organoids suggests a potential strategy for basic cancer research and exploration in clinical application. We expect to see the development of this application in RC field.

Discussion

In recent decades, organoid cultures of various types of tumors have been widely used in basic and translational cancer research, which is a major boost for the field. PDOs can truly reflect an individual's condition and possess the capacity to predict individual responses to diverse clinical therapies, allowing clinicians to tailor treatment strategies for each patient and practice precision medicine. The establishment of living biological sample banks enables us to detect a wide range of molecular and different patterns of one cancer entity to further promote new medicine development and fully exploit existing drugs. The presented studies also reveal the possible applications in cocultivation assays with

stromal, immune, or CAR cells, which may facilitate research in targeting stromal compositions to lead immune cells to their points.

However, current PDO systems do show some limitations, and there are gaps together with challenges between knowledge and the translational application of PDO models. First, culturing PDOs still costs a great deal, and the materials and culturing conditions used vary between laboratories. We present a summary in Table 3 with methodologic differences and limitations of selected studies (Table 3). An agreement needs to be reached. Second, successful establishment of a PDO platform depends on many factors, such as the size of the biopsy samples, amount of living tumor cells, and cancer cell proliferation rate. Contamination and residue of other cell types could also be problems in the biopsy material. In addition, as for rectal cancer, successful PDOs biobanks are precious resources since most RC patients have received neoadjuvant therapy prior to surgical resection, which may affect its role as prognosis tool in preclinical and clinical practice. At the same time, the frequency of eligible organoids for RC entities, such as sarcomas, is far from 80%–90%, suggesting the existence of relevant subtype conditions that may require specific growth factors. The greatest current challenge in patient-derived RC organoid culture is the absence of a TME. PDOs cannot recapitulate the full structure and function (such as the ability to grow blood vessels) of primary tissue for lacking CAFs, stroma cells, and immune cells in their culture system. Breakthroughs are being made in this field with continuous improvement of existing culture methods (e.g., an air–liquid interface PDO model enabling coculture with endogenous tumor-infiltrating lymphocytes). Finally, in view of inter- and intratumor heterogeneity, PDOs may need more *in vivo* model practice compared with PDX. Thus, the RC PDO model used in precision medicine requires extensive optimization and more focused research along with prospective study.

New technology, such as genomic, transcriptomic, and proteomic analyses, are used on organoids and help us further understand tumor biology to define targeted treatment. It is promising to see that PDOs allow direct prediction of the individual response to identified RC therapies. In a nutshell, patient-derived cancer organoids show the potential to bridge the gap between the present basic research and clinical practice in the near future, but more detail is needed before we can really identify the areas of most impact in terms of research and clinical application.

Methods

Data sources and literature search strategy

This review followed the PRISMA guidelines. Two investigators (IC and YY) independently conducted a literature search using as combined keywords rectal cancer and organoid, patient-derived organoid on Pubmed (https://www.ncbi.nlm.nih.gov/pubmed/) and Web of Science (v. 5.35). The database search was run on published articles in the last two years, all languages, from database inception until May 20, 2022. In both databases, the following search strategy was used: terms were searched as follows: organoid AND rectal cancer; organoid AND rectal carcinoma; patient-derived organoid AND rectal cancer; patient-derived organoid AND rectal carcinoma. It is thought that these terms would identify the majority of manuscripts within a narrow definition of rectal cancer and organoid, patient-derived organoid, though it remains likely that relevant sections might be embedded within the methodology sections of particular projects and, thus, more challenging to identify. In addition, we conducted a search on ClinicalTrials (https://clinicaltrials.gov/) using the keywords colorectal cancer organoid or rectal cancer organoid.

Study selection and data synthesis

All studies reporting information on RC and organoids, PDOs were included. One hundred twenty-four articles were identified and reviewed independently by two authors (IC and YY), and after all duplicates were removed, 41 articles were considered. After removing articles that were not in English and those that had simply a mention of the words with no further expansion, 21 articles were considered. Sixteen articles (of the 21) devoted a considerable amount of the manuscript to expand on those topics, and five articles (of the 21) are reviews or systematic reviews.

As for the search on ClinicalTrials, 24 studies were identified using the term “colorectal cancer organoid” and “rectal cancer organoid.” Eighteen studies were considered after all duplicates and studies unrelated to CRC or organoid were removed.

Thematic groupings and analyses were reviewed by an additional author (IC). All outcomes were included due to the wide range of use of the terminologies.

Results

The manuscripts identified in this review ($n = 21$) followed six loosely defined thematic groups: a) analysis of the mutational landscape using high-throughput sequencing technologies ($n = 7$), b) drug screening to delineate novel treatment strategies ($n = 4$), c) personalized medicine based on the testing of individual PDOs ($n = 10$), d) investigation of intratumoral heterogeneity and tumor evolution ($n = 1$), e) studying the TME with PDOs ($n = 2$) and f) cancer modeling by genetic engineering of organoids ($n = 2$).

In terms of the search on ClinicalTrials ($n = 18$), 13 studies (of the 18) were excluded for the following reasons: a) the study did not mention whether RC patients were involved in the studies ($n = 10$), b) studies related to CC PDO ($n = 2$), c) RC study used a malignant colonic organoid model ($n = 1$). Five

TABLE 3 Methodologic differences and limitations of selected studies.

Reference	Methods used for isolation	Composition of the extracelluar matrix	Type and number of cells seeded	Media and growth factors used	Purity of cell composition of the organoid
(42)	• Dissociating cells using 2 mg/ml collagenase I • Filtering with a 70 µm nylon cell strainer • Centrifuging at 250*g* for 5 min at room temperature	15 µl Matrigel Matrix	50,000 live cells	50% advanced Dulbecco's modified Eagle's medium (DMEM);50% L-WRN conditioned media; Both supplemented with: 20% fetal bovine serum (FBS), 2 mM GLUTamax, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 10 µM SB 431542 (TGF-beta/Smad inhibitor), 10 µM Y-27632 (ROCK Inhibitor), 50 µg/ml gentamicin. Media were changed every other day.	Not mentioned
(43)	• Tumor biopsies were cut in small pieces, washed with PBS supplemented with 1x penicillin/ streptomycin several times. • Incubating with 5mM PBS/EDTA for 10 min on ice with several vortex cycles • Incubating with PBS/ EDTA 5 mM for 30 min on ice with several vortex cycles • Collecting the supernatant and filtering through 70 µm filters • Centrifuging at 800 rpm for 5 min	Matrigel polymerized at 37°C for 30 min	Not mentioned	Advanced DMEM F12 supplemented with: 1x Glutamax, 1x HEPES, 1x Penicillin/ Streptomycin, 2.5% B27 supplement, 20% R-spondin, 10% Noggin condition media, 50 ng/ml human EGF, 2 mM N-Acetylcysteine, 500 nM TGF-β inhibitor A-83-01, 10 µM p38 MAPK inhibitor SB202190. 1000 µl media was added to each well (12 wells suspension plate)	Not mentioned
(45)	• Tumor tissues were incubated with digestion buffer (Advanced DMEM/ F12 medium with 2% FBS, 100 U/mL penicillin/ streptomycin, 500 U/mL collagenase, and 125 µg/ mL Dispase type II) for 30 minutes at 37°C, shaking every 5 minutes. • Samples were washed with 10 mL of ADF-FBS medium and spun at 300 × *g* for 5 minutes.	40 ul Matrigel cell suspension per well overlaid with 500 µL of EN medium in 24-well culture plates.	Not mentioned	ADF basal medium: Advanced DMEM/F12 medium plus 1 mmol/L GlutaMAX, 1 mmol/L HEPES, and 100 U/mL penicillin/ streptomycin. EN medium for tumor PDOs: ADF medium was supplemented with 10% Noggin conditioned medium (conditioned media were collected from cultures of HEK293 cells expressing recombinant Noggin proteins), 10 nmol/L gastrin I, 500 nmol/L A83–01, 10 mmol/L SB202190, 10 mmol/L nicotinamide, 1X B27 supplement, 1X N2 supplement, 1 mmol/L N-acetyl cysteine, and 50 ng/mL human recombinant EGF.	Not mentioned
(38)	• Tumor tissues were washed with 10 mL of Hanks Balanced Salt Solution containing antibiotics, minced with scissors. • Digesting with 5 mL of 5 mg/mL collagenase type II in Advanced DMEM/ F12 for ≈4 h at 37 °C with gentle shaking and intermittent pipetting. • Samples were filtered with a 70 µm cell strainer and centrifuged at 300*g*.	Incubated with 30 ul Matrigel basement membrane matrix and polymerizing at 37°C for 30 min	For drug response analyses, organoids were resuspended in 2% Matrigel/organoid culture medium with 200–1000 clusters per milliliter	Advanced DMEM/F12 without other factors mentioned	Not mentioned about cell position. Success rate:69.77% in the pilot study;80.21% in the blinded study

(Continued)

TABLE 3 Continued

Reference	Methods used for isolation	Composition of the extracelluar matrix	Type and number of cells seeded	Media and growth factors used	Purity of cell composition of the organoid
	• Red blood cells were lysed using lysis buffer for 5 minutes.				
(48)	• Tumor tissues were incubated with collagenase type II, dispase type II and Y-27632 for 30 min at 37°C. • Isolated cells were washed with PBS and centrifuged at 300 × g for 3 min at room temperature.	Matrigel (growth factor reduced, phenol red free)	Not mentioned	1× B27 supplemented with: 1.25 mM *N*-acetyl cysteine, 50 ng/ml human epidermal growth factor, 50 ng/ml human Noggin, 10 nM gastrin, 500 nM A83-01 and 100 mg/ml primocin, CHIR99021, R-spondin1. 10 uM Y-27632 was added to the culture medium to prevent anoikis.	PDTOs differentiated into enterocytes, goblet cells, and enterochromaffin cells and contained amplifying cells. Success rate:70%
(41)	• Tumor pieces were digested in mixed medium consisted of advanced DMEM/F12 with 2% FBS, Pen/Strep, 100 U/mL collagenase type XI, and 125 μg/mL dispose type II at 37°C for 40 min. • Adding TrypLE Express and DNase I medium for further digestion for 10 min • Samples were filtered through a 70 μm cell strainer, and centrifuged at 300 × g for 5 min.	Matrigel without mentioned about the dosage	Not mentioned	Used the same protocol described in Yao et al. study (16)	Not mentioned
(49)	• Tumor tissues were incubated in collagenase type II, dispase type II and Y-27632 for 30 min at 37°C	Matrigel on ice (growth factor reduced, phenol red free)	2 mm^3-sized tumor piece was implanted into Central Institute for Experimental Animals NOG mice. Not mentioned about the type and number of cells seeded *in vitro*.	1xB27 supplemented with: 1.25 mM N-acetyl cysteine, 50 ng/mL human epidermal growth factor, 50 ng/mL human Noggin, 10 nM gastrin, 500 nM A83-01 and 100 mg/mL primocin. During the first 2–3 days, 10 uM Y-27632 was added to the culture medium to prevent anoikis.	Not mentioned
(51)	• Tumor specimens (2.5-6 × 7 mm in size) were minced into 0.1- 0.5 mm fragments, washed with ice-cold basal culture media. • Straining with a 70 μm pore mesh, and collected by centrifugation at 400*g* 4°C for 5 min.	25 μl drops Matrigel (Growth Factor Reduced) overlaid with 3 ml organoid growth media in pre-warmed 6-well tissue culture plates	Not mentioned	1xB27 supplemented with: 10 nM [Leu15]-Gastrin I, 1 mM N-acetyl-l-cysteine, 50 ng/mL for EGF, 100 ng/mL for Noggin, 500 nM for TGF-β receptor type I inhibitor A83-01 and 10 μM for p38 MAP kinase inhibitor SB202190. Organoid growth media without Y-27632 was refreshed every two to four days.	Not mentioned
(16)	• Tumor tissues were washed in the cold PBS with penicillin/streptomycin for 5 × 5 minutes, and then minced into tiny fragments. • Tissue fragments were digested in 8 mL digestion medium containing 7 mL DMEM medium, 500 U/mL collagenase IV, 1.5 mg/	Incubated with Matrigel and polymerizing at 37°C, 5% CO_2 for 5-8 min.	For irradiation response and drug tests, organoids were seeded in 48-well plate and density was adjusted to 10-15/μL Matrigel before seeding. Every well contained about 200 ± 50 organoids in 15 μL Matrigel with 300 μL culture medium. They applied organoid size (100 μm in diameter) to	Advanced DMEM/F12 medium, supplemented with: 500ng/mL R-spondin 1, 100ng/mL Noggin, 50ng/mL EGF, HEPES, Glutamax, Normocin, Gentamicin/amphoteritin B, N2, B27, n-Acetylcysteine, Niacinamide, Alk 4/5/7 inhibitor, p38 inhibitor, Gastrin and Prostaglandin E2.	Not mentioned about cell position. Success rate:77-85.7%

(Continued)

TABLE 3 Continued

Reference	Methods used for isolation	Composition of the extracelluar matrix	Type and number of cells seeded	Media and growth factors used	Purity of cell composition of the organoid
	mL collagenase II, 20 μg/mL hyaluronidase, 0.1 mg/mL dispase type II, 10 μM RHOK inhibitor ly27632 and 1% fetal bovine serum on an orbital shaker at 37°C for 30-60 minutes. • Tumor cells were collected after centrifugation at 300-500 g for 5 minutes.		define right time for *in vivo* treatments.		
(54)	Tumor tissues were incubated with collagenase type II, dispase type II and Y-27632 for 30 min at 37°C.	Matrigel on ice (growth factor reduced, phenol red free)	For viability test 5,000 cells/10 μl Matrigel per well	1× B27, CHIR99021 supplemented with: R-spondin1, 1.25 mM *N*-acetyl cysteine, 50 ng/ml human epidermal growth factor, 50 ng/ml human Noggin, 10 nM gastrin, 500 nM A83-01 and 100 mg/ml primocin.	Not mentioned
(27)	• Tumor tissues were cut into <1 mm^3 tumor fragments, washed with HBSS. • Samples were resuspended in Ammonium–Chloride–Potassium (ACK) Lysis Buffer to eliminate blood cells. • Digesting with 0.2 μ/ml of Liberase DH containing 10 μm Y-27632 for 60 min at 37°C. • Cells were filtered through a 250 μm sieve followed by a 100 μm cell strainer.	2 ml BME (reduced growth factor basement membrane extract) mixed with tumor cells. 40 μl droplets were seeded into a prewarmed six-well plate with seven droplets per well and incubated at 37°C for 10 min to solidify BME. Three milliliters of complete organoid culture medium were added to each well.	1×10^6 tumor cells	Advanced DMEM/F12 supplemented with: 10 mM HEPES, 1× GlutaMAX and 1× penicillin/streptomycin, 500 nM A83-01, 1XB27 supplement, 50 ng/ml epidermal growth factor, 10 nM gastrin, 1 mM *N*-acetyl-l-cysteine, 10 mM nicotinamide, 10 nM prostaglandin E2, 6 μM SB20219 and 10 μM Y27632 Fresh medium was added every 3 days and PDTOs were passaged every 7 days.	100% cancer epithelial cellularity based on CDX2 and CK20 staining Pleomorphic single cells (n = 1), small solid cell clusters (n = 3), small patent glands (n = 1), medium patent glands (n = 1) and large cribriform glands (n = 3) Success rate:90%
(55)	• Tumor biopsies were washed in PBS and incubated with antibiotics for 30 min at RT. • Biopsies were cut into small pieces and digested with collagenase type IV (1 mg/mL in PBS for 30 min at 37 °C with continuous shaking in a water bath. • Disaggregating by passing the suspension through a 18 G needle and filtering through a 200-μm mesh into a 50 mL conical tube and centrifuged at 250× *g* for 5 min at 4°C.	Matrigel without mentioned about the dosage	Not mentioned	Advanced DMEM/F12 supplemented with: 10 mM HEPES, 10mM GlutaMAX and 1× N2, 1XB27 supplement,1:500 primocin, 1ug/ml gastrin, 0.1ug/ml Noggin, 1 mM *N*-acetyl-l-cysteine, 10 mM nicotinamide, 50ng/ml EGF, 0.02uM PGE2, 1 μM LY-2157299 and 10 μM Y27632 Culture medium was changed every second day.	Not mentioned about cell position. Success rate:55%
(56)	Not mentioned	40ul Matrigel	10000 cells	Intesticult™ Organoid Media supplemented with: 1% Penicillin-streptomycin and 10 μM Y27632 dihydrochloride, Rho kinase (ROCK) inhibitor	Not mentioned
(39)	• Tumor tissues were washed with ice-cold PBS-Abs buffer and chopped	Samples derived from biopsies were embedded in 800 μL Matrigel and	Not mentioned	Advanced DMEM/F12 was supplemented with: antibiotic-antimycotic, 1×B27, 1×N2, 2 mM	RC PDOs retained Alcian blue-positive and

(Continued)

TABLE 3 Continued

Reference	Methods used for isolation	Composition of the extracelluar matrix	Type and number of cells seeded	Media and growth factors used	Purity of cell composition of the organoid
	into 1 mm pieces in ice-cold PBS-DTT buffer. • Digesting with digestion medium (advanced DMEM/F12 with 2% FBS, Pen/Strep, 100 U/mL collagenase type XI, and 125 µg/mL dispase type II) at 37 °C for 40 min and further digested for 10 min by adding a half-volume of TrypLE Express, and 3 mg of DNase I per sample. • Samples derived from resected tumors were filtered through a 70 µm Cells Strainer, centrifuged at 300×g for 5 min.	samples derived from resected tumors were embedded in 1–2 mL of Matrigel. 50 µL/well overlaid with 500 µL culture medium after the Matrigel balls were polymerized. (24-well suspension plate)		GlutaMAX, 10 nM gastrin I, 10 mM HEPES, 1 mM N-acetylcysteine, and 10 mM nicotinamide, 50% Wnt-3A conditioned medium, 20% R-spondin conditioned medium (media collected from HEK293 cell lines expressing recombinant Wnt3a and R-spondin1, kindly provided by Kevin P. O'Rourke and the S. Lowe laboratory), 100 ng/mL mouse recombinant noggin, 50 ng/mL human recombinant EGF, 500 nM A83–01, and 10 µM SB 202190. Upon expansion, RC PDOs were passaged and then cultured in medium without Wnt-3A, R-spondin, and noggin.	MUC-2-positive goblet cells, CK20 and CDX2-positive enterocytes, robust expression of E-cadherin (epithelial marker), and cytoplasmic/nuclear patterns. Success rate:77% (65/84)
(40)	• Chopped tissues were digested with 4 ml tissue digestion solution, 37°C water bath for 30 minutes; • Centrifuging at 1200 rpm and 4°C for 4 minutes, discarding the supernatant; • Suspending with Dulbecco's modified eagle medium/nutrient mixture F-12 (DMEM-F12), and filtering with 70 mu m and 40 mu m filters • Adding filtrate to low-adsorption dish, incubating for 1 hour in a 37°C and 5% carbon dioxide; • Centrifuging the liquid at 1200 rpm, 4°C for 4 minutes, discarding the supernatant; • Adding 1 ml phosphate buffered saline to wash, taking an appropriate amount and counting, and centrifuging the remaining portion at 1200 rpm and 4°C for 4 minutes, discarding the supernatant	50ul Matrigel (RTM: Gelatinous protein mixture) per well (48-well cell culture plate) solidifying for 20 minutes, overlaid with 500 ul preheated self-made culture medium to each well.	(2-4) *10^4 cells/ ul	Not mentioned Culture medium was changed every 3 days.	Not mentioned

studies associated with patient-derived RC organoid application were finally considered.

All publications or clinical studies that met the criteria were included in Table 2, and some of the publications and clinical studies excluded are shown in Table 1 (28–37). They are presented subsequently in this order, reflecting the scientific continuum, moving from the characterization and acquisition of knowledge to the interpretation and finally toward clinical implementation.

Author contributions

YY and IC conducted the review and applied the eligibility selection criteria for the identified manuscripts. IC validated the selected manuscripts and arbitrated any queries. CP participated in literature collecting. YY and IC wrote the manuscript. HW, XL and XW supervised the whole article. 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.

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SAMHD1 as a prognostic and predictive biomarker in stage II colorectal cancer: A multicenter cohort study

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Background: The identification of high-risk population patients is key to the personalized treatment options for the stage II colorectal cancers. The use of proteomics in the prognosis of patients with stage II colorectal cancer remains unclear.

Methods: Using quantitative proteomics, we analyzed proteins that are differentially expressed in the tumor and adjacent normal tissues of 11 paired colorectal cancer patients with and without recurrence selected by a nested case-control design. Of the 21 identified proteins, we selected one candidate protein. The association of the corresponding gene of the selected protein with overall survival (OS) and adjuvant chemotherapy was analyzed using two independent cohorts of patients with stages II colorectal cancer.

Results: Sterile α motif and histidine-aspartate domain-containing protein 1 (SAMHD1) was selected as the candidate biomarker. A group of 124 patients (12.5%) were stratified into SAMHD1-high subgroup. The 5-year OS rate of SAMHD1-high patients was lower than that of SAMHD1-low patients with stage II colorectal cancer (discovery cohort: hazard ratio [HR] = 2.89, 95% confidence interval [CI], 1.17-7.18, P = 0.016; validation cohort: HR = 2.25, 95% CI, 1.17-4.34, P = 0.013). The Cox multivariate analysis yielded similar results. In a pooled database, the 5-year OS rate was significantly different between patients with and without adjuvant chemotherapy among stage II SAMHD1-low tumors than in patients with stage II SAMHD1-high tumors (88% vs. 77%, P = 0.032).

Conclusions: SAMHD1-high expression could help in identifying patients with stage II colorectal cancer with poor prognosis and less benefit from adjuvant chemotherapy.

KEYWORDS

SAMHD1, colorectal cancer, Cox model, prognostic markers, nested case-control design, MSI

Introduction

Globally, colorectal cancer is the third most common malignant tumor and the second leading cause of cancer-related deaths (1). In China, colorectal cancer poses a huge health burden, with more than 290,000 deaths reported annually (2). Local recurrence and distant metastasis are the major reasons for the high mortality rate in patients with resectable colorectal cancer (3). The rate of recurrence for stages II and III colorectal cancer is approximately 20% and 48%, respectively (4). To reduce the incidence of recurrence, adjuvant chemotherapy following total meso-rectal excision is the standard of care for stage III patients according to international guidelines (5–8); wherein patients with stage III colorectal cancer who received adjuvant chemotherapy showed significant improvement in survival (9). However, patients with stage II colorectal cancer showed minimal improvement in the 5-year overall survival (OS) rate (2%–5%) (10). There is no consensus on whether patients with stage II colorectal cancer could benefit from adjuvant chemotherapy; therefore, recommending adjuvant chemotherapy for those patients is still controversial (5–8). Therefore, it is crucial to identify patients with stage II colorectal cancer who could benefit from adjuvant chemotherapy.

Prognostic risk factors are essential to help clinicians make better-informed decisions while selecting the best treatment strategy for patients with stage II colorectal cancer and determining the need for adjuvant treatment (11). At present, the major well-known prognostic risk factors for patients with colorectal cancer are stage pT4, bowel perforation or occlusion, lymphatic-vascular-perineural invasion, poorly differentiated histology (excluding microsatellite instability-high [MSI-H] tumors), inadequate lymph node sampling, and positive margins after surgery (5–8). However, all these factors, except stage pT4, are insufficient to identify patients with stage II colorectal cancer who could benefit from adjuvant chemotherapy (12). In the last few years, several efforts have been made to identify novel biomarkers that are able to predict a higher risk of relapse in patients with stage II colorectal cancer, such as identifying their gene expression signatures (Oncotype, ColoPrint, ColDX) (13–15), microRNA signatures (16), circulating tumor DNA (17–21), immune-related signatures (22–24), and deep learning signatures (25). However, high costs or complexity in the techniques of these approaches have prevented their successful translation into routine clinical practice. This has led to the emerging need for the identification of novel and more feasible biomarkers.

Over the past few years, mass-spectrometry-based proteomics has emerged as the method of choice for identifying possible prognostic indicators of outcome and disease response to therapy (26–29). We used proteomics to identify and select sterile α motif and histidine-aspartate domain-containing protein 1 (SAMHD1) as the candidate biomarker based on literature reviews and experiments. Using subgroup analysis involving retrospective patient cohorts, we evaluated the association between the SAMHD1 biomarker and the benefits from adjuvant chemotherapy and survival in patients with stage II and III colorectal cancer.

Methods

Patients and samples

The study protocol was approved by the Yunnan Cancer Hospital Ethics Committee (No. KY2019141). The requirement for informed consent was waived by the ethics committee owing to the retrospective nature of the study. The data were anonymized. For proteomic analysis, surgically resected biopsies of patients with colorectal cancer and paired non-cancerous tissues (collected 10 cm from the tumor) were collected from 11 pairs of patients with stage II and III colorectal cancer with and without recurrence, from Yunnan Cancer Hospital. These 11 pairs of patients were selected by propensity score matching (PSM) from the original cohort, including consecutive patients with stage I–III colorectal cancer who underwent radical resection at Yunnan Cancer Hospital between December 2010 and February 2019 (referred to as the Yunnan colorectal cancer cohort). The association between the expression levels of SAMHD1 messenger RNA (mRNA) and OS was tested in a discovery dataset of 335

patients from The Cancer Genome Atlas (TCGA) and a validation dataset of 465 patients from the National Center for Biotechnology Information Gene Expression Omnibus (NCBI-GEO). Patients who received neoadjuvant treatment were excluded from the analysis. The flowchart of the study is shown in Figure 1.

Propensity score matching

We performed PSM (30, 31) to strictly balance the critical variables between postoperative and non-postoperative metastatic patients within 3 years after surgery in the Yunnan colorectal cancer cohort. Propensity scores were generated using a logistic regression model with age, sex, body mass index, surgical pathological type, site of primary carcinoma, and pathological stage as the independent variables. Each metastatic patient was matched 1:1 to two patients in the non-metastasis group using a 0.001 caliper width (propensity scores must be within 0.1% of each other to create a match), and the resulting matches were used in the following selection.

Subsequently, we selected matched patients according to the following inclusion criteria: (1) patients with stage II or III colorectal cancer; (2) available formalin-fixed paraffin-embedded (FFPE) specimens; (3) available data on recurrence-free survival (RFS) and OS; (4) patients without recurrence whose duration of OS is longer than that of patients with recurrence; and (5) data including cancerous and paired non-cancerous tissues. Tandem mass tag (TMT)-labelled quantitative proteomics was performed on the matched patients.

TMT-labelling quantitative proteomics

For each patient, quantitative proteomics was performed on the tumor and tumor-adjacent tissues, and the protein was

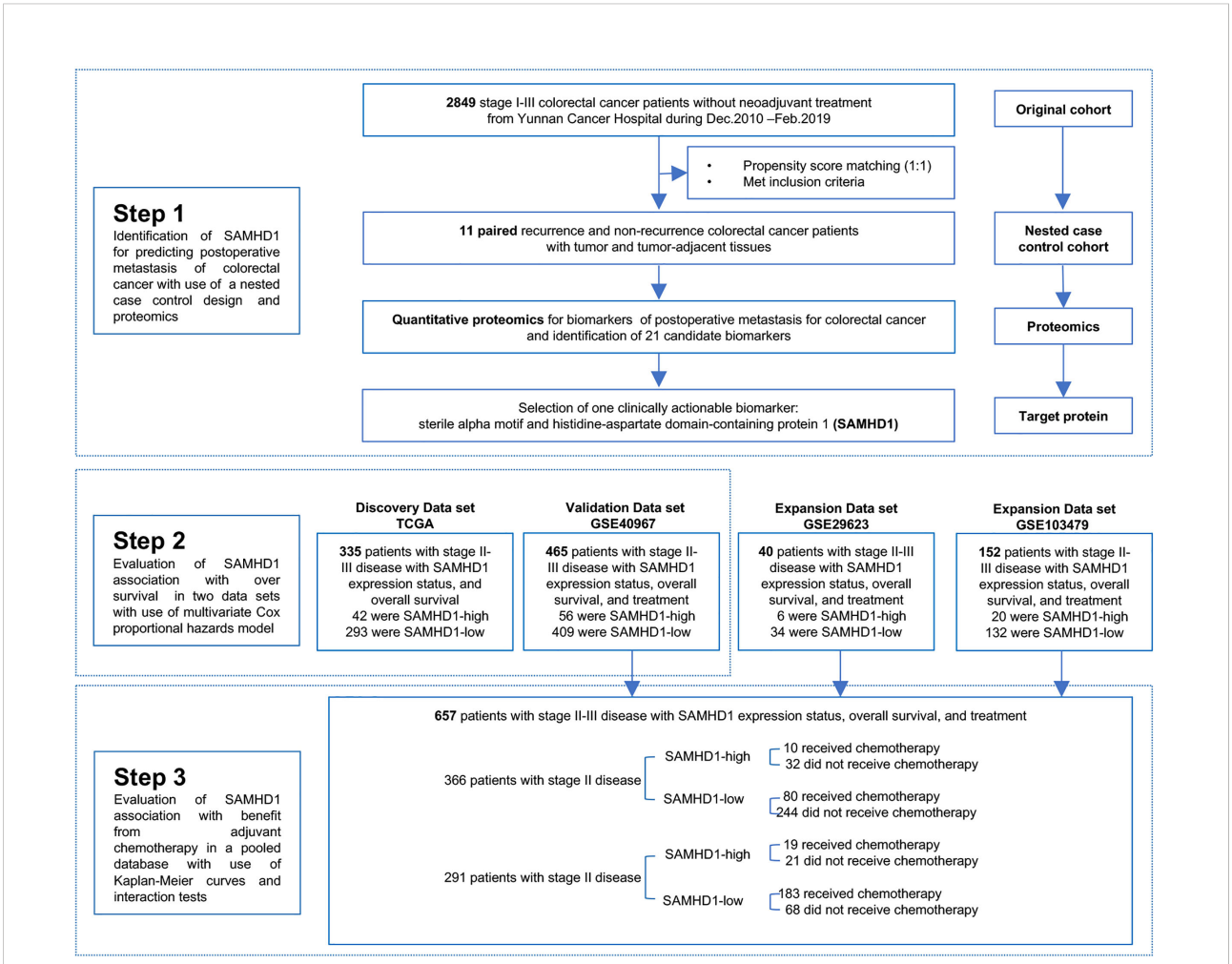

FIGURE 1
Study Design. PSM, propensity score matching; SAMHD1, sterile α motif and histidine-aspartate domain-containing protein 1; TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus.

extracted using the FFPE Total Protein Extraction Kit (Sangon Biotech, NO. C500058, Shanghai, China), according to the manufacturer's instructions. The extracted proteins were quantified using a BCA protein assay kit (Bio-Rad, USA). Protein digestion was performed according to the FASP procedure described by Wisniewski et al. (32), and the resulting peptide mixture was labeled using the 6-plex TMT reagent according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, USA). Detailed procedures for TMT labeling, peptide fractionation, and LC-MS/MS analysis are described in the Supplementary material A.

Identification of the target protein

The differentially expressed proteins between tumor and tumor-adjacent tissues were identified using the Student's t-test ($P < 0.05$). Proteins associated with metastasis were verified using the univariate Cox regression analysis ($P < 0.01$). Subsequently, we focused on the intersection of the differentially expressed and metastasis-related proteins. Proteins that had rarely been reported in most cancers, according to the literature search and our basic research, were selected for further analysis.

Analysis of tissue microarrays in the discovery and validation datasets

Gene expression profiles for colorectal cancer tissues, fully annotated with clinical and pathological information, were obtained from two independent sources; TCGA (Figure S1) and NCBI-GEO, including GSE40967 (Figure S2). A detailed description of the patient cohorts represented by the two independent sources is provided in Table S1.

Due to the considerable variation in the coverage of the sequencing platforms, pipelines, assays, and tools/algorithms between the TCGA and GEO datasets, the frequency of the identified variants was impacted (33). Taking these constraints into consideration, we used the Z-score (34) to standardize data across different experiments and to normalize the expression data of SAMHD1 from these two datasets prior to data analysis.

Subsequently, SAMHD1 expression levels were stratified into SAMHD1-high and SAMHD1-low subgroups according to the SAMHD1 expression, the threshold of which was identified in patients with stage II colorectal cancer using X-tile from the discovery dataset (35). We explored the association between the expression levels of SAMHD1, the OS outcomes, and the interaction between SAMHD1 expression level and adjuvant chemotherapy in stage II and stage III colorectal cancer.

SAMHD1 expression and benefit from adjuvant chemotherapy

To evaluate whether patients with SAMHD1-high tumors could benefit from adjuvant chemotherapy, we investigated the association between SAMHD1 status (assessed at the mRNA level) and OS among patients who either did or did not receive adjuvant chemotherapy in the NCBI-GEO dataset by pooling the following three datasets: GSE40967, GSE29623 and GSE103479. The three datasets were found to satisfy our criteria (i.e., knowledge of pathological stage, available information on SAMHD1 expression, adjuvant chemotherapy, duration of OS, and follow-up duration) (Figures S3, S4, and Table S1).

Statistical analysis

We downloaded the transcriptome profiles in FPKM format and the corresponding clinical information from the TCGA portal (https://portal.gdc.cancer.gov/) and NCBI-GEO dataset (https://www.ncbi.nlm.nih.gov/geo/). The NCBI-GEO datasets recruited for multiple dataset analysis and were based on different platforms. Therefore, we combined the three datasets to expand the sample size and avoid generating less reliable results by normalization using the robust multi-chip average (RMA) algorithm and removed the batch effect using the affy and sva R packages. Probes corresponding to the same gene were averaged.

Patient subgroups were compared with respect to survival outcomes using Kaplan–Meier curves, log-rank tests, and multivariate analyses based on the Cox proportional hazards method adjusting for patient age, sex, and adjuvant chemotherapy.

All analyses were conducted using R software (version 3·6·3; http://www.R-project.org). Statistical significance was set at $P < 0.05$.

Results

The clinical characteristics of 11 paired-patients

Eleven pairs of patients were selected through PSM. The clinical and pathological characteristics of patients with stage II or III colorectal cancer are shown in Table S2. The age of the patients in the non-metastasis group ranged between 46 and 74 years. In the non-metastatic group, 5 patients were males, 8 patients were in stage II, one patient died, and no recurrence occurred. The median OS and RFS follow-up times were both 51.6 months. The age of patients in the metastatic group ranged between 42 and 75 years. In the metastatic group, five patients

were males, eight patients had stage II disease, five patients died, and recurrence occurred in five patients. The median OS and RFS follow-up times were 31.8 and 21.4 months, respectively. Based on the univariate analysis, there was a significant difference in the OS follow-up time (51.6 months vs 31.8 months, respectively, $P = 0.002$), RSF event (100% vs 54.5%, respectively, $P = 0.042$), and RFS follow-up time (51.6 months vs 21.4 months, respectively, $P < 0.001$) between the non-metastatic and metastatic groups. Figure S5 shows the OS and RFS curves for all patients.

Identification of SAMHD1

A total of 5,197 proteins were identified using TMT-labelled quantitative proteomics. We processed the protein expression data by deleting proteins in which more than 50% of the samples had missing values. Among the remaining 2,760 proteins, which were retained for further analysis, 1,409 proteins showed significance with the $P < 0.05$ t-test threshold (Table S3); 38 proteins were associated with metastasis ($P < 0.01$) in the univariate Cox regression analysis (Table S4). The volcano plot shows the distribution of *P*-values of the t-test and the univariate Cox regression analysis (Figure S6A). In our study, 28 candidate proteins were found to be common between the 1,409 differentially expressed proteins and the 38 metastasis-related proteins (Figure S6B). Of the 28 candidate proteins, 7 proteins were not annotated with coding genes. The information regarding the remaining 21 proteins is shown in Table S5.

Based on previous literature reviews and basic experiments, we screened these 21 proteins and finally yielded the protein SAMHD1 (36–39). Figure S7 shows the different distributions of SAMHD1 expression in the cancer tissues between the non-metastatic and metastatic groups.

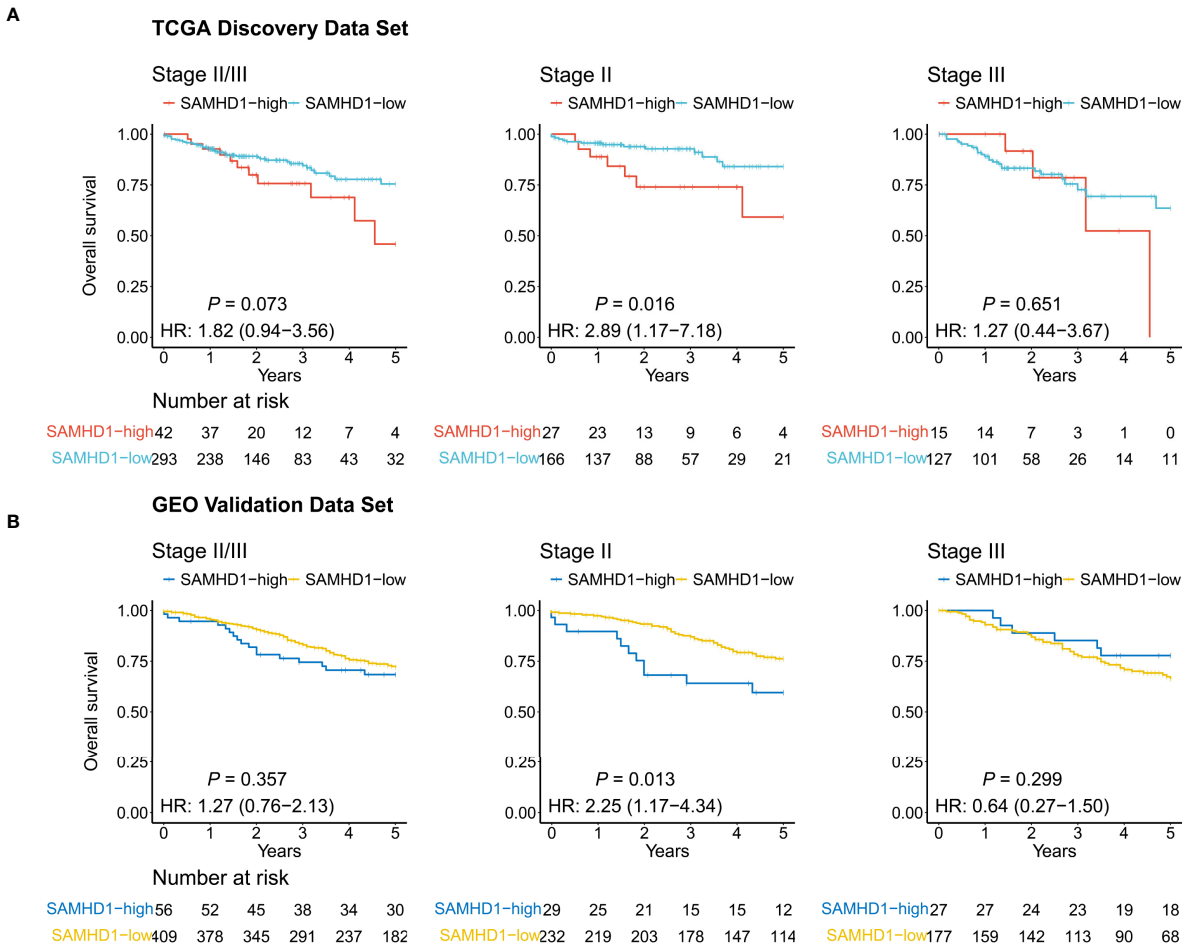

FIGURE 2
Kaplan–Meier curves. The curves show the relevance between the 5-year overall survival and SAMHD1 gene expression status in colorectal cancer, using The Cancer Genome Atlas data (TCGA) **(A)** and Gene Expression Omnibus data (GEO) **(B)**. Left: Patients with stage II and III disease. Middle: Patients with stage II disease. Right: Patients with stage III disease.

The Pearson's correlation coefficients between the expression of the SAMHD1 gene and the genes associated with microsatellite instability (MLH1, MSH2, MSH6, and PMS2) were -0.012, 0.17, 0.19, and 0.28, the results indicated there was a weak correlation between SAMHD1 expression and microsatellite instability-related genes (Figure S8), and SAMHD1 has a good complementary effect with those genes. Additionally, we compared and contrasted SAMHD1 expression according to KRAS mutation status, BRAF mutation status, tumor location, and defective DNA mismatch repair status, as they were frequently mutated genes or risk parameters in colorectal cancer. Statistical significance was detected using t-test for comparisons between all those groups. The results showed that SAMHD1 gene expression only partially overlapped with tumors defined by those factors (Figure S9).

SAMHD1 expression and OS in the discovery dataset

The optimum cutoff score for SAMHD1 expression generated by the X-tile plot was 1.15 (Figure S10). In total, the 335 patients were stratified into SAMHD1-low (n = 293, 87.5%) and SAMHD1-high (n = 42, 12.5%) groups, according to the expression of SAMHD1 in the discovery dataset. The baseline characteristics and known molecules of stage II and III in the discovery dataset were shown in Table S6.

Kaplan–Meier curves were used to compare the 5-year OS of the two groups. As shown in Figure 2A, the expression level of SAMHD1 tended to be associated with the 5-year OS (SAMHD1-high vs. SAMHD1-low; HR = 1.82; 95% CI, [0.94–3.56]; and P = 0.073) among patients with stage II and III colorectal cancer. With respect to stage II, the 5-year OS rate of the 27 patients (13.99%) with SAMHD1-high expression level was higher than that that of the 166 patients (86.01%) with SAMHD1-low expression level (HR = 2.89; 95% CI, [1.17–7.18]; and P = 0.016). However, there was no significant difference in the 5-year OS between the SAMHD1-low (n = 127) and SAMHD1-high (n = 15) groups with stage III colorectal cancer (HR = 1.27; 95% CI, [0.44–3.67]; and P = 0.651). In the multivariate analysis, with adjustment of age and sex as confounding variables, the HR for OS among stage II patients with SAMHD1-high versus SAMHD1-low was 2.99 (95% CI, [1.17–7.65]; and P = 0.023) (Table 1).

SAMHD1 expression and OS in the validation dataset

To evaluate the robustness of our findings, we performed an analysis in the validation dataset including 56 SAMHD1-high patients (12.04%) and 409 SAMHD1-low patients (87.96%). The baseline characteristics and known molecules of stage II and III in the validation dataset were described in Table S7. As shown in Figure 2B, we observed that the high expression of SAMHD1 (n = 29) was associated with a lower 5-year OS rate than a low expression of SAMHD1 (n = 232) among stage II patients (HR = 2.25; 95% CI, [1.17–4.34]; and P = 0.013), but not in stage III patients (n = 204; HR = 0.64; 95% CI, [0.27–1.50]; and P = 0.299) (Table 1). After adjusting for sex, age, and adjuvant chemotherapy, multivariate analysis also confirmed that high SAMHD1 expression status was associated with shorter OS in stage II patients (HR = 2.81; 95% CI, [1.43–5.50]; and P = 0.003) (Table 2).

The SAMHD1 expression groups had similar hazard ratios among stage II patients compared with the classical risk parameter such as age and T stage in the multivariate Cox regression analyses (Figure S11), which is based on analyses about the relative importance of each risk parameter for OS using the x^2 proportion test in stage II patients.

TABLE 1 Univariate and multivariable Cox analyses for overall survival among patients in the discovery data set.

Subgroup	Variable	Univariate Analysis		Multivariate Analysis	
		HR (95% CI)	*P* value	HR (95% CI)	*P* value
Stage II/III	SAMHD1_high vs. SAMHD1_low	1.82 (0.94-3.56)	0.078	1.55 (0.77-3.09)	0.217
	Age[a]	1.04 (1.01-1.07)	0.002	1.05 (1.02-1.07)	0.001
	Male vs. Female	0.79 (0.46-1.37)	0.409	0.75 (0.43-1.32)	0.327
	Stage III vs. Stage II	2.20 (1.25-3.85)	0.006	2.58 (1.47-4.55)	0.001
Stage II	SAMHD1_high vs. SAMHD1_low	2.89 (1.17-7.18)	0.022	2.99 (1.17-7.65)	0.023
	Age[a]	1.12 (1.05-1.18)	<0.001	1.12 (1.05-1.20)	<0.001
	Male vs. Female	1.09 (0.46-2.58)	0.849	1.20 (0.49-2.94)	0.686
Stage III	SAMHD1_high vs. SAMHD1_low	1.27 (0.44-3.67)	0.653	0.92 (0.30-2.80)	0.888
	Age[a]	1.03 (1.00-1.06)	0.086	1.03 (1.00-1.06)	0.055
	Male vs. Female	0.70 (0.34-1.45)	0.334	0.59 (0.28-1.27)	0.180

[a]continuous variable.
CI, confidence interval; HR, hazard ratio; SAMHD1, sterile alpha motif and histidine-aspartate domain-containing protein 1.

TABLE 2 Univariate and multivariable Cox analyses for overall survival among patients in the validation data set.

Subgroup	Variable	Univariate Analysis		Multivariate Analysis	
		HR (95% CI)	*P* value	HR (95% CI)	*P* value
Stage II/III	SAMHD1_high vs. SAMHD1_low	1.27 (0.76-2.13)	0.359	1.14 (0.66-1.95)	0.634
	Age[a]	1.03 (1.02-1.05)	<0.001	1.03 (1.01-1.05)	0.001
	Male vs. Female	1.28 (0.88-1.86)	0.195	1.47 (1.01-2.16)	0.046
	Stage III vs. Stage II	1.30 (0.90-1.88)	0.156	1.70 (1.08-2.67)	0.021
	Adjuvant Chemotherapy[b]	0.71 (0.49-1.03)	0.073	0.64 (0.40-1.04)	0.071
Stage II	SAMHD1_high vs. SAMHD1_low	2.25 (1.17-4.34)	0.015	2.81 (1.43-5.50)	0.003
	Age[a]	1.03 (1.01-1.05)	0.016	1.04 (1.01-1.06)	0.006
	Male vs. Female	1.20 (0.71-2.04)	0.496	1.32 (0.78-2.25)	0.301
	Adjuvant Chemotherapy[b]	0.76 (0.40-1.47)	0.414	1.07 (0.53-2.16)	0.850
Stage III	SAMHD1_high vs. SAMHD1_low	0.64 (0.27-1.50)	0.304	0.43 (0.18-1.02)	0.056
	Age[a]	1.03 (1.01-1.06)	0.002	1.03 (1.01-1.05)	0.017
	Male vs. Female	1.43 (0.84-2.44)	0.185	1.60 (0.93-2.75)	0.088
	Adjuvant Chemotherapy[b]	0.40 (0.24-0.68)	0.001	0.40 (0.22-0.71)	0.002

[a]continuous variable. [b]yes vs. no.
CI, confidence interval; HR, hazard ratio; SAMHD1, sterile alpha motif and histidine-aspartate domain-containing protein 1.

SAMHD1 expression and benefit from adjuvant chemotherapy

Figure 3 shows the relationship between the expression levels of SAMHD1 and benefit from adjuvant chemotherapy in 657 patients with stage II or III colorectal cancer. In the SAMHD1-low patient population, treatment with adjuvant chemotherapy was associated with higher OS in the stage II subgroup (88% with chemotherapy vs. 77% with no chemotherapy; HR = 0.49; 95% CI, [0.25–0.95], and $P = 0.032$) and in the stage III subgroup (73% with chemotherapy vs. 44% with no chemotherapy; HR = 0.34; 95% CI, [0.22–0.51], and $P < 0.001$) (Figure 3). In the SAMHD1-high patient population, treatment with adjuvant chemotherapy was not associated with higher OS in either the stage II subgroup (chemotherapy vs. no chemotherapy; HR = 0.67; 95% CI, [0.19–2.35], and $P = 0.523$) or the stage III subgroup (chemotherapy vs. no chemotherapy, HR = 0.50; 95% CI, [0.12–1.99], and $P = 0.312$) (Figure 3).

Discussion

Using proteomics analysis, SAMHD1 was identified as a potential biomarker displaying a significant prognostic value. It was differentially expressed in the paired colorectal cancer groups with and without recurrence as selected by a nested case-control design from a large retrospective cohort. Using public colorectal cancer datasets for biomarker discovery, we illustrated that SAMHD1 had prognostic and predictive powers that could be helpful for patients with stage II colorectal cancer and had a predictive power in those with stage III colorectal cancer. Validation was performed using tissue microarrays on different cohorts of patients. Hence, SAMHD1 could complement MSI/MMR status as a molecular marker involved in the high-risk definition for patients with stage II colorectal cancer and help in making clinical decisions for adjuvant chemotherapy for patients with stages II and III colorectal cancer.

This study showed that the high expression of SAMHD1 in stage II colorectal cancer tissues was correlated with poor prognosis. We speculated that a higher rate of mutations may occur in patients with high expression of SAMHD1 resulting in disease progression because mutations in SAMHD1 that alter its dNTPase activity are associated with colon cancer (40). Moreover, a previous study reported that SAMHD1 upregulation was found in the colorectal cancer tissue of the patients with advanced colorectal cancer compared to their normal counterparts (41). Additionally, the role of SAMHD1 in numerous types of cancer, such as chronic lymphocytic leukemia, lung cancer, and colorectal cancer, has been extensively studied (42). Moreover, the high expression level of SAMHD1 had an independent significant association with unfavorable OS in some types of cancer (37, 43, 44). Hence, the expression level of SAMHD1 could be a prognostic biomarker for stage II colorectal cancer.

This study is the first to demonstrate that SAMHD1 is a predictive biomarker for adjuvant chemotherapy in patients with stage II and III colorectal cancer. Several studies have reported that high expression of SAMHD1 negatively impacts the efficacy of nucleoside-based chemotherapies in different cohorts of patients with leukemia (36, 37, 43, 45–47). The negative role of SAMHD1 in the sensitivity to chemotherapy can be attributed to various reasons. SAMHD1 is a dNTPase that hydrolyzes dNTPs into deoxyribonucleosides (dNs) and triphosphates (48). It has been identified as a restriction factor that blocks infection by a broad range of retroviruses, including HIV-1, in noncycling myeloid-lineage cells and quiescent CD4+ T lymphocytes (49–54). Owing to its dNTPase activity, SAMHD1

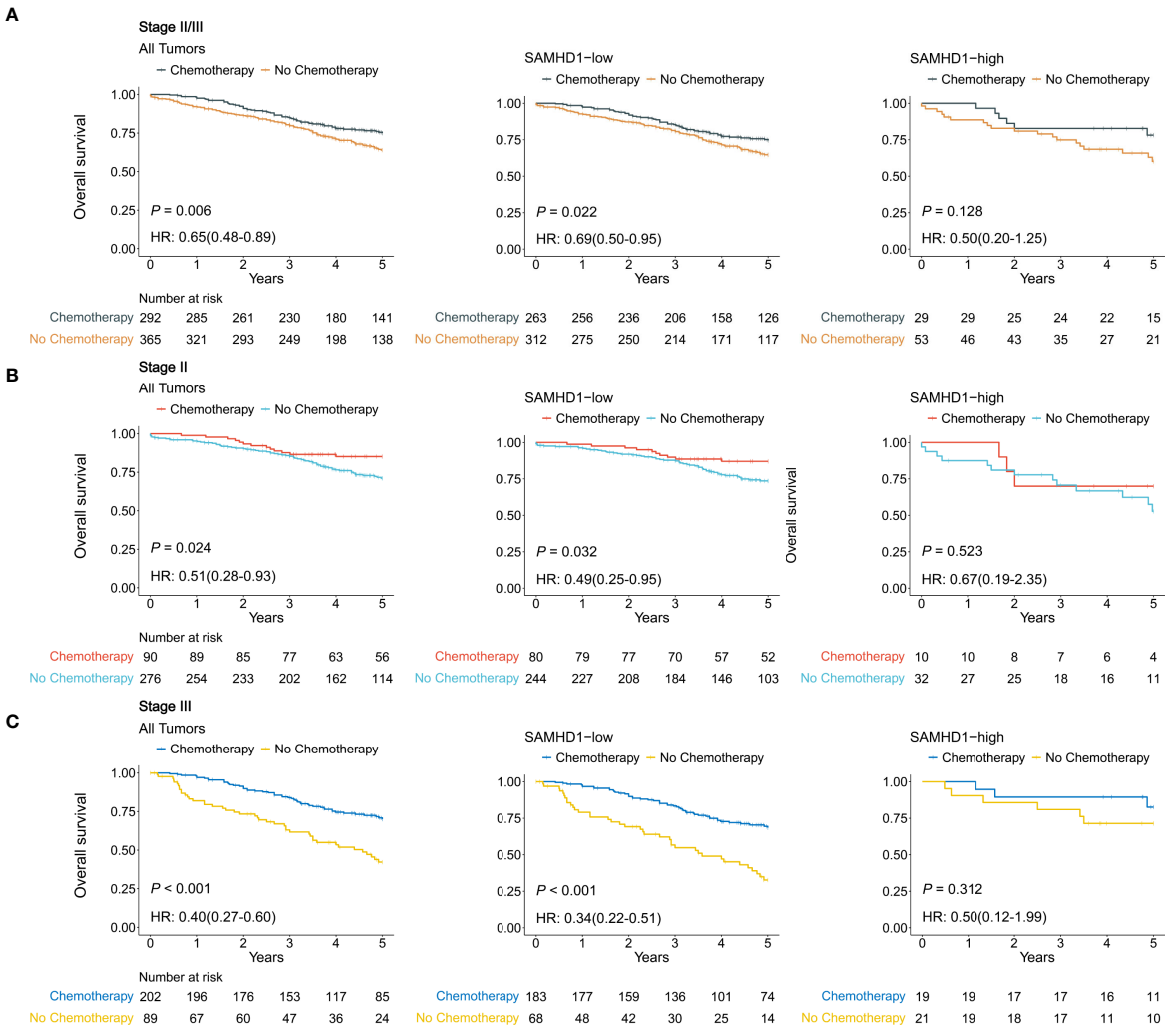

FIGURE 3
Relationship between SAMHD1 expression and the benefit from adjuvant chemotherapy using Gene Expression Omnibus data. **(A)** Patients with stage II and stage III disease. **(B)** Patients with stage II disease. **(C)** Patients with stage III disease.

can degrade the analog cytarabine triphosphate and reduce its concentrations in cells, such as the patient-derived acute myeloid leukemia blasts, thereby posing a significant barrier to the effective analog cytarabine-based treatment (45). However, SAMHD1 can hydrolyze several active triphosphate (TP) nucleoside analogs used for anti-cancer therapies (47). Therefore, evaluation of the expression levels of SAMHD1 in patients with stages II and III colorectal cancer before adjuvant chemotherapy is warranted.

SAMHD1 could complement MSI/MMR status as a promising molecular marker, leading to more accurate treatment decisions in patients with stage II colorectal cancer. The MSI/MMR status of the tumor is the only molecular marker involved in adjuvant chemotherapy decisions for stage II colorectal cancer (11). However, the MSI/MMR rate is 10%–15%, while 20% of patients with stage II colorectal cancer experience relapse after surgery (3). This leads to the emerging need to identify novel biomarkers for the effective treatment of colorectal cancer. Our results show that SAMHD1 expression only partially overlaps with tumors defined by the MSI/MMR status. In this study, high expression of SAMHD1 was approximately 12% and conferred poor prognosis and less benefits from adjuvant chemotherapy for stage II disease in both the discovery and validation datasets. We will further assess the prognostic and predictive value of SAMHD1 using immunohistochemistry in a prospective multicenter cohort before clinical practice.

The major strength of our study is its nested case-control design combined with proteomics. The nested case-control design is an efficient method to identify novel prognostic biomarkers using the available, large sets of clinical data storing biological samples and taking both feasibility and economic factors into account (55). We

identified 21 proteins associated with the prognosis of patients with colorectal cancer by using quantitative proteomics in a nested case-control cohort within a large cohort of patients with colorectal cancer. Among these 21 proteins, five proteins showed a promising role as potential biomarkers for the identification of high-risk populations and chemo-sensitive patients with stage II colorectal cancer. Therefore, further studies are required to validate these results.

Each omics discipline has its own advantages and disadvantages, and can give information about many aspects of disease from transcriptomics signatures to proteomic profiles. By comparison, colorectal cancer-related protein-coding genes have little overlap with known cancer genes, this is one of the advantages of proteomics over other omics (29, 56). It is logical therefore to examine this extensive information in parallel with the aim of revealing those attributes that can be considered robust and sensitive enough to work as a biomarker of patient risk (57).

While the results are promising, this study has several limitations. Firstly, this study was lack of immunohistochemical validation of SAMHD1 due to the retrospective design, we could not obtain effective FFPE specimen from many patients because of the long storage time. We will further validate these results using the prospective, multicenter clinical trials. Secondly, since there were few stage III patients in our cohort, we do get lose the predictivity in stage III patients, the specific reasons are not clear, and further research is needed in the future. Thirdly, we did not perform more detailed analysis about adjuvant chemotherapy regimens due to the lack of specific treatment information in public datasets, so SAMHD1 should be a predictive parameter for a group of drugs, and much more clinical data should be available until SAMHD1 could be an add-on to clinical practice.

In conclusion, our research showed that SAMHD1 can effectively stratify patients with stage II colorectal cancer into subgroups with good and poor prognosis, thereby complementing the prognostic value of the MSI/MMR status that is used to evaluate the prognosis of these patients. Moreover, our results showed that the expression levels of SAMHD1 can identify stages II and III patients who could benefit from adjuvant chemotherapy. Thus, SAMHD1 may potentially be used as an easy and useful tool in clinical practice to develop more accurate treatment decisions for patients with stages II and III colorectal cancer.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Ethics statement

The studies involving human participants were reviewed and approved by Yunnan Cancer Hospital Ethics Committee (No. KY2019141). The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

DY, SZ, and SY helped design the statistical approach, performed all the analyses, interpreted the results, and drafted the manuscript. YD, XC, LW, and WW helped with the collection of patient-related data. CL helped with the statistical methodology. TZ, ZL, and YH conceptualized and led the study, helped design the study and interpret the results, and revised and submitted the manuscript. All authors 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.

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Recent advances in the diagnostic and therapeutic roles of microRNAs in colorectal cancer progression and metastasis

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Colorectal cancer (CRC) is the third most common malignancy in the world and one of the leading causes of cancer death; its incidence is still increasing in most countries. The early diagnostic accuracy of CRC is low, and the metastasis rate is high, resulting in a low survival rate of advanced patients. MicroRNAs (miRNAs) are a small class of noncoding RNAs that can inhibit mRNA translation and trigger mRNA degradation, and can affect a variety of cellular and molecular targets. Numerous studies have shown that miRNAs are related to tumour progression, immune system activity, anticancer drug resistance, and the tumour microenvironment. Dysregulation of miRNAs occurs in a variety of malignancies, including CRC. In this review, we summarize the recent research progress of miRNAs, their roles in tumour progression and metastasis, and their clinical value as potential biomarkers or therapeutic targets for CRC. Furthermore, we combined the roles of miRNAs in tumorigenesis and development with the therapeutic strategies of CRC patients, which will provide new ideas for the diagnosis and treatment of CRC.

KEYWORDS

colon cancer, microRNAs, progression, metastasis, therapeutic strategies

Abbreviations: CRC, colorectal cancer; 3'-UTR, 3'-untranslated regions; VEGF, vascular endothelial growth factor; PGE2, prostaglandin E2; EMT, epithelial-mesenchymal transition; MET, mesenchymal-to-epithelial transition; RASA1RASp21, protein activator 1; PGRN, progranulin; PD-1, programmed death-1; KEGGKyoto, Encyclopedia of Genes and Genomes; PD-L1, Programmed death ligand 1; MSCs, mesenchymal stem cells; TME, tumor microenvironment; TAMs, tumor-associated macrophages; 5-Fu, 5-Fluorouracil; OXA, Oxaliplatin; OAZ2O, rnithine Decarboxylase Antizyme 2; MTX, methotrexate; MSS, microsatellite stability; MSI, microsatellite instability; MSI-H, microsatellite instability-high; MMR, mismatch repair; lncRNAs, long non-coding RNAs; ncRNAs, non-coding RNAs; ICI, simmune checkpoint inhibitors; mRNAs, messenger RNAs; circRNAs, circleRNAs; PTCs, patient-derived tumor-like cell clusters.

Introduction

Colorectal cancer(CRC) is the third leading cause of cancer deaths in humans, with an overall incidence of approximately 5% and a 5-year survival rate of 40% to 60% (1). In 2018, there were approximately 1.8 million new CRC cases and 860,000 deaths. It is estimated that by 2040, the global CRC burden will increase by 72% to more than 3 million new cases, which will pose a serious threat to human health (2). Environmental and genetic factors play an important role in the pathogenesis of CRC (3). Dietary habits, smoking, low levels of physical activity, population ageing, and obesity are also factors that affect the pathogenesis of CRC (3). In recent years, although some progress has been made in the screening and treatment of CRC, the overall survival rate of patients with advanced stage disease remains low. Because the symptoms of CRC patients are not obvious in the early stage, and the prognosis is poor when it develops to the advanced stage, early detection and treatment are particularly important.

MicroRNAs are a group of single-stranded small noncoding RNAs of 21-23 nucleotides (nt) in length. They were first discovered and reported in 1993 (4), and an increasing number of studies have focused on the regulatory role of microRNAs since then. MicroRNAs play important roles in biological and pathological processes such as metabolism, apoptosis, differentiation, cell proliferation, cell cycle, invasion and metastasis, and are closely related to the occurrence and development of tumours. They regulate the expression of their target genes post transcriptionally and they may be involved in various physiological and pathological processes, including CRC metastasis, by affecting various factors in the human body (5).

Recent studies have shown that dysregulated microRNAs play an important role in the development and metastasis of CRC, and the abnormal expression of microRNAs may act as potential oncogenes or suppressors in the development of tumours. Disordered microRNAs may have carcinogenic or tumour suppressor functions, and can regulate some oncogenes and tumour suppressor genes. Similarly, they are also regulated by oncogenes and tumour suppressor genes (6). Studies have shown that alterations in the Wnt/β-catenin, EGFR, TGF β and TP53 signalling pathways can affect CRC survival, proliferation and metastasis, and specific miRNAs can lead to changes in these signalling pathways, thereby promoting or inhibiting tumorigenesis (7). The same microRNAs may act as a tumour promoter in one cancer and a tumour suppressor in another, so there is no need to study the role of the same microRNAs in different cancers. For example, miR-146a may have a carcinogenic effect in thyroid cancer and a tumour inhibitory effect in CRC (8).

As microRNAs could be used for the diagnosis and prognostic monitoring of CRC, their high tissue specificity and role in tumorigenesis make them novel biomarkers for diagnosing cancer and predicting patient outcomes (9). Meanwhile, due to the role of abnormal expression of microRNAs in tumour development and the therapeutic response, correcting miRNA deficiency or restoring miRNA function may be a new cancer treatment strategy.

In addition, the association of microRNAs with tumour angiogenesis, cell proliferation, metastasis, and apoptosis suggests that the related microRNAs may serve as potential targets for CRC therapy (10). This article reviews the roles of microRNAs in the occurrence, development and metastasis of CRC and provides new ideas for the diagnosis and treatment of CRC.

Colorectal cancer

CRC is one of the most common gastrointestinal malignancies, the incidence of CRC in young adults is rapidly increasing (11). Patient survival is closely related to tumour stage at diagnosis, with approximately 50% of patients dying from distant metastases (12). The diagnosis of CRC is generally based on the evaluation of symptoms or screening. However, because CRC has no obvious symptoms in the early stage, most tumours have already metastasized at the time of diagnosis.

The treatment of CRC includes primary tumour resection, radiotherapy, chemotherapy, targeted therapy, immunotherapy and so on. Despite advances in surgery and adjuvant therapy, cure rates and long-term survival have barely changed over the past few decades (13). Decreased chemotherapy sensitivity remains a major obstacle preventing effective treatment of advanced disease. The development of cancer resistance to chemotherapy also often leads to treatment failure. Although there are targeted therapies for CRC, there are still relatively few ways to improve survival (14). Therefore, we need to clarify the mechanism of tumour progression and find new therapeutic targets. CRC patients are still at risk of recurrence after surgical removal of the tumour. Routine surveillance of postoperative patients to detect recurrence during the early asymptomatic period is one of the ways to improve survival (15).

MicroRNAS

MicroRNAs are the most abundant small RNAs in animals and play a key role in the regulation of gene expression. They are involved in mRNA degradation by binding to the 3'-untranslated region (3'-UTR) and play important roles in cell differentiation, development, cell cycle regulation and apoptosis (6). It is estimated that microRNAs can regulate up to 30% of protein-coding genes in the human genome (16). Most microRNAs are detected in the cellular microenvironment, but circulating microRNAs or extracellular microRNAs can be detected in extracellular environments such as biological fluids. Circulating microRNAs exist as proteins or lipoprotein

complexes in exosomes, microvesicles, apoptotic bodies, Argonaut protein complexes, and high-density lipoprotein complexes (17). These molecules are transported to recipient cells and regulate various physiological and pathological processes (18).

MicroRNAs are involved in the development and progression of cancer. Under specific conditions, microRNAs can act as both tumour promoters and tumour suppressors. Dysfunctional microRNAs can affect tumour progression, including maintaining proliferative signals, escaping growth inhibitors, resisting cell death, activating invasion and metastasis, and inducing angiogenesis (19). In recent years, an increasing number of studies have shown that microRNAs are not only potential biomarkers for CRC diagnosis and prognosis, but also potential therapeutic targets, and have broad application prospects in clinical diagnosis and treatment.

The role of microRNAs in tumour progression

Angiogenesis

Angiogenesis, the process of growing new blood vessels from venules of the existing capillaries, is an important step in tumour cell proliferation and metastasis (20). Studies have found that microRNAs can regulate all stages of angiogenesis (21). Approximately 33 different microRNA families have been reported to play a role in angiogenesis (22).

Zeng et al. (23) found that miR-25-3p secreted by CRC can be transferred to vascular endothelial cells through exosomes, destroy the integrity of the endothelial barrier, induce angiogenesis, and promote CRC metastasis. MTDH is a target gene of miR-375 in CRC. Han et al. (24) proved that the expression level of MTDH is negatively correlated with the expression of miR-375 in CRC. Inhibition of miR-375 expression in CRC can regulate cell proliferation and angiogenesis by increasing the expression of MTDH. Meanwhile, overexpression of miR-218 can significantly inhibit angiogenesis (25). In addition, miR-17~92 can inhibit CRC progression by inhibiting angio-genesis in tumours (26). Hu et al. (27) showed that exomiR-1229 has a positive effect on angiogenesis by activating the vascular endothelial growth factor (VEGF) pathway and may be a therapeutic target for inhibiting tumour angiogenesis. The recent findings of He et al. (28) revealed that miR-21-5p secreted by CRC cells is a key switch for cancer-induced angiogenesis and vascular permeability, and may also serve as a new target for cancer therapy. Moreover, hypoxia is closely related to angiogenesis. Targeting hypoxia-related microRNAs, such as miR-145, can inhibit CRC metastasis and may also help control tumour metastasis (29). In conclusion, the pathogenesis of cancer is related to the imbalance of angiogenesis, and miRNAs can regulate the related pathways of angiogenesis. Therefore, they are expected to become potential therapeutic targets for CRC.

Premetastatic niche formation

The primary tumour creates a favourable microenvironment for subsequent metastasis in the secondary organs and tissues, that is, the premetastatic niche. The premetastatic niche can increase angiogenesis and vascular permeability, thereby promoting metastasis (30). Therefore, analysis of the molecular and cellular components of the premetastatic niche in blood may contribute to the diagnosis and prognosis of cancer metastasis. The study by Shao et al. (31) showed that during the development of CRC, miR-21 secreted by primary CRC cells is phagocytosed by macrophages in the liver, thereby forming a premetastatic niche in the liver, and circulating CRC cells can settle there and survive. A recent study demonstrated that upregulated miR-135a-5p plays a key role in CRC liver metastasis by promoting the formation of a premetastatic niche through dual regulation of immunosuppression and cell adhesion (32). Furthermore, circulating tumour-derived exosomal miR-203 can promote distant metastasis by inducing host M2 macrophages to form a premetastatic niche (33). Exosomal miR-25-3p is also involved in the formation of the premetastatic niche and may serve as a blood-derived biomarker for CRC metastasis (23). These studies show that miRNAs can participate in the formation of the premetastatic niche and promote CRC metastasis. Quantitative blood detection of the level of relevant miRNAs in circulating exosomes may be helpful for the diagnosis of CRC metastasis and the preventive treatment of high-risk metastatic patients.

Cell proliferation and metastasis

Immortal proliferation of CRC cells is the basis of cancer development. MicroRNAs play an important role in the process of cell proliferation. Previous studies have shown that many microRNAs can affect the proliferation of CRC cells in different ways. For example, Huang et al. (34) found that upregulation of miR-17 could promote CRC proliferation. In contrast, miR-22 can inhibit the proliferation of CRC cells and slow the growth rate of tumours (35). In prostaglandin E2 (PGE2)-induced tumour cells, overexpression of miR-206 can reduce the proliferation of CRC cells (36), which may be a potential therapeutic target for PGE2-induced CRC cells. In addition, upregulation of miR-1258 and miR-500a-5p both inhibited tumour cell proliferation by blocking the cell cycle in G0/G1 (37, 38).

MicroRNAs can control multiple aspects of epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET) and support tumour progression and metastasis (39) (Figure 1). Exosomes from tumour cells can

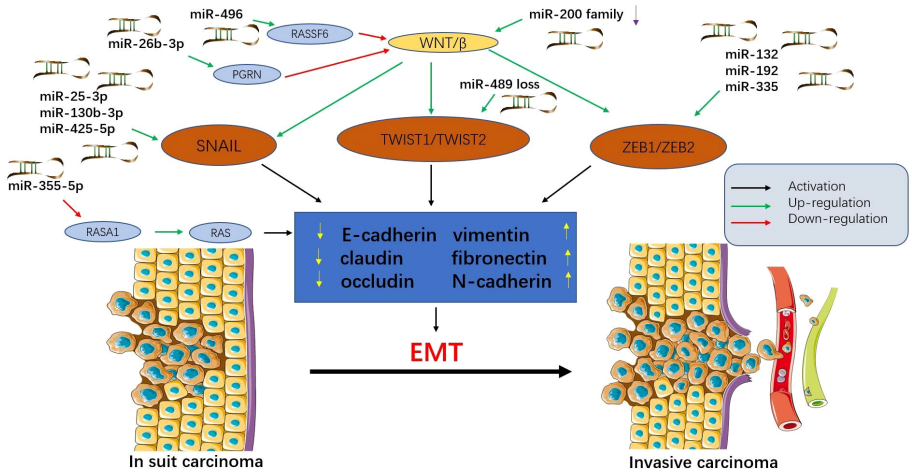

FIGURE 1
Epithelial-mesenchymal transition (EMT) is regulated by microRNAs in colorectal cancer (CRC). MicroRNAs affect multiple signalling pathways and participate in EMT by decreasing epithelial markers (e-cadherin, claudin, and occludin) and increasing interstitial markers (vimentin, fibronectin, and N-cadherin). During this process, epithelial cells acquire mesenchymal phenotypes, which play an important role in the progression and metastasis of CRC. Tumour cells that have undergone EMT can invade the local stroma and enter the vasculature, travel in the circulation, and finally establish a secondary tumour at a distant site. This figure summarizes some microRNAs involved in the EMT process in CRC.

transfer miRNAs to normal cells, stimulating carcinogenesis and promoting metastasis (40). Exosomes promote EMT by targeting RASp21 protein activator 1 (RASA1) to deliver miRNA-335-5p, thereby promoting CRC cell invasion and metastasis (41). In addition, miR-29b-3p can directly target progranulin (PGRN) to alter the downstream Wnt signalling pathway and promote EMT (42). The miR-496/RASSF6 axis can also promote EMT and CRC migration through Wnt signalling (43). The study by Wang et al. (44) showed that miR-25-3p, miR-130b-3p, and miR-425-5p can induce tumour cell proliferation and metastasis, and may be potential therapeutic targets for blocking CRC metastasis.

In addition to cancer-promoting microRNAs, there are also cancer-suppressing microRNAs. Upregulation of miR-200c can inhibit EMT, thereby inhibiting tumour progression (45). Furthermore, the expression of miR-382-5p was significantly down-regulated in CRC tissues and cell lines. Upregulation of miR-382-5p expression can target NR2F2 and PD-L1, thereby inhibiting CRC cell proliferation and metastasis (46, 47). Przygodzka et al. (48) reported that miR-192 and miR-194 can inhibit snail-induced EMT and metastasis. Therefore, prevention of EMT may be a promising approach to block CRC metastasis. In a word, under normal physiological conditions, miRNAs can maintain the normal regulation of some cellular processes, and their abnormality will lead to abnormal growth and biosynthesis of cells, thus promoting or inhibiting the spread and metastasis of tumors.

Apoptosis

Apoptosis is a programmed death process that occurs during normal cell development and senescence. Chemotherapy forces cancer cells to undergo apoptosis by causing DNA damage or cell damage. Abnormal apoptosis is one of the pathogenic mechanisms of CRC and plays a role in the resistance to chemotherapeutic drugs and radiotherapy (49). MicroRNAs play an important role in tumour cell apoptosis and drug resistance. Activation of the caspase family of proteases is the main pathway for inducing apoptosis (50). MiR-433 can increase the expression of caspase-3 and caspase-9, thereby promoting apoptosis (51). Overexpression of miR-218 can also promote CRC cell apoptosis by increasing caspase-8 levels (52). In the Kyoto Encyclopedia of Genes and Genomes (KEGG) apoptosis pathway, miR-92a is associated with two apoptosis-related genes, CSF2RB and BCL2L1. Moreover, increased expression of miR-92a-3p in tumour tissue can improve patient survival time (53). Overexpression of miR-766 reduces CRC cell growth and induces apoptosis by inhibiting the MDM4/p53 pathway (54). MiR-27a-3p increases apoptosis through the ERK-MAPK pathway, while miR-422a induces apoptosis in CRC cells through the p38-MAPK pathway (55, 56). In contrast, mi-421 exerts an anti-apoptotic effect in CRC by downregulating caspase-3 (57). Therefore, the regulation of microRNAs will help to regulate the occurrence and development of CRC, promote cancer cell apoptosis, and alleviate drug resistance.

Immune system activity

Escape from immune system surveillance is an important link in tumorigenesis and development. Studies have shown that microRNAs may be involved in the immune escape process of CRC and are significantly associated with tumour survival. MicroRNAs may be involved in the differentiation of monocytes into M2 macrophages, which have been implicated in playing key roles in colon cancer (58, 59). Exosomes derived from M2 macrophages transfer miR-21-5p and miR-155-5p to CRC cells, promoting cell migration and invasion (60). The results of Ma et al. showed that M2 macrophage-derived exosomal miR-155-5p could promote immune escape by colon cancer, enhancing the progression of CRC (61). Studies have shown that miR-203-containing exosomes released by CRC cells can be internalized by monocytes, thereby promoting the expression of M2 markers (33). However, the PD-1/PD-L1 pathway, as an important immune checkpoint, is dysregulated in various human malignancies, including CRC, and is involved in tumorigenesis by inhibiting antitumor immune response. MiR-124 inhibits PD-L1 expression in CRC cells, which in turn promotes T-cell mediated anti-cancer responses (62). In conclusion, the interaction between miRNAs and immune checkpoints has great application prospects in the personalized treatment of CRC in the future.

Impact on the tumour microenvironment

Tumour growth and metastasis are highly dependent on the interaction between tumour and relevant microenvironment, and several miRNAs have been shown to play a key role in the interaction between tumour and tumour microenvironment (TME). In every step of tumour growth and metastasis, complex molecular interactions occur between cells in the tumour microenvironment, such as fibroblasts and immune-related cells (63). Cancer-associated fibroblasts (CAFs) affect tumour growth by regulating inflammation or direct cell-to-cell communication. Studies have shown that miRNA can alter chemokines secreted by fibroblasts to alter TME, thereby promoting migration and invasion (64). Tumour-derived microRNAs affect the matrix and immune cell components of the tumour microenvironment. In TME, miRNA is considered to be an important molecular mechanism for the interaction between tumour cells and immune cells. For example, miRNAs can control the production of chemokines or cytokines by tumour cells, which in turn affect the aggregation and expansion of immune cells (65). Tumour-associated macrophages (TAMs) are the key components of TME, and miRNAs play an important role in the regulation of TAMs on tumour progression. TAMs have been shown to be associated with a poor prognosis of CRC. TAMs can induce EMT in CRC cells by regulating the STAT3/miR-506-3p/FoxQ1 axis, thereby promoting metastasis (66). However, miR-195-5p could inhibit the polarization of M2-like TAMs, and patients with low miR-195-5p levels have significantly shorter overall survival times (67).

Mesenchymal stem cells (MSCs) are also an important part of the TME and play a key role in promoting tumour progression (68). In the TME, microRNAs generally have tumour-promoting effects and are an important direction for future cancer therapy. Although MSCs have some antitumor activity, microRNAs mediate immunosuppressive activity (69), which provides ideas for future cancer therapy. Intestinal microRNAs can influence the growth and composition of the intestinal microbiota (70). The pathogenesis of CRC is also associated with disorder in the microbiota, termed ecological disorder (71). Imbalances in microRNAs can affect the survival or gene expression of some beneficial bacteria in the microbiota. Dysfunctional microRNAs in tumour cells can be transmitted to stromal cells and immune cells, creating a more favourable microenvironment for tumour cells (72). Thus, microRNAs can modulate the microbiota, promoting the growth of beneficial bacteria and inhibiting the growth of cancer-causing bacteria. In short, the interaction between microRNAs and the TME may also be one of the entry points for antimetastatic treatment in the future.

The role of microRNAs in tumour tumourigenesis

MiRNAs also play an important role in the initiation of human cancer. MiRNAs are related to the pathogenesis of various types of human malignant tumours. In several types of cancer, the decreased expression of miR-34 and let-7 can trigger tumorigenesis, and the up-regulation of miR-34 and let-7 can lead to tumour growth inhibition (73). Moreover, there is ample evidence that miRNAs are closely related to the dysregulation of several key pathways in CRC. miR-31 is a potential driver of colon tumorigenesis by targeting EphB2 and EphA2 signalling pathways (74). Mamoori et al. (75) demonstrated that miR-21 expression was increased many times in colonic cancer stem cells compared to parental cells. Moreover, since the expression of miR-21 is increased, the expression level of PTEN in the colon bulb is decreased, and the Akt signalling pathway is activated, miR-21 is considered to play an important role in the tumorigenic regulation of colon cancer stem cells.

Inflammation also drives the steps of tumorigenesis. Jeffries et al. (76) found that miR-223 can regulate tumorigenesis at multiple levels, including by inhibiting the inflammatory tumour microenvironment and regulating the malignancy of cancer cells. And some studies have proved that the level of miR-223 can be used to predict the probability of CRC by sequencing

circulating exosomal miRNAs (77). MiRNAs can be used as therapeutic targets and prediction means, and are potential tools for cancer management and treatment in the future. However, more research is needed before they can be applied to clinic.

Clinical applications

Early diagnosis

Increasing evidence suggests that miRNAs can serve as non-invasive biomarkers for CRC diagnosis and prognosis (Figure 2). They exist in the bloodstream in a highly stable form by binding to specific proteins or vesicles (78, 79). Karimi et al. (80) showed that miR-23a and miR-301 were upregulated in patients compared with healthy individuals, which can be used to distinguish CRC patients from normal subjects. Zhu et al. (81) found that miR-19a-3p, miR-21-5p and miR-425-5p were significantly upregulated in CRC patients compared with healthy individuals. Cheng et al. (82) found that the circulating abundance of exocrine miR-146a correlated with high levels of CD66 neutrophils. However, the proportion of tumour-infiltrating TCD8 cells decreased. MiR-146a is the main miRNA in the exosomes of CRC stem cells and can be used as a diagnostic biomarker. In addition, both miR-486-5p and miR-18b-5p have potential for use as non-invasive biomarkers for the early diagnosis of CRC (83, 84). Min et al. (85) found that miR-92b was differentially expressed in CRC patients and healthy individuals but could not be used to differentiate between CRC and adenoma. Even so, it has promise as a minimally invasive tool for the early diagnosis of CRC. In addition, miR-21, miR-155, and miR-221, which are expressed differently in colon and rectal cancers, can be used to distinguish colon and rectal cancer (86). In addition, the levels of miR-17-5p and miR-92a-3p isolated from serum exosomes were found to correlate with the pathological stage and grade of patients with CRC (87).

Numerous studies have shown that microRNAs in serum, exocrine and even faeces have the potential for early diagnosis. Decreased expression of miR-4478 and miR-1295-p in stool specimens is a noninvasive and effective diagnostic marker for CRC patients, which can be detected at an early stage of CRC, suggesting that it may be a promising CRC screening approach (88). Moody et al. (89) found that miR-20a in the faeces of CRC patients also serves as a potential prognostic biomarker. Furthermore, stool miR-135b-5p is not only a potential biomarker but also an ideal candidate intervention strategy for CRC patients (90). The establishment of appropriate miRNA biomarkers is very important for the early diagnosis of CRC. Of course, prospective studies with larger patient cohorts are needed to confirm the diagnostic value of these microRNAs. Further efforts are required before microRNAs in faeces can be used clinically.

Treatment options for patients with CRC require accurate assessment of TNM staging. Therefore, biomarkers that can accurately predict preoperative TNM staging will significantly improve the treatment efficiency of CRC. Bjørnetrø et al. (91) found that low levels of miR-486-5p and miR-181a-5p were associated with locally advanced dis-ease and lymph node metastasis, while high levels of miR-30d-5p were associated with metastatic progression. Orosz et al. (86) also evaluated

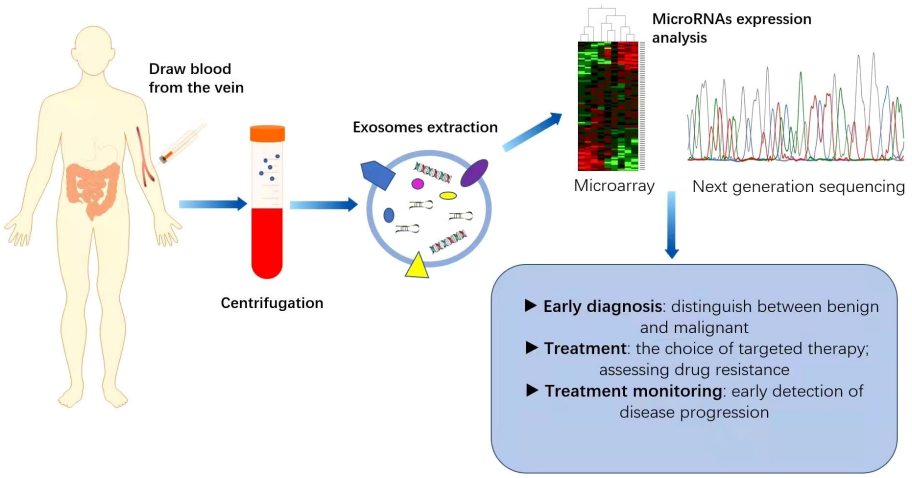

FIGURE 2
Application of miRNAs as biomarkers for colorectal cancer (CRC). MicroRNAs are generally expressed abnormally in CRC patients. Blood samples collected from CRC patients are used as a source of circulating exosomes and after their isolation, we can analyse the pattern of microRNA expression, which is helpful for the diagnosis and treatment of CRC. This can play an important role in the early diagnosis of cancer, identification of high-risk patients requiring intensive treatment, monitoring of drug efficacy and real-time monitoring of the effectiveness of treatment.

the potential of several microRNAs to distinguish individual TNM stages. The results showed that the expression levels of miR-155, miR-34a, and miR-29a in the serum of TNMII, III, and IV patients were downregulated.

Treatment of CRC

Drug resistance

Although good progress has been made in the systemic treatment of tumours in recent years, in addition to surgery, chemotherapy is still the main treatment for CRC. The resistance of cancer cells to chemotherapy is a major factor leading to chemotherapy failure, often resulting in a poor prognosis. Many studies have shown that there is a certain relationship between tumour drug resistance and microRNA imbalance. Tumour drug resistance can occur through a variety of mechanisms, including apoptosis inhibition (92). Studies have shown that ectopic expression of miR-520 g resists 5-FU-induced apoptosis by inhibiting the expression of p21 (6). Decreased levels of miR-125b-5p have also been shown to contribute to tumour cell metastasis and 5-FU chemotherapy resistance (93). Similarly, miR-22 and miR-206 can also promote apoptosis induced by 5-FU (94, 95). Recent studies have shown that the tumour suppressor miR-27b-3p can increase the sensitivity of CRC cells to 5-FU (96). Oxaliplatin (OXA) resistance is also a major obstacle to the treatment of advanced CRC. Li et al. (97) reported that miR-34a was significantly downregulated in OXA-resistant patients, which could reduce OXA resistance by targeting OAZ2. In addition, studies have shown that miR-128-3p can enhance tumour sensitivity to chemotherapy and may become a promising OXA chemotherapy marker (98). In contrast, mir-5000-3p, mir-135b-5p, and mir-208b were associated with decreased sensitivity to OXA chemotherapy (99–101). Recent studies have shown that miR-24-3p can enhance the resistance of CRC cells to methotrexate (MTX) (102).

The hypothesis that drug resistance is the result of tumour-host interactions has been proposed, suggesting new strategies for overcoming the development of cancer chemotherapy resistance (103). Studies have shown that miR-21 and 5-FU combined with engineered exosomes can effectively reverse the drug resistance of 5-FU-resistant colon cancer cells and improve therapeutic efficiency (104). More efforts are needed to prevent cancer cells from developing resistance to chemotherapy and to try to resensitize cancer cells to chemotherapy drugs (Table 1).

There are two different types of CRC: “microsatellite stability” (MSS) and “microsatellite instability” (MSI). Cancers of MSS and MSI types promote tumorigenesis and progression through two distinct molecular pathways (105). Microsatellite stability-high (MSI-H) is caused by functional defects in the DNA mismatch repair (MMR) system. MSI-HCRC immune checkpoint molecules, such as PD-1 and PD-L1, have been shown to be resistant to the antitumor immune response (106). MicroRNAs can play a role in cancer-related immune responses by targeting immunosuppressive or immunostimulatory factors. It has been proven that miR-140-3p, miR-382-3p, miR-148a-3p, miR-93-5p, miR-200a-3p, miR-200c-3p, miR-138-5p and miR-15b-5p can regulate immune escape by inhibiting tumour PD-L1. They can also transform the immunosuppressive tumour microenvironment into a proinflammatory tumour microenvironment, enhancing the chemosensitivity of tumour cells (107). Therefore, it may be possible to alleviate the drug resistance of MSI-H CRC by regulating microRNAs.

Long noncoding RNAs (lncRNAs) are noncoding RNAs (ncRNAs) and microRNAs. Studies have shown that lncRNAs, as precursors of microRNAs, are also associated with drug resistance. For example, the lncRNA MIR100HG, a precursor of miR-100 and miR-125b, can lead to cetuximab resistance (108). The lncRNA-XIST/miR-125b-2-3p axis can also induce chemoresistance in CRC, but the specific mechanism by which it affects chemosensitivity has not been elucidated (109). The complex feedback loop between lncRNAs and microRNAs may provide new perspectives for the reversal of CRC drug resistance. In contrast, the lncRNA-XIST/miR-137 axis can enhance CRC glycolysis and chemotherapy resistance, providing a possible alternative to improve chemotherapy efficacy in CRC patients (110).

Therapeutic target and microRNA therapy

The aberrant expression of microRNAs plays an important role in the development of cancer and the response to anticancer drugs. Correcting microRNA defects or restoring microRNA function can be used as a new cancer treatment strategy. MicroRNAs have been proven to be therapeutic targets for CRC (111). For example, miR-135b has been shown to be upregulated in CRC and associated with tumour progression and a poor clinical prognosis. Therefore, tumour growth can be inhibited by reducing miR-135b. Studies have shown that blocking exocrine miR-25-3p in CRC can reduce the vascular permeability and metastasis of CRC, suggesting that miR-25-3p can be used as a therapeutic target for interfering with CRC metastasis (23).

With the development of high-throughput sequencing technology, the interaction of the gene expression network system comprised of messenger RNAs (mRNAs), miRNAs, lncRNAs and circular RNAs (circRNAs) in CRC progression has been discovered. It has been proven that lncRNA-miRNA cross-talk is a novel mechanism affecting CRC cell proliferation, invasion and metastasis (112). For example, lncRNA TUG1 can promote the growth and migration of CRC cells by secreting miR-145-5p, and the TUG1/miR-145-5p/TRPC6 pathway can serve as a target for CRC diagnosis and therapy (113). Liu et al. showed that the circIFT80/hsa-miR-370-3p/WNT7B signalling

TABLE 1 Several microRNAs are involved in CRC resistance.

microRNA	Effect on drug resistance	Type of drug	Target(s)	Reference
miR-520g	Inhibit	5-FU	P21	(6)
miR-125b-5p	Inhibit	5-FU	Sp1, CD248	(93)
miR-22	Inhibit	5-FU	HDACs	(94)
miR-206	Inhibit	5-FU	Bcl-2	(95)
miR-34a	Inhibit	OXA	OAZ2	(97)
miR-128-3p	Inhibit	OXA	Bmi1,MRP5	(98)
miR-5000-3p	Promote	OXA	USP49	(99)
miR-135b-5p	Promote	OXA	MUL1	(100)
miR-208b	Promote	OXA	PDCD4	(101)
miR-24-3p	Promote	MTX	CDX2	(102)

axis might also play a role in carcinogenesis (114). CircIFT80 inhibits the expression of hsa-miR-370-3p in CRC cell lines, thereby inhibiting apoptosis. Therefore, in addition to research on microRNAs, research on lncRNAs and circRNAs may also provide new ideas for the targeted therapy of CRC.

In recent years, the application of immune checkpoint inhibitors (ICIs), especially anti-PD-1 therapy, has greatly improved the efficiency of tumour treatment. However, the role of ICIs in CRC is generally limited to MSI-H tumours. The latest study by Liu et al. (115) found that miR-15b-5p downregulated the expression of PD-L1 at the protein level, inhibited tumorigenesis, and improved the sensitivity to anti-PD-1 therapy. Elevating the level of miR-15b-5p can improve the sensitivity of MSS CRC patients to ICI treatment. Blocking oncogenic microRNAs may adversely affect the physiological functions regulated by these microRNAs, thus requiring specific sites or cellular targets to avoid potential adverse effects. At the same time, extensive clinical trials are needed to evaluate the efficacy and safety of microRNAs as therapeutic targets in patients.

Despite advances in the application of immune checkpoint blockade therapy in malignancies, CRC patients usually only benefit if they have tumours with mismatch repair deletions or severe mutations in MSI-H (116). However, most tumours are MSS, so immunotherapy has a low response rate in treating CRC. Many studies have shown that microRNAs can modulate immune responses, and some of these microRNAs can inhibit the progression of CRC and are expected to be effective antineoplastic drugs. Since the disorder of microRNAs was first discovered in cancer, it has been studied extensively and uncovered new therapeutic possibilities. MiRNAs can regulate multiple signalling pathways of the immune system and have the advantage of multiple targets (117). Previous studies have shown that restoration of miR-34 expression can reduce the proliferative potential of CRC cells; thus, miR-34 can be used as a therapeutic drug (118). In addition, miR-34 can also increase tumour sensitivity to 5-Fu, thereby reversing drug resistance (119). Unfortunately, the therapeutic application of microRNAs is limited by technical barriers. MicroRNA molecules are unstable and are rapidly cleared from the blood, with only a small fraction absorbed by cells (120).

In some studies, exosomes have been used as transporters for microRNA drugs, and the lipid bilayer membrane of exosomes can protect exosomes from being degraded during blood circulation. Han et al. (121) used CBMSC-derived exosomes to infiltrate anti-miRNA-221 into solid tumours and significantly inhibited tumour growth. As a tumour suppressor microRNA, miR-124 can regulate several oncogenes and signalling pathways closely related to tumour growth and promote T-cell dependent immune responses. The study by Rezaei et al. (1) used CT-26-derived exosomes as a natural vehicle for miR-124-3p delivery, which elicited potent antitumor immune responses and reduced tumour growth. In the future, the response rate of immunotherapy may be significantly improved by increasing the technology of exosomes carrying microRNAs. However, the source of exosomes is limited and lacks targeting, there are still many challenges in future applications, and further research is needed. In addition, the efficacy and safety of microRNA therapy in patients need to be studied.

Prognosis of colon cancer

Predicting recurrence

Approximately one-third of patients with CRC undergoing radical surgery will experience disease recurrence (13). Studies have shown that miRNAs can be used as biomarkers for predicting CRC recurrence, which is beneficial to the prognosis of CRC patients. The serum levels of exocrine miR-1229, miR-1224-5p, miR-223, let-7a, miR-150 and miR-21 in CRC patients were significantly increased, and then they decreased after resection (122). Plasma miR21-5p could be used to predict recurrence and disease progression after surgical resection (123). Studies have shown that serum exocrine miR-21 could be used to predict CRC recurrence and a poor outcome in TNM stage II, III, or IV (124). In addition,

postoperative plasma miR-31, miR-141, and miR-16 have also been shown to be biomarkers of disease recurrence after surgical resection (125). In general, for patients with stage II CRC, surgical resection of the primary tumour is effective and may not require other treatment, but whether adjuvant chemotherapy should be used in patients with stage II CRC remains controversial (111). Yamazaki et al. (126) proposed that high expression of miR-181c plays a role in predicting recurrence of stage II CRC. Through the study of microRNAs, it is possible to assess which postoperative patients with stage II CRC may benefit from adjuvant therapy (Table 2).

Aberrant expression of microRNAs as biomarkers may contribute to individualized treatment of patients. A study by D'Angelo et al. (128) showed that miR-194 was a potential predictive biomarker of chemotherapy response. Meanwhile, other studies have found that miR-33a-5p, miR-21, miR-99b, and miR-375 can predict clinical response and outcomes in patients treated with radiotherapy and chemotherapy (127, 129). Yin et al. (130) established an *in vitro* tumour model called patient-derived tumour-like cell clusters (PTCs), which has been shown to be useful for assessing tumour sensitivity to drugs. By incorporating microRNAs as markers into this predictive model, real-time efficacy monitoring can be achieved to assess the benefit of chemotherapy or targeted therapy.

Metastasis of colon cancer

Approximately 50% of CRCs will metastasize in the advanced stage of malignant tumours, and distant metastasis is the main cause of death of CRC patients (Figure 3). Early detection and treatment of distant metastasis is of great significance to improve the long-term survival of CRC patients. MicroRNAs are significantly associated with tumour metastasis. Several microRNAs, including members of the miR-34 and miR-200 families, have been found to target the mRNAs of EMT transcription factors, such as ZEB1, ZEB2, and SNAIL (131). Downregulation of these microRNAs is associated with distant metastasis and advanced tumours. The liver is the most common metastatic site of CRC. The study by Hur et al. (132) showed that elevated serum miR-203 levels are closely associated with liver and systemic metastasis. Teng et al. (133) detected significantly elevated plasma miR-193a levels in CRC patients with liver metastasis. Lan et al. (60) found that miR-21-5p and miR-155-5p were transferred to CRC cells *via* exosomes and were key factors in promoting CRC metastasis. Preventing such messages may be a new strategy to suppress CRC metastasis. These microRNAs can be used as biomarkers to determine prognosis and predict distant metastasis. MiR-181a is significantly upregulated in CRC tissues of patients with liver metastases and promotes tumour cell growth and proliferation, which is closely associated with distant metastasis and poor survival (134). In contrast, miR-802 is negatively correlated with lymphatic and distant metastasis of CRC (135), and may be a regulatory target for suppressing metastasis.

Induction of muscular dystrophy

Cachexia is a complex metabolic and behavioural syndrome associated with underlying disease and is characterized by loss of skeletal muscle. Previous studies have found a significant correlation between skeletal muscle mass and circulating miR-21 expression in CRC patients, suggesting that assessment of serum miR-21 levels can be used to assess the risk of sarcopenia and cancer cachexia in patients with CRC (136). The results of Miao et al. (137) suggest that abundant microRNAs in tumour exosomes may induce muscle atrophy mainly by targeting Bcl-2-mediated apoptosis. In addition, the detection of serum miR-203 expression can be used to evaluate the risk of sarcopenia, and miR-203 may be a new therapeutic target for inhibiting sarcopenia in patients with CRC (138).

Conclusion and perspectives

MicroRNAs have a wide range of biological functions and are involved in many physiological and pathological processes, including cancer. An increasing number of studies have shown

TABLE 2 Examples of microRNAs associated with CRC prognosis.

microRNA	Clinical application	reference
miR21-5p	Predicting recurrence after surgical resection as well as disease progression	(123)
miR-21	Prediction of CRC recurrence and poor prognosis when stratified by TNM stage II, III or IV; Prediction of clinical response and outcome in patients treated with chemoradiotherapy	(124, 127)
miR-31	Biomarkers of disease recurrence after surgical resection	(125)
miR-141	Biomarkers of disease recurrence after surgical resection	(125)
miR-16	Biomarkers of disease recurrence after surgical resection	(125)
miR-181c	Prediction of CRC recurrence with TNM stage II	(126)
miR-194	Potential predictive biomarkers of chemotherapy response	(128)
miR-33a-5p	Predictive markers of chemotherapy efficacy	(129)
miR-99b	Prediction of clinical response and outcome in patients treated with chemoradiotherapy	(127)
miR-375	Prediction of clinical response and outcome in patients treated with chemoradiotherapy	(127)

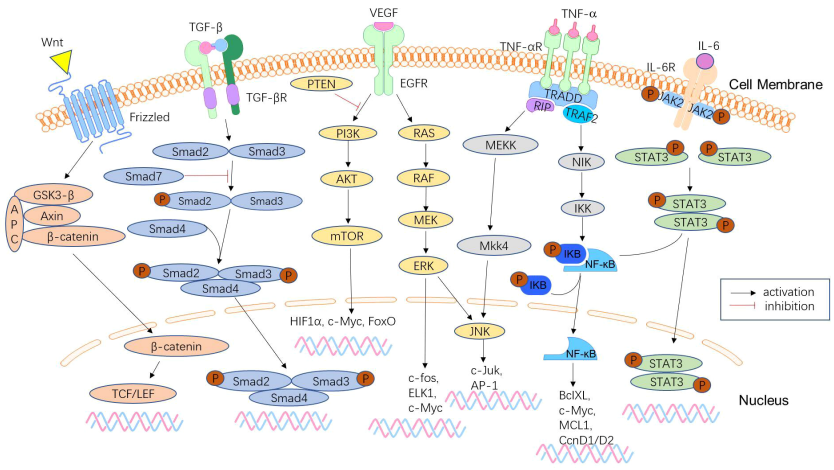

FIGURE 3
Signalling pathways involved in CRC metastasis. CRC metastasis is mediated by a complex network of signalling pathways, which include the Wnt/β-catenin signalling pathway, TGF-β/Smad pathway, phosphoinositide 3-kinase (PI3K)/phosphatase and tensin homologue (PTEN)/AKT pathway, KRAS-ERK signalling pathway, NF-κB signalling pathway, and JAK/STAT3 signalling pathway. These pathways lead to tumour anti-apoptosis, EMT, proliferation, and invasion.

that microRNAs play an important role in the progression and metastasis of CRC. Specific microRNAs can be used to overcome diagnostic and therapeutic challenges of different types of tumours. The combination of novel microRNA markers with traditional biomarkers may help to improve the specificity and sensitivity of detection. Using microRNAs as new therapeutic targets to correct maladjusted microRNAs would be a promising approach for CRC therapy. In future studies, we should determine which biological fluids and assays are most suitable for CRC screening and which microRNA combinations have the best diagnostic performance. We should maximize the specificity of these microRNA biomarkers. At the same time, we should increase our understanding of the role of microRNAs in the molecular pathogenesis and treatment of cancer. This will facilitate the clinical application of microRNAs.

At present, some progress has been made in the study of microRNAs reversing drug resistance, but there are still few studies on immunotherapy resistance in MSI-H CRC. In addition, the biggest problem facing microRNA therapy is the choice of carrier. Nanoparticles or exosomes are used as carriers in the current studies. Both of these carriers have certain limitations, and more research is needed to overcome these difficulties and allow for their application in clinical practice. The roles and functions of individual microRNAs in CRC remain unclear and more research is needed. Investigating the effects of microRNAs on the occurrence, development and metastasis of CRC is of great significance for the diagnosis and treatment of CRC.

lncRNAs, circRNAs and microRNAs are all ncRNAs and have great potential in clinical applications. Accumulating evidence suggests that a complex regulatory net-work exists between lncRNAs, circRNAs, and microRNAs. They have great biological potential and may regulate CRC initiation, progression and metastasis. However, the exact mechanisms of how these interactions affect tumorigenesis and progression have not been fully revealed. Future analysis of different RNA molecules with potential crosstalk may provide new insights into the diagnosis and treatment of CRC, contributing to the improvement of biomarker prediction and the development of new treatments.

Author contributions

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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A novel DNA methylation marker to identify lymph node metastasis of colorectal cancer

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Lymph node metastasis (LNM) of colorectal cancer (CRC) is an important factor for both prognosis and treatment. Given the deficiencies of conventional tests, we aim to discover novel DNA methylation markers to efficiently identify LNM status of CRC. In this study, genome-wide methylation sequencing was performed in a cohort (n=30) using fresh CRC tissue to discover differentially methylated markers. These markers were subsequently validated with fluorescence quantitative PCR in a cohort (n=221), and the optimal marker was compared to conventional diagnostic methods. Meanwhile, immunohistochemistry was used to verify the effectiveness of the antibody corresponding to this marker in a cohort (n=56). *LBX2* achieved an AUC of 0.87, specificity of 87.3%, sensitivity of 75.7%, and accuracy of 81.9%, which outperformed conventional methods including imaging (CT, PET-CT) with an AUC of 0.52, CA199 with an AUC of 0.58, CEA with an AUC of 0.56. *LBX2* was also superior to clinicopathological indicators including the depth of tumor invasion and lymphatic invasion with an AUC of 0.61and 0.63 respectively. Moreover, the AUC of *LBX2* antibody was 0.84, which was also better than these conventional methods. In conclusion, A novel methylation marker *LBX2* could be used as a simple, cost-effective, and reliable diagnostic method for LNM of CRC.

KEYWORDS

colorectal cancer, lymph node metastasis, DNA methylation marker, immunohistochemistry, diagnostic method

Abbreviations: LNM, Lymph node metastasis; CRC, colorectal cancer; IHC, Immunohistochemistry; NCCN, National Comprehensive Cancer Network; CT, Computed tomography; PET-CT, Positron emission tomography-computed tomography; CEA, Carcinoembryonic antigen; CA199, Carbohydrate antigen 199; FF, Fresh frozen; FFPE, Paraffin-embedded; qPCR, Quantitative PCR; LVI, Lymphatic vessel invasion; BVI, Blood vessel invasion;NI, Neural invasion; CI, Confidence interval; OR, Odds ratio; ROC, Receiver operating characteristic (ROC); PPV, Positive predictive value; NPV, Negative predictive value; DMR, Differential methylation region.

Introduction

Colorectal cancer (CRC) is the third most common cancer in the world. Until now, the incidence and mortality rate have increased to the third and the second among all cancers. However, lymph node metastasis (LNM) is the main cause of the increasing mortality in CRC (1). According to National Comprehensive Cancer Network (NCCN) guidelines on the treatment of CRC, surgical operation is still the preferred treatment for CRC, meanwhile, lymph node dissection is recommended whenever there is an opportunity to remove the tumor (2). Although lymph node dissection could reduce the recurrence of CRC, the patients without LNM could not benefit from lymph node dissection, but it could bring many complications such as postoperative intestinal adhesion, intestinal obstruction, lymphatic leakage, sexual dysfunction, and postoperative bleeding, which lead to excessive medical treatment (3).

Currently, the clinical diagnosis of LNM of CRC mainly relies on imaging including computed tomography (CT) and positron emission tomography-computed tomography (PET-CT), or clinicopathological characteristics including depth of tumor invasion, ulceration, lymphatic vascular invasion, etc. (4, 5). In addition, some clinical serological indicators such as carcinoembryonic antigen (CEA) and carbohydrate antigen 199 (CA199) could also be used as a basis for LNM of CRC (6). However, the accuracy and reliability of these methods is not ideal. This may be the primary reason why the NCCN guidelines on surgical treatment of CRC recommend lymph node dissection, despite its potential for postoperative complications.

DNA methylation is one of the important epigenetic modifications. It has been proved that abnormal DNA methylation is related to cancer. During tumorigenesis, changes in DNA methylation patterns may be easily detected, thus tumor-related methylation markers have more accurate and direct effects on cancer diagnosis (7). So far, many studies on DNA methylation of CRC are based on early diagnosis and prognosis. In terms of early diagnosis, methylation of the promoter of *RASSF1A* (8), methylation of the CpG of *Caveolin-1* (9), hypomethylation of transcription suppressor *HES1* (10), hypomethylation of histone lysine methyltransferase encoding gene *SMYD3* (11) are proved to be associated with CRC. In terms of prognosis assessment, methylation of the promoter of *CDX2* is an independent indicator of prognosis of CRC (12), and methylation of the promoter of RAI2 is a poor indicator of prognosis of CRC (13).

Our previous work has demonstrated that the methylation markers of *KCNJ12*, *VAV3-AS1*, and *EVC* could be used as the basis for stage and stratification of CRC, with an area under curve (AUC) of 0.87, sensitivity of 83.0%, and specificity of 71.2% (14). Currently, it is common to take CRC tissue under colonoscopy for preoperative diagnosis in clinical practice. By obtaining CRC tissue samples, this study aims to identify LNM status of CRC by discovering novel DNA methylation markers, which could be used for the formulation of clinical treatment plans and prognosis evaluation of CRC.

Methods

Study design and patient recruitment

In this study, a three-phase strategy was designed (Figure 1), which included a marker discovery cohort (n = 30, fresh frozen (FF) tissue samples) and a marker validation cohort (n =221, FF and formalin-fixed paraffin-embedded (FFPE) tissue samples). The proportion of tumor in all tissue samples was more than 60%, which was obtained by two qualified pathologists on

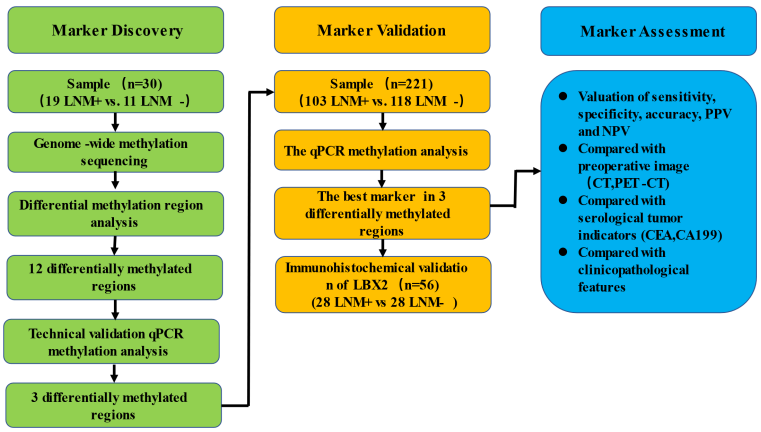

FIGURE 1
Schematic workflow of the study design.

observing paraffin sections with high-power microscopy. Genome-wide methylation sequencing was performed on 30 FF tissue samples from the marker discovery cohort to identify LNM-specific methylation markers. These methylation markers were verified by fluorescence real-time quantitative PCR (qPCR) from the marker validation cohort (n=221). The optimal methylation markers were selected to compare with imaging (CT and PET-CT), serological indicators (CEA and CA199) and clinicopathological characteristics in a validation cohort. All CRC patients were recruited from Zhujiang Hospital, Southern Medical University. CRC samples (FF, n=76; FFPE, n=182) were derived from January 2017 to March 2020. Samples with less than twelve lymph nodes (15) and failed DNA quality control (n=37) were excluded from the study. Tissue sample of CRC patients was tumor surgical specimens before radiotherapy or chemotherapy. And these samples corresponding to pathological reports and LNM status were confirmed by at least two gastrointestinal pathologists. The clinicopathological characteristics containing age, gender, depth of invasion (t-stage of TMN), tumor size, lymphatic vessel invasion (LVI), blood vessel invasion (BVI), neural invasion (NI) and ulceration were shown in Table 1.

Discovery of methylation markers

30 FF tissue samples of CRC (19 LNM+, 11 LNM-)were collected to identify differential methylation markers. Next, we independently constructed a genome-wide methylation library using TruSeq® Methyl Capture EPIC Library Prep Kit (Illumina, USA, Catalog No. Fc-151-1002). After EPIC library was quality-assured with Agilent High-Sensitivity DNA Kit (Agilent, USA, Catalog No. 5067-4626), high-throughput sequencing was performed on Illumina X-TEN platform.

DNA extraction, bisulfite treatment

Genomic DNA was extracted from FF tissue samples and FFPE tissue samples with AllPrep DNA/RNA Mini Kit (Qiagen, Germany, Catalog No. 80204) and AllPrep DNA/RNA FFPE Kit (Qiagen, Germany, Catalog No. 80234). Subsequently, the extracted DNA was quantified by the qubit dsDNA Customs Assay Facility (Thermal Fisher Science, USA, Catalog No.Q32851). The quality controlled criteria of CRC samples

TABLE 1 Characteristics of CRC patients in the validation cohorts.

Characteristics		LNM-	LNM+	P value
		n = 118 (%)	n = 103 (%)	
Age				0.700
	<55	25 (21.2)	26 (25.2)	
	≥55	93 (78.8)	77 (74.8)	
Gender				0.910
Depth of invasion (t-stage)	Male	69 (58.5)	42 (40.8)	0.003
	Female	49 (41.5)	61 (59.2)	
	T1	4 (3.40)	1 (1.00)	
	T2	19 (16.1)	8 (7.80)	
	T3	57 (48.3)	43 (41.7)	
	T4	38 (32.2)	51 (49.5)	
Tumor size (cm)				0.203
	<5	59 (50.0)	62 (60.2)	
	≥5	59 (50.0)	41 (39.8)	
Lymphatic vessel invasion				<0.001
	Yes	14 (11.9)	39 (37.9)	
	No	104 (88.1)	64 (62.1)	
Blood vessel invasion				<0.001
	Yes	17 (14.4)	38 (36.9)	
	No	101 (85.6)	65 (63.1)	
Neural invasion				0.003
	Yes	34 (28.8)	50 (48.5)	
	No	84 (71.2)	53 (51.5)	
Ulceration				0.361
	Yes	65 (55.1)	63 (61.2)	
	No	53 (44.9)	40 (39.8)	

LNM+, samples of CRC patients with lymph node metastasis; LNM–, samples of CRC patients without lymph node metastasis.

required that DNA content was more than 100 nanograms and the main band of agarose gel electrophoresis exceeded 500 bps. 50 nanograms of genomic DNA was taken from each sample, and EZ-96-DNA Methylation Direct MagPrep Kit (Zymo Research, USA, Catalog No. D5044) was used for bisulfite treatment of DNA.

Methylation analysis by fluorescence qPCR

The primer and probe sequences of the selected genes were designed through the biological software Beacon Designer V8.14. Fluorescence qPCR was used for methylation analysis in a validation cohort (n=221,103 LNM+ and 118 LNM-) (16). qPCR methylation analysis was performed on the Quant Studio 3 Real-Time PCR System (Thermo Fisher, USA). Based on our previous study (14), *ACTB* was selected as an internal reference gene. △Ct value obtained by fluorescence qPCR was used to indicate the methylation level of the target gene (△Ct= Ct value of the target gene - Ct value of the reference gene). If the Ct value is not present, the Ct value was set to 40.

Immunohistochemical analysis

Immunohistochemistry (IHC) was subsequently performed on the optimal genes validated by fluorescence qPCR. A total of 56 CRC paraffin sections (28 LNM+, 28 LNM-) were colllected for immunohistochemical analysis. First, the 2μm thick paraffin sections were roasted at 65°C for 1 hour, dewaxed with xylene and 100% ethanol, repaired with citrate buffer solution (PH 6.0) for 3 minutes under high pressure, and incubated with 3% H202 for 10 minutes. Next, the paraffin sections were sealed with goat serum for 30 minutes, and the primary antibody *LBX2* (Bioss, Beijing, China) was diluted at 1:100 and incubated in a metal bath at 37°C for 1 hour. After washing with phosphate buffered saline PBS (PH 7.6), enzyme-labeled sheep anti-mouse/rabbit IgG polymer (Second Antibody, GeneTech, Shanghai, China) was selected to incubate at 37°C for 30 minutes. Peroxidase activity was cultured with 3, 3-diaminobenzidine hydrochloride (DAB) in sterile H2O2 solution for 2 minutes. Finally, nuclear re-staining was performed with Mayer hematoxylin solution. All the slices were independently examined by two observers. The positive composite score was used in this study, which was the staining intensity multiply the percentage of positive cells. The staining intensity is classified into four levels. No staining was rated 0 point, light yellow was rated 1 point, pale brown was rated 2 points, brown was rated 3 points (Figures 3A-D). In addition, the percentage of positive cells was evaluated as 0 points for 0 ~ 5%, 1 point for 6% ~ 25%, 2 points for 26% ~ 50%, 3 points for 51% ~ 75% and 4 points for >75%.

Comparison of DNA methylation markers and clinicopathological features, imaging, serological indicators

Eight clinicopathological variables were included in univariate analysis to explore their correlation with LNM. Variables with P value less than 0.05 were included in a multivariate analysis. Stepwise regression was used to assess 95% confidence interval (CI) of odds ratio (OR) values to identify independent predictors. DNA methylation marker was compared to the selected clinicopathological indicators, imaging and serological indicators (CEA and CA199) by the area under the receiver operating characteristic (ROC) curve including the specificity, sensitivity, accuracy, positive predictive value (PPV), and negative predictive value (NPV).

Statistical analysis

R package DSS(2.0.16) of ComplexHeatmap and Corrplot were used for unsupervised hierarchical clustering and correlation analysis, pROC (1.16.1) was used for ROC, AUC and AUC confidence interval calculations, ggplot2 (3.2.1) and RColorBrewer (1.1.2) were used for visualization of figures. Differences between 2 groups were analyzed with the unpaired Student's t test (2-tailed tests), and 1-way ANOVA followed by Dunnett's multiple comparisons tests when more than 2 groups were compared. Pearson's $\chi 2$ test was used to analyze the clinical variables on sensitivity and specificity. Univariate and multivariate logistic regressions were used to evaluate the clinicopathological variables. Comparison of AUC values were conducted by Hanley and McNeil tests or DeLong test, when appropriate. The AUC values, sensitivity, specificity, and accuracy of methylation maker *LBX2*, clinicopathological features, serologic tumor markers and image in detecting LNM of CRC were used for comparison. A p value < 0.05 on two sides of all hypothesis tests were considered statistically significant.

Results

Genome–wide screening of DNA methylation markers to detect LNM in CRC tissue samples

A schematic workflow of the study design is shown in Figure 1. To identify LNM-specific DNA methylation markers in CRC, we

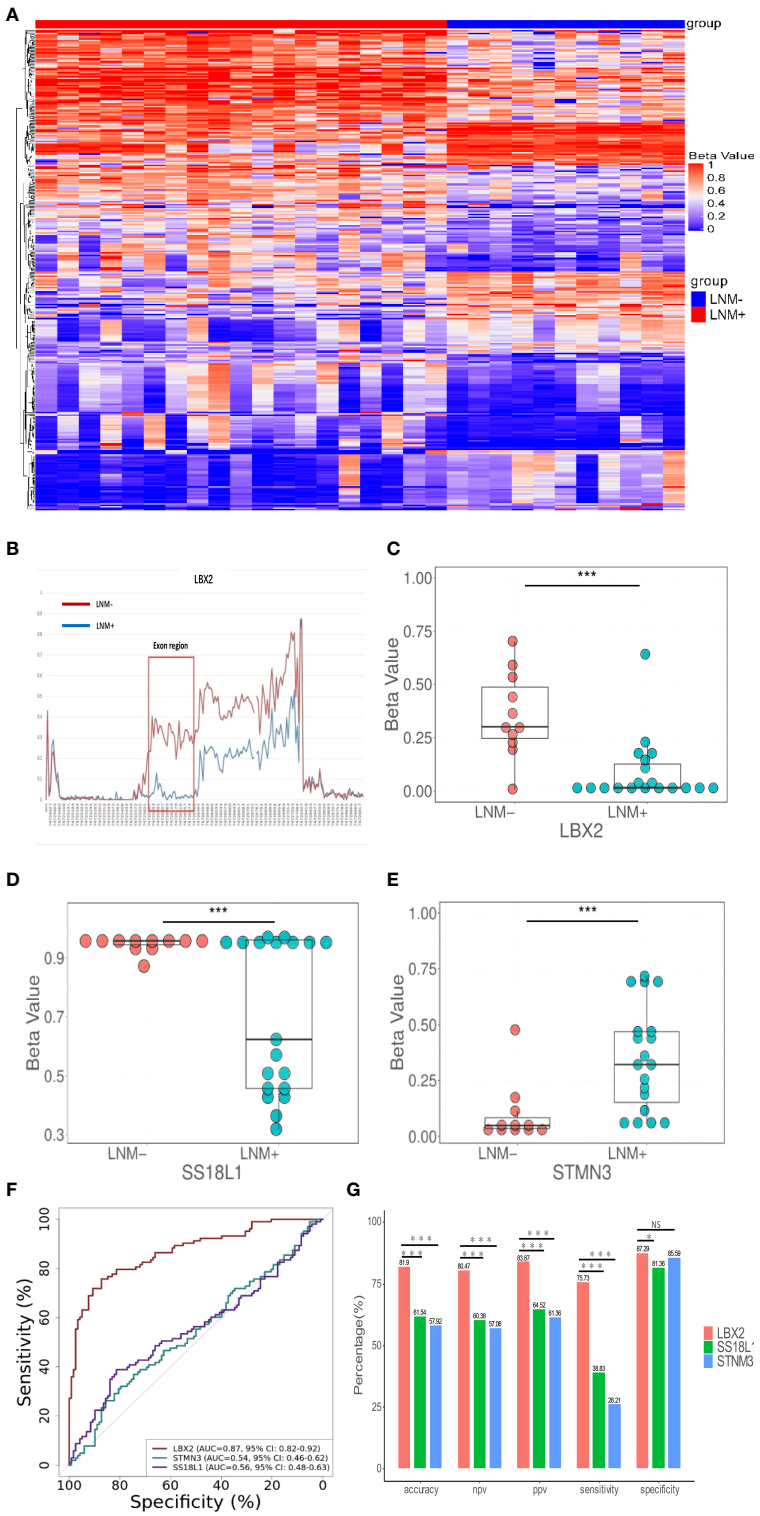

FIGURE 2
The discovery of DNA methylation markers to detect LNM in CRC tissue. **(A)** In the discovery cohort, an unsupervised hierarchical cluster analysis was based on differential methylation between LNM+(n=19) and LNM-(n=11), with each column representing a patient and each row representing a CpG marker. **(B)** Methylation levels of exon region of *LBX2* in LNM- and LNM+ groups in CRC **(C-E)**. The methylation level distribution of *LBX2, STMN3*, and *SS18L1* between LNM+(n=19) and LNM-(n=11) was represented by the β value from genome-wide methylation sequencing in the discovery cohort. **(F)** ROC curve of three methylation markers. The effectiveness of three DNA methylation methods was evaluated by comparing AUC values. **(G)** The accuracy, NPV, PPV, sensitivity and specificity of three methylation marker were compared respectively. *p < 0.05, **p < 0.01 and ***p < 0.001. NS, not statistically significant.

TABLE 2 Designed primer and probe sequences of target genes.

Gene	Forward primer 5'→3'	Reverse primer 5'→3'	Probe 5'→3'
LBX2	CGTTTAGTGTTGCGTTAAGGGTTT	AAAATCGAATCTTTCCGAATAACCAAA	TCCGCTCCAAACCACTCTCTTCTCGAAA
STMN3	TATCGTTTTGGGTTTATTACGGTTATCG	AACGTAAAACGCGATCCCTCG	ACAAACACCAAACCGAACGCGACTAAATCC
SS18L1	GGTTTTGAGCGTCGTTTATATGTTTT	CGAACAACATAACGCATCTATATATAAAAC	AAACCACGACACACCCTCTACTTCCTCAAA

first performed genome-wide methylation analysis containing 3.34 million CpG sites on fresh tissue samples from LNM+ group (n=19) and LNM- group (n=11). A total of 734 CpG sites with differential methylation were found ($p<0.001$, β value difference ≥0.15). The unsupervised heretical clustering showed that LNM+ and LNM- were clearly distinguished by majority of specific DNA methylation markers (Figure 2A). Based on these methylation sites, we further analyzed the differential methylation region (DMR) status and screened out twelve markers with DMR status. There were three hypomethylation markers including *LBX2, SS18L1, CYTH2* in LNM + group, meanwhile, there were nine hypermethylation markers including *ACHE*、*RPS15*、*APC2*、*BAHCC1*、*LEFTY1*、*RTN4RL2*、*KCNQ1*、*STMN3*、*LINC01072* in LNM + group.

Our primary goal was to develop a simple methylation-specific qPCR to detect LNM status (16). These twelve markers were further verified by qPCR in a cohort (n=65) (Supplemental material: Figure S2). However, nine markers were excluded due to low AUC value and inconsistent methylated patterns. Only three markers including *LBX2, STMN3, SS18L1* showed higher AUC values and consistent methylated patterns in sequencing and methylation-specific qPCR analysis, and significantly differentiated LNM+ from LNM- in the same samples (Figures 2C–E). In addition, we found exons of *LBX2* included significant methylation differences between LNM+ and LNM- (Figure 2B). To sum up, these results indicated that these three methylation markers and qPCR-based analysis were reliable and could be used for large-scale cohort analysis.

LBX2 had the best test performance in three target methylation makers

Through the biological software Beacon Designer V8.14, the information about three target genes (*LBX2, STMN3, SS18L1*) was input to set appropriate conditions. The primer and probe sequence were shown in Table 2. Based on validation of 221 tissue samples, *LBX2* achieved an AUC of 0.87 (95%CI 0.82-0.92, $p<0.001$), specificity of 87.3%, sensitivity of 75.7%, accuracy of 81.9%. *STMN3* achieved an AUC of 0.54 (95%CI 0.46-0.61, $p=0.30$), specificity of 85.6%, sensitivity of 26.2%, and accuracy of 57.9%. *SS18L1* achieved an AUC of 0.56 (95%CI 0.48-0.63, $p=0.15$), specificity of 81.4%, sensitivity of 38.8%, and accuracy of 61.5%. The comparison of these three methylation markers was shown in Figure 2F, G. Obviously, *LBX2* had higher efficiency in accuracy, specificity, sensitivity and AUC compared to other two methylation markers.

LBX2 antibody could identify LNM status by IHC

IHC of *LBX2* antibody was performed on CRC tissue section. These sections were amplified by 400 times, the staining of cancer cells was mainly observed. LNM- tissue sections (Figure 3E) showed light staining of cancer cells. However, LNM+ tissue section (Figure 3F) showed deep staining of cancer cells, especially in the nucleus where there was dark brown, presenting strong positive. In addition, the immunohistochemical score of LNM+ group was significantly higher than that of LNM- group ($p<0.001$) (Figure 3G). Comparing IHC with qPCR in a cohort (LNM+, n=28 and LNM-,n=28), the AUC of IHC was 0.84 (95%CI 0.74-0.94, $p<0.001$), but qPCR achieved an AUC of 0.93 (95%CI 0.89-0.97, $p< 0.001$) (Figure 3H). Moreover, the specificity, sensitivity and accuracy of the two methods were compared. The specificity, sensitivity and accuracy of immunohistochemical method were 92.9%, 64.3% and 78.6%, respectively. However, the qPCR method achieved a specificity of 100%, sensitivity of 82.1% and accuracy of 91.1% (Figure 3I). Obviously, both methods had good discrimination efficiency, despite the qPCR method was better than IHC.

LBX2 showed stable table discriminative efficacy in different classification

DNA methylation marker *LBX2* could identify LNM of CRC well in both male and female populations ($P < 0.001$) (Figure 4A). Similarly, *LBX2* had good discrimination effect in the group under 55 years old or the group over 55 years old ($P < 0.001$) (Figure 4B). *LBX2* could identify LNM of CRC well at T3 and T4 stages ($P < 0.001$) and T2 stage ($P < 0.05$). However, the strength of evidence was weak due to the small sample size at T1 stage (Figure 4C). *LBX2* also could identify LNM of CRC in tumor diameter less than 5cm and tumor diameter more than 5cm ($P < 0.001$) (Figure 4D). In different clinicopathological groups, *LBX2* could identify LNM of CRC well in both ulcerated and non-ulcerated groups, both lymphatic and non- lymphatic invasion groups, both vascular and non-vascular invasion

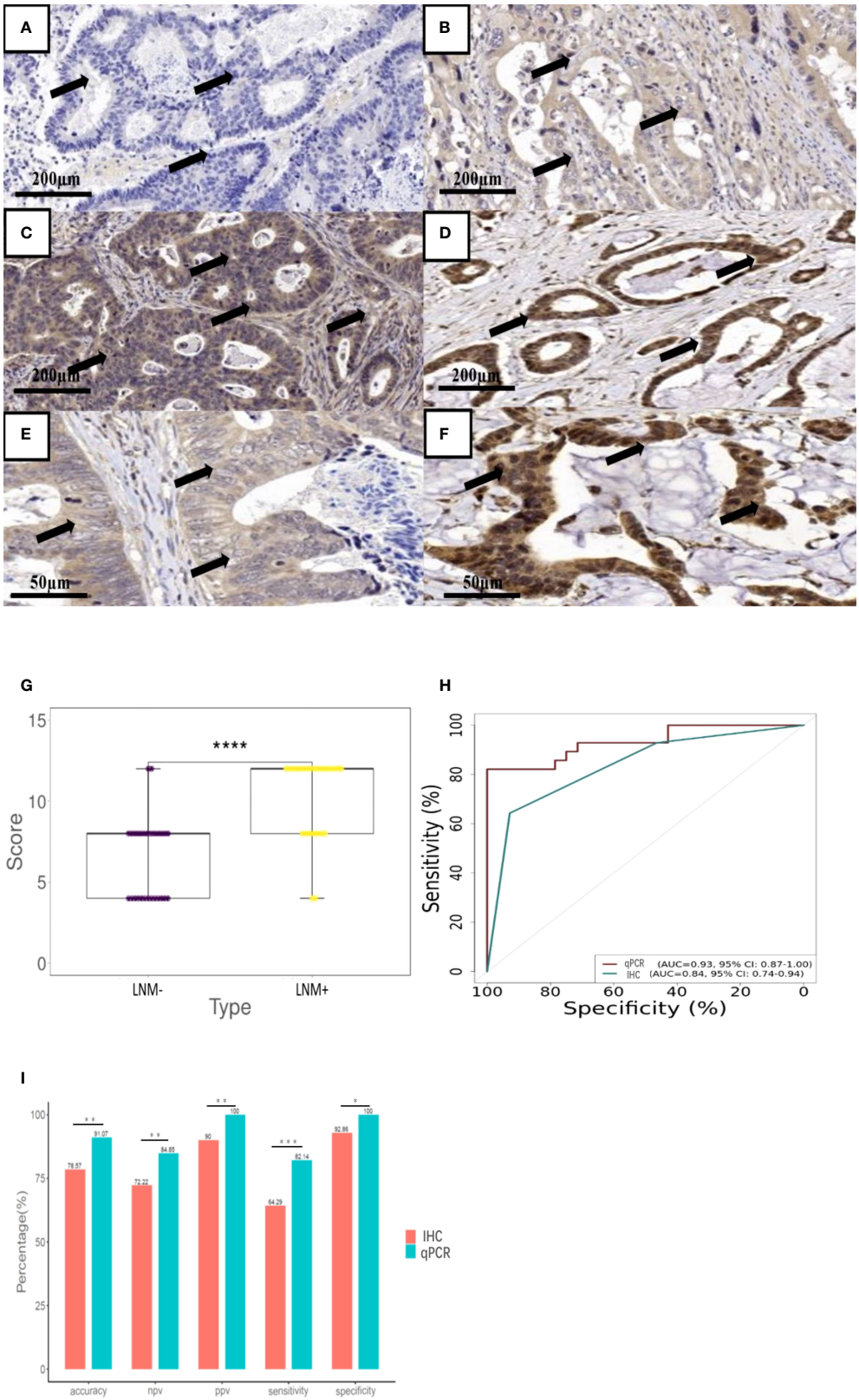

FIGURE 3 (Continued)

FIGURE 3 (Continued)
The performance of antibody *LBX2* to detect LNM in CRC tissue. **(A–D)** Immunohistochemical staining depth corresponding to the score. Stained samples (1:100 diluted concentration & 100X Magnification) were divided into four grades. (**A** 0=non-staining **B** 1=light yellow, **C** 2=pale brown **D** 3=brown). **(E)** Faint yellow stained samples (1:100 diluted concentration & 400X Magnification) was considered as LNM- CRC. **(F)** Brown staining samples (1:100 diluted concentration & 400X Magnification) was considered as LNM+ CRC. **(G)** The level of *LBX2* antibody was compared between LNM+(n=28) and LNM-(n=28). **(H)** ROC curve of two methods (IHC&. qPCR). The effectiveness of these two methods was evaluated by comparing AUC values. **(I)** The accuracy, NPV, PPV, sensitivity and specificity of two methods (IHC&. qPCR) were compared respectively. *p < 0.05, **p < 0.01, ***p < 0.001 and ****p<0.0001.

groups, and both neural and non-neural invasion groups (P < 0.001) (Figures 4E-H). In conclusion, DNA methylation marker *LBX2* had stable performance in different groups of each factor.

LBX2 was superior to clinicopathological features in distinguishing LNM

The relation between clinicopathological features and LNM status was further analyzed. Clinicopathological features included gender, age, depth of tumor invasion (t-stage of TMN), tumor size (demarcated by 5cm), ulcerative, LVI, BVI, and NI. In univariate analysis, there were four factors associated with LNM, including t-stage (OR 1.78, 95% CI 1.23-2.63, p<0.01), LVI (OR 4.52,95%CI 2.33-9.63,p<0.001), BVI (OR 3.47,95%CI 1.84-6.80, p<0.001), NI (OR 2.33,95%CI 1.34-4.09, p<0.01). (Table 3); Taking these four factors into account in multifactorial analysis, only LVI (OR 6.41,95%CI 1.31-47.66, p<0.05) and t-stage (OR 1.71,95%CI 1.15-2.61, p<0.01) were related to LNM. Therefore, among these eight clinicopathological features, LVI and t-stage were closely associated with LNM. Next, compared to LVI and t-stage, the *LBX2* achieved an AUC of 0.87 (95%CI 0.82-0.92, p<0.001), accuracy of 81.9%, sensitivity of 75.7%, and specificity of 87.3%. Turning to other two clinicopathological features, the LVI achieved an AUC of 0.63 (95%CI 0.57-0.69, p<0.001), accuracy of 64.7%, sensitivity of 37.9%, and specificity of 88.1%. while the t-stage achieved an AUC of 0.61(95%CI 0.54-0.67, p<0.01), accuracy of 59.3%, sensitivity of 49.55%, and specificity of 67.8% were 59.3%, 49.5%, and 67.8%. Compared to *LBX2*, *LBX2* was clearly superior to clinicopathological features (Figures 5A, B).

LBX2 was superior to CEA, CA199, imaging in distinguishing LNM

The relation between CA199, CEA, imaging and LNM status was analyzed. The AUC of CA199 was 0.58 (95%CI 0.51-0.66), with the specificity of 49.1%, the sensitivity of 70.6%, and the accuracy of 59.3%. Moreover, the AUC of CEA was 0.56 (95%CI 0.48-0.64), with the specificity of 43.4%, the sensitivity of 71.8% and the accuracy of 56.9%. In addition, the AUC of imaging (CT and PET-CT) was 0.52 (95%CI 0.45-0.59), with the specificity of 46.2%, the sensitivity of 58.2%, and the accuracy of 51.8%. Compared to these three conventional methods, the AUC of *LBX2* was 0.87 (95%CI 0.82-0.92, p<0.001),with the specificity of 87.3%, the sensitivity of 75.7%, and the accuracy of 81.9%, which was better than these current clinical examination (Figures 5C, D).

Discussion

DNA methylation profiles may represent relatively stable long-term programming of the genome and underlying cellular functions, which is a reliable method of the diagnosis of cancer occurrence and progression (17). Therefore, in this study, genome-wide methylation sequencing on CRC tissues (n=30) was performed and three LNM related specific methylation markers were selected. These three methylation markers were further validated by a large retrospective cohort of 221 tissue samples. We found that a qPCR-based methylated marker *LBX2* had the best discriminative performance for the diagnosis of LNM, which was superior to traditional clinicopathological features, as well as imaging, CEA, and CA199. *LBX2* also had stable discriminative efficacy in different groups including age, sex, tumor size, depth of tumor invasion and clinicopathological feature. At the same time, the antibody corresponding to *LBX2* also showed good performance in differentiating LNM of CRC in immunohistochemical validation. In addition, a more comprehensive approach was used to analyze CRC-associated LNM differential methylation sites, which covered more than 3.34 million CpG sites, accounting for 97.3% of the CpG islands in the genome. To date, few studies have used such a wide range of genome-wide methylation strategies to discover methylation markers for the diagnosis of LNM in CRC.

In previous similar studies, Tsuyoshi Ozawa et al. used microRNA sequencing data from the Cancer Genome Atlas (TCGA) and analyzed a five microRNAs model (*MIR32, MIR181B, MIR193B, MIR195, and MIR411*) that could distinguish LNM in t1-t2 CRC. This verified model achieved an AUC of 0.74 (18), lower than that of the single DNA methylation marker *LBX2* (AUC 0.87) in this study. Moreover, Ailin Qu et al. found a four microRNAs model (*Mir-122-5p, Mir-146B-5p, Mir-186-5p and Mir-193a-5p*) related to LNM status of CRC from high-throughput sequencing data of CRC tissues (n=20), and it showed that the AUC of the four microRNAs model was 0.88 through a verification cohort (n=198) (19), which was similar to the detection efficiency of DNA methylation sites in our

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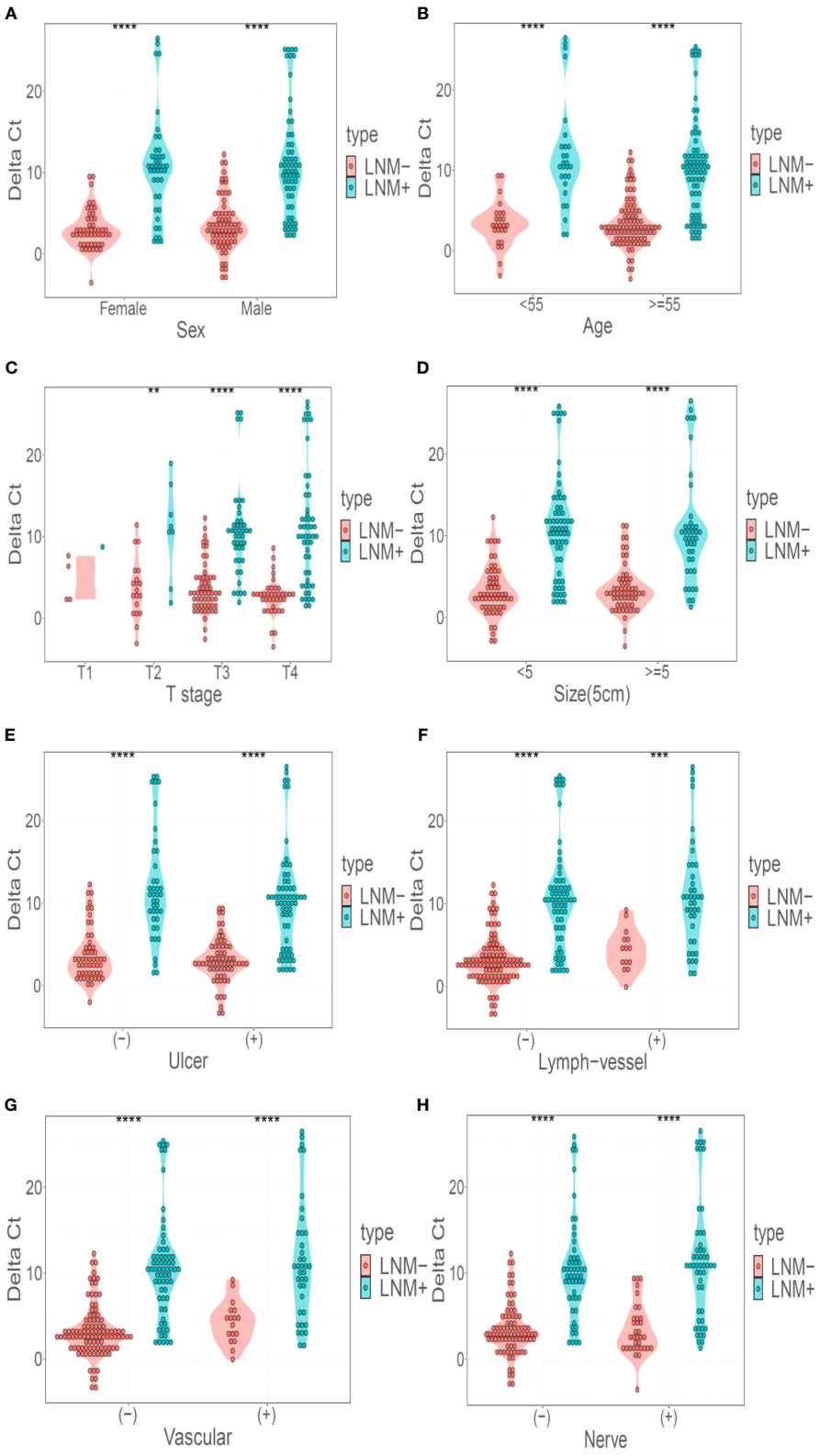

FIGURE 4 (Continued)

FIGURE 4 (Continued)
LBX2 has a good discriminative effect in different classification. **(A)** Performance of *LBX2* in the male and female groups. **(B)** Performance of *LBX2* in age less than 55 years and age more than 55 years groups. **(C)** Performance of *LBX2* in t_1, t_2, t_3, t_4-staging. **(D)** Performance of *LBX2* in tumor size less than 5cm and tumor size more than 5cm groups. **(E)** Performance of *LBX2* in ulcerative and non- ulcerative groups. **(F)** Performance of *LBX2* in lymph-vessel invasion and non-lymph-vessel invasion groups. **(G)** Performance of *LBX2* in vascular invasion and non- vascular invasion groups. **(H)** Performance of *LBX2* in nerve invasion and non-nerve invasion. *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

experiment. However, compared with RNA markers, DNA methylation markers are more stable as diagnostic biomarkers and relatively stable clinical specimens, which are not easily degraded. Therefore, it is easier to be applied to clinical practice.

Interestingly, *LBX2* could be used to identify the LNM status of CRC not only in qPCR verification, but also in immunohistochemical verification, which indicated that *LBX2* played a significant role in the differentiation of both molecular level and protein level. *LBX2* is located on chromosome 2, which starts at 74725882 and terminates at 74726332, with a total length of 451bp and containing 34 CG. In the LNM- and LNM+ groups, there were significant differences in the methylated levels of *LBX2* in exon region (Figure 2B). Due to the low methylated level of *LBX2* in the LNM+ group, it would be overexpressed in the process of protein translation. On the contrary, *LBX2* in the LNM- group has a high methylated level, which results in low expression in the process of protein translation. This view is well explained by our immunohistochemical verification. Subsequently, compared qPCR with IHC in 56 CRC samples, it is clearly that qPCR had better performance (AUC 0.93 vs. 0.84). Apparently, qPCR method had better differentiated efficiency because of more sophisticated level quantification from the qPCR instrument, while IHC was manually assessed and divided into only four grades according to the depth of staining. Because IHC examination is cheaper and easier to generalize, both methods could be applied flexibly for clinical practice.

Currently, there are few studies on gene *LBX2* related to CRC. Some researchers have found that *LBX2-AS1* could promote cell proliferation, migration and invasion through *Mir-4766-5P* mediated *CXCL5* upregulation in gastric cancer (20). In addition, it has been reported that knockout of *LBX2-AS1* in hepatoma cells could reduce its proliferation (21). Moreover, it has been proved that Zinc-finger E-box binding homeobox 1 (*ZEB1*) could induce upregulation of *LBX2-AS1* to enhance the stability of *ZEB1* and *ZEB2*, which could promote the migration and mesenchymal transformation of esophageal squamous cell carcinoma (22). The potential biological pathways of *LBX2* upregulation remain to be proved further. It is widely accepted that *LBX2* may be involved in the positive regulation of Wnt signaling pathway, which is active in the nucleus. Meanwhile, Wnt signaling pathway may be a complex protein action network, whose function not only participates normal physiological processes and embryonic development, but also induces cancer (23). Wnt signaling pathway mainly occurs in intestinal epithelial cells. Under normal conditions, colonic epithelial cells could bind secretory Frizzled related proteins (SFRP) to inhibit Wnt signaling. Once SFRP is silenced under epigenetic regulation, the Wnt signaling pathway would be activated and other molecules in the signaling pathway may mutate, which promotes cell proliferation and inactivation of cells into differentiation and results in the occurrence and invasion of tumors (24).

Although there are a few clinical methods to identify LNM of CRC, the discrimination efficiency of these methods is generally limited. In this study, it had been found that the AUC of imaging, CEA, and CA199 were only 0.52, 0.56 and 0.58, respectively. In addition, the AUC of LVI and the depth of

TABLE 3 Univariate and multivariate logistic regression analyses associated with LNM.

Characteristics	Univariate analysis		Multivariate analysis	
	OR (95%CI)	P value	OR (95%CI)	P value
Gender (Male vs. Female)	1.03 (0.60-1.76)	0.91		
Age (≤ 55 vs. > 55)	1.00 (0.97-1.02)	0.70		
t-stage (1,2 vs.3,4)	1.78 (1.23-2.63)	<0.01	1.71 (1.15-2.61)	<0.01
Tumor size cm (> 5 vs. ≤ 5)	1.00 (0.99-1.00)	0.20		
Ulceration (Presence vs. Absence)	1.28 (0.75-2.20)	0.36		
LVI (Presence vs. Absence)	4.52 (2.33-9.24)	<0.001	6.41 (1.31-47.66)	<0.05
BVI (Presence vs. Absence)	3.47 (1.84-6.80)	<0.001	0.57 (0.08-2.71)	0.51
NI (Presence vs. Absence)	2.33 (1.34-4.09)	<0.01	1.52 (0.82-2.81)	0.18

OR, odds ratio; CI, confidence interval.

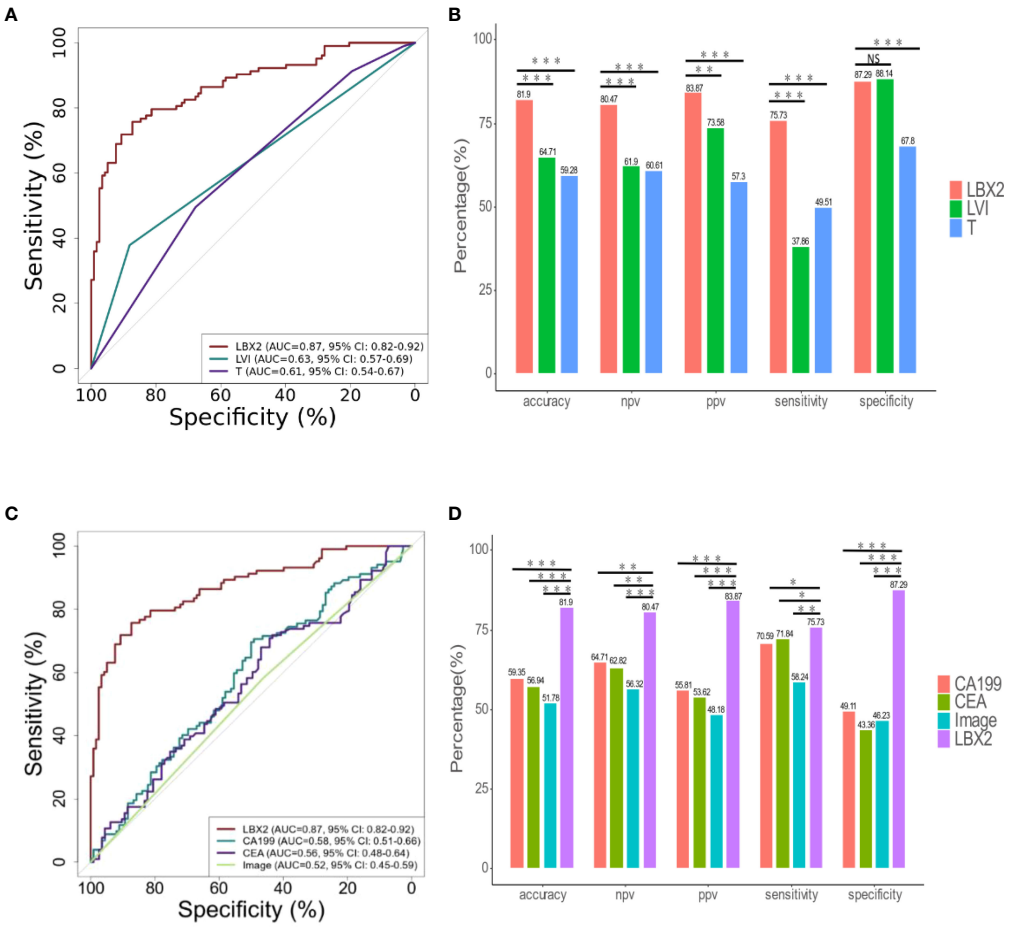

FIGURE 5
The comparison of *LBX2* and traditional indicators (clinical pathology, CA199, CEA, and image) to detect LNM of CRC. **(A)** ROC curve of three indicators (*LBX2*, LVI, and T). The effectiveness of three indicators was evaluated by comparing AUC values. **(B)** The accuracy, NPV, PPV, sensitivity and specificity of these three indicators were compared respectively. **(C)** ROC curve of four indicators (*LBX2*, CA199, CEA and image). The effectiveness of four indicators was evaluated by comparing AUC values. **(D)** The accuracy, NPV, PPV, sensitivity and specificity of these four indicators were compared respectively. *p < 0.05, **p < 0.01 and ***p < 0.001. NS: not statistically significant.

tumor invasion in clinicopathology was 0.63 and 0.61. This may be the main reason that the CRC surgical treatment guidelines suggest we should remove intact tumor with lymph node dissection (2). In fact, the incidence of LNM in many CRC patients, especially those in t1-t2 stage, is only 16% (18). Therefore, excessive medical treatment frequently exists on many CRC patients. Since *LBX2* achieved an AUC of 0.87, which is significantly superior to the current clinical diagnostic methods, meanwhile, DNA samples are more stable than RNA samples. Therefore, DNA methylation marker *LBX2* is easy to be transformed into clinical application and it has the opportunity to become a novel clinical indicator for the identification of LMN of CRC.

Turning to clinical application of *LBX2* in the future, the LNM status of CRC could be determined by immunohistochemical analysis or qPCR analysis of biopsy tissue obtained by colonoscopy. In addition, because CRC tumor cells are easily shed into stool and blood, we could also extract DNA of stool and ctDNA of blood and detect LNM of CRC by *LBX2* probe. This makes it possible to identify LNM of CRC early by minimally invasive or noninvasive methods.

Conclusion

In conclusion, a novel DNA methylation marker *LBX2* could be used as a simple, cost-effective, easy-to-implement, and reliable diagnostic method for LNM of CRC compared to traditional methods, it holds the potential to provide a better clinical diagnosis for the precise treatment of CRC.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of Zhujiang Hospital, Southern Medical University; Southern Medical University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

J-BF and CC conceived, designed, and directed the study. YY designed the experiments and developed the methodology. YY and WX completed experimental work. YY, JW, LX, and XL performed the analyses and interpretation of data. YY, ZL, SC, and QP acquired the patient samples and information. YY and J-BF wrote and critically reviewed the manuscript. All authors reviewed and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interests

The authors J-BF, JW, XL, LX are current employees of AnchorDx Medical Co., Ltd or AnchorDx, Inc.

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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.1000823/full#supplementary-material

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Detection of circulating *KRAS* mutant DNA in extracellular vesicles using droplet digital PCR in patients with colon cancer

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Background: Extracellular vesicles secreted by tumor cells contain double-stranded DNA called extracellular vesicle DNA (evDNA). EvDNA is genomic DNA that reflects cancer driver mutations. However, the significance of evDNA analysis in the diagnosis and surveillance of colon cancer remains unclear. This study aimed to investigate the clinical utility of extracellular vesicles and evDNA isolated from the plasma of colon cancer patients harboring *KRAS* G12D and G13D mutations.

Methods: Cell-free DNA (cfDNA) and evDNA were collected from the plasma of 30 patients with colon cancer. *KRAS* mutation status (G12D and G13D) was detected using a droplet digital polymerase chain reaction assay (ddPCR). Sensitivity and specificity were evaluated in patients with wild-type *KRAS* tumors. Mutation status was correlated with carcinoembryonic antigen (CEA) levels and overall survival (OS).

Results: Thirty cfDNA and evDNA pairs showed a *KRAS* fractional abundance (FA) ranging from 0 to 45.26% and 0 to 83.81%, respectively. When compared with eight wild-type *KRAS* samples, cfDNA exhibited 70% sensitivity and 100% specificity, whereas evDNA achieved 76.67% sensitivity and 100% specificity. The concentration of evDNA was significantly lower than that of cfDNA, but it obtained a higher FA than cfDNA, while showing a positive correlation with CEA.

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Conclusions: Our findings demonstrate the feasibility of evDNA as a complementary tool to aid current methods of patient evaluation in the diagnosis and surveillance of colon cancer.

KEYWORDS

cancer, colon cancer, liquid biopsy, ddPCR, extracellular vesicle, exosome, exosomal DNA, cell-free DNA

Introduction

Liquid biopsy is a noninvasive method for analysis of tumor-derived materials circulating in a patient's body fluid, primarily blood (1). It is used in the diagnosis and surveillance of cancer by monitoring treatment response and resistance-conferring mutations (2). One of the most used sources for liquid biopsy would be nucleic acids that are shed from the tumor and circulate in the bloodstream (3). DNA fragments are especially important in the detection of cancer driver mutations and are often found in the form of cell-free DNA (cfDNA), which is located in circulating tumor cells and extracellular vesicles (EV) (4–7).

Extracellular vesicles, 50–150 nm in size, are secreted by essentially all types of cells. They contain DNA, RNA, and proteins encapsulated in a lipid bilayer that can be transferred from cell to cell as signals of intracellular communication (8–10). Their secretion is exacerbated in cancer cells by active interactions with peripheral cells in the tumor microenvironment (TME) (11). The double-stranded DNA fragments found in extracellular vesicles in the size range up to a few kb are called extracellular vesicle DNA (evDNA) and represent the whole genomic DNA, making them a valuable source for the detection of mutations (12, 13).

A representation of genomic DNA in double-stranded evDNA highlights its significance as a novel source for liquid biopsy for the detection of cancer (14–16). Unlike pieces of cfDNA that are shed from apoptotic or necrotic cancer cells, extracellular vesicles are released from actively proliferating cells and are thus expected to be used in the early detection of developing disease and probable metastasis (17, 18).

KRAS is an important molecular switch that regulates cell survival and proliferation. A mutation in *KRAS* results in the constitutive activation of downstream signaling pathways, thereby leading to tumorigenesis (19). An aberration in the *KRAS* gene is the most frequent type of driver mutation found in cancer, occurring in approximately 20% of all cancer cases and up to 40% of colon cancer cases (20–22). In particular, point mutations in codons 12 and 13 have been validated as critical negative predictors of response to chemotherapy (22). Therefore, determining the *KRAS* mutation status of tumors is a significant step in managing patients with colon cancer.

In this study, we aimed to investigate the clinical utility of extracellular vesicles and evDNA isolated from the plasma of colon cancer patients harboring *KRAS* G12D and G13D mutations. We compared them with cfDNA and matched clinical data to determine whether they are indeed a credible tool for the diagnosis and surveillance of colon cancer.

Materials and methods

Patient sample collection and preparation

A total of thirty patients with colon cancer were prospectively examined. Up to 4 mL of plasma samples were extracted from each patient for the isolation of cfDNA and evDNA. Their clinical information includes age, sex, tumor TNM stage (the eighth edition of the American Joint Committee on Cancer [AJCC] cancer staging), and *KRAS* mutational status. The study was approved by the institutional review board of Severance Hospital (4-2019-0811). To isolate cfDNA, the blood was centrifuged twice at 1900 × *g* for 15 min. For ultracentrifugation, the mixture was centrifuged at 1900 × *g* for 15 min, 500 × *g* for 10 min, and 3000 × *g* for 20 min, as previously described (23). The centrifuged plasma samples were stored at -80°C for subsequent cfDNA and extracellular vesicle isolation.

Extracellular vesicle isolation and characterization

The extracellular vesicles were isolated from plasma by ultracentrifugation. Plasma samples were centrifuged at 12,000 × *g* for 20 min. The supernatants were centrifuged twice at 100,000 × g for 70 min. The pellets were then resuspended in 200 μL of PBS and stored at -80°C. The particle number and size distribution of the isolated extracellular vesicles were measured using a Nanosight NS300 (Malvern Panalytical, Worcestershire, UK).

Western blot analysis

The extracellular vesicles were lysed with RIPA lysis and extraction buffer (Thermo Fisher Scientific, Waltham, MA, USA), 1X protease cocktails, and phenylmethylsulfonyl fluoride (Sigma-Aldrich, Burlington, MA, USA). Denatured proteins were mixed with NuPAGE LDS Sample Buffer (Invitrogen, Waltham, MA, USA) and β-mercaptoethanol, and then heated at 95°C for 5 min. Proteins were electrophoresed on Bolt Bis-Tris Plus gels (Invitrogen) and electroblotted onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked and incubated overnight at 4°C with the following primary antibodies: flotillin-1, CD9 (Cell Signaling Technology, Danvers, MA, USA), CD81 (Novus Biologicals, Centennial, CO, USA), and β-actin (Santa Cruz Biotechnology, Dallas, TX, USA). The membranes were then washed four times with PBS-T. Immunoblots were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and ImageQuant LAS4000 mini (GE Healthcare, Chicago, IL, USA).

CfDNA and evDNA extraction

cfDNA was extracted from 2 mL of plasma using a NextPrep-Mag cfDNA Automated Isolation Kit (PerkinElmer, Waltham, MA, USA). Plasma samples were incubated with the binding solution, proteinase K, and magnetic beads at 56°C. The beads were then separated on a magnetic stand, and cfDNA was eluted with an elution solution. The concentration of cfDNA was measured using the Qubit dsDNA High Sensitivity Assay Kit and a Qubit 4 Fluorometer (Invitrogen). EvDNA was extracted from the extraceullar vesicle samples using AMPure XP beads (Beckman Coulter, Brea, CA, USA). Plasma samples were incubated with lysis buffer and proteinase K. Then, they were bound with magnetic beads, polyethylene glycol, and isopropyl alcohol at 56°C. Finally, the beads were separated on a magnetic stand and evDNA was eluted with nuclease-free water. The isolated evDNA was analyzed using an Agilent High Sensitivity DNA Kit and an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. The amount of cfDNA and evDNA isolated from each patient is listed in Supplementary Table 1.

EvDNA pre-processing

The isolated evDNA was amplified through whole genome amplification (WGA) using the REPLI-G UltraFast Mini Kit as previously described (24), followed by nested PCR to enrich the *KRAS* region. The primer sequences for nested PCR were as follows: forward primer: 5'-AAAGGTACTGGTGGAGTATTTG-3' and reverse primer: 5'-CCTGCACCAGTAA TATGCATA-3', respectively. Thermal cycling was performed using a SimpliAmp thermal cycler (Thermo Fisher Scientific). The following PCR conditions were used: an initial cycle at 95°C for 120 s, followed by 30 cycles of 95°C for 15 s and 60°C for 30 s, with a final cycle of DNA melting from 60°C to 95°C at a ramping rate of 0.2°C/s.

The 10 μL of Dynabeads M-270 Streptavidin (Invitrogen) was washed three times with 1X binding/washing buffer (5 mmol/L Tris-HCl, pH 7.5, 0.5 mmol/L EDTA, and 1.0 mol/L NaCl) and resuspended in 40 μL of 2X binding/washing buffer. The hybridization mixture (80 μL) was captured by mixing 10 μL of processed Dynabeads and incubating the mixture on a shaker for 30 min at room temperature. The beads were washed three times with 1X binding and washing buffer supplemented with 0.05% Tween-20 and twice with 1X binding and washing buffer only. Finally, the beads were resuspended in 20 μL water, denatured at 95°C for 2 min, and immediately placed on DynaMag magnets (Invitrogen). The suspension was then recovered for further analysis.

Droplet digital PCR

A ddPCR was designed to recognize specific mutations in codons 12 and 13 of the *KRAS* gene (e.g., G12D, G13D), which account for the majority of *KRAS* mutations found in colon cancer. This assay was performed on QX200 Droplet Digital PCR System (BioRad, Hercules, CA, USA). The oil droplets containing up to 66 ng of cfDNA or evDNA were generated using Droplet Generation 8 (DG8) Cartridge and Droplet Generator. The generated droplets went through a PCR reaction under the following conditions: an initial cycle at 95°C for 10 min, followed by 40 cycles at 94°C for 30 s and 55°C for 1 min, and a final cycle of 98°C for 10 min and 4°C for 4 min. The droplets were analyzed in the QX200 droplet reader. The interpretation of the results was performed under the Rare Mutation Detection Best Practice Guidelines provided by Bio-Rad Laboratories. The fractional abundance (FA) was calculated as follows:

$$FA = \frac{\text{Absolute quantification of mutant alone}}{(\text{Absolute quantification of mutant} + \text{Wild-type clones})}$$

Positivity was determined using a threshold set to more than 10000 total droplets, five or more positive droplets, or FA of at least 0.1%.

Statistical analysis

Normality and lognormality were assessed using the D'Agostino & Pearson test, Shapiro-Wilk test, and Kolmogorov-Smirnov test. Analysis of paired samples of cfDNA and evDNA was performed using the Wilcoxon

matched-pairs signed-rank test. The Mann–Whitney U test was used to assess the association between unpaired samples. Statistical significance was set at $p< 0.05$. Statistical analyses were performed using the GraphPad Prism software (version 8.0). Survival curves were generated using the Kaplan-Meier method and compared using a log-rank test. Survival curves were generated using the R statistical software version 4.2.0.

Results

Patient characteristics

Patient characteristics are summarized in Table 1. A total of 30 patients were included in the study. Blood samples of the patients were extracted within 30 days at the time of the first chemotherapy. Their median age was 60 years (range: 43 – 88 years). As for staging, 12.5% (n = 4) of patients were classified as TNM stage I, 18.8% (n = 5) as stage II, 15.6% (n = 5) as stage III, and 53.1% (n = 16) as stage IV. More than half of the patients (n = 19) had a *KRAS* G12D mutation, and the rest (n = 11) had a *KRAS* G13D mutation.

TABLE 1 Patient characteristics (n = 30).

Characteristic	n = 30	(%)
Median age (range) - yr	60	(43-88)
Male sex - no. (%)	20	(67)
Tumor stage - no. (%)		
T1	2	(7)
T2	5	(17)
T3	17	(56)
T4	6	(20)
Nodal stage - no. (%)		
N0	13	(43)
N1	6	(20)
N2	11	(37)
Stage, TNM (AJCC[1], 8th)		
I	4	(13)
II	5	(17)
III	5	(17)
IV	16	(53)
Tumor grade or histology		
Well	2	(7)
Moderate	25	(83)
Poor	1	(3)
Mucinous or signet-ring cell	2	(7)
Microsatellite instability (MSI)		
MSI-high	3	(10)
MSS	27	(90)
KRAS mutation[2]		
KRAS G12D	19	(63)
KRAS G13D	11	(37)
Tumor site		
Right	10	(33)
Left	20	(67)

[1]AJCC, The American Joint Committee on Cancer.
[2]The corresponding KRAS mutation statuses were acquired by tissue biopsy.

Characterization of extracellular vesicles, extracellular vesicle DNA, and cell-free DNA

All extracellular vesicle samples used in this study were isolated by differential ultracentrifugation. Nanoparticle tracking analysis (NTA) was used to measure the size of extracellular vesicles isolated from the plasma of patients with colon cancer, with a size distribution of 50 to 150 nm. (Figure 1A). Their common protein markers, such as CD9, CD81, and Flotillin-1, were identified in the samples harvested from the two patients by western blot analysis (Figure 1B). Cell-free DNA (cfDNA) isolated from plasma and extracellular vesicle DNA (evDNA) extracted from extracellular vesicles were analyzed using the Bioanalyzer 2100 system. CfDNA fragments were enriched at an average size of 177 bp (Figure 1C), whereas evDNA fragments were enriched at an average size of 4,500 bp (Figure 1D). 2D intensity scatter plots generated by droplet digital PCR (ddPCR) analysis of wild-type *KRAS* (Figure 1E) and *KRAS* G13D mutant (Figure 1F) showed distinguishable scatter patterns.

Comparing the mutation detection rates of cell-free DNA and extracellular vesicle DNA

CfDNA and evDNA isolated from 30 blood samples of colon cancer patients with *KRAS* mutations were profiled using ddPCR. CfDNA yielded a median *KRAS* mutant fractional abundance (FA) of 0.3% ranging from 0 to 45.26%, while evDNA yielded a median FA of 0.78% ranging from 0 to 83.81%. When paired, the mean of evDNA FA (5.17%) was significantly higher than that of cfDNA (3.57%) ($P = 0.0408$, Wilcoxon matched-pairs signed-rank test) (Figure 2A). A value of FA greater than or equal to 0.1% was considered a detection. When compared with eight additional plasma samples from patients with wild-type *KRAS* and their tissue biopsy results, the *KRAS* detection rate of cfDNA showed 70% sensitivity and 100% specificity, whereas evDNA achieved a higher detection rate of 76.67% sensitivity and 100% specificity (Figure 2B). We then compared FAs with the TNM stage and *KRAS* mutation status of patients (Figure 2C). Among the 28 out of 30 (93%) samples that yielded a detection, 16 samples (53%) were detected in both types, while 12 samples (40%) were detected in only one of the DNA types. This suggests that evDNA can be a complementary source of mutant *KRAS* detection. Furthermore, a positivity was not associated with the TNM stage or type of *KRAS* mutation (G12D or G13D).

Representation of patients' clinical status in cell-free DNA and extracellular vesicle DNA

The 30 patient samples were sorted according to TNM stage, and their FAs from cfDNA and evDNA were compared (Figure 3A). Mean FAs of cfDNA in each TNM stage were 0.17% (Stage I; n = 4), 0.17% (Stage II; n = 5), 0.46% (Stage III; n = 5), and 6.45% (Stage IV; n = 16), while mean FAs of evDNA were 6.84% (Stage I; n = 4), 2.17% (Stage II; n = 5), 5.83% (Stage III; n = 5), and 8.16% (Stage IV; n = 16). A significant difference was observed between cfDNA and evDNA in TNM stage I (P = 0.0286, Mann-Whitney U test), highlighting the detection capability of evDNA, even in the early stage of the tumor (Figure 3B). To more profoundly associate ddPCR profiling of *KRAS* mutations using cfDNA and evDNA with the actual clinical status of patients, we analyzed the correlation between FAs and carcinoembryonic antigen (CEA) levels. The patient cohort was divided into two groups using a cutoff value of 5 ng/mL CEA (CEA ≤ 5 ng/mL and CEA > 5 ng/mL). In both groups, the concentration of evDNA was significantly lower than that of cfDNA (P = 0.0005 and P< 0.0001, respectively, Mann-Whitney

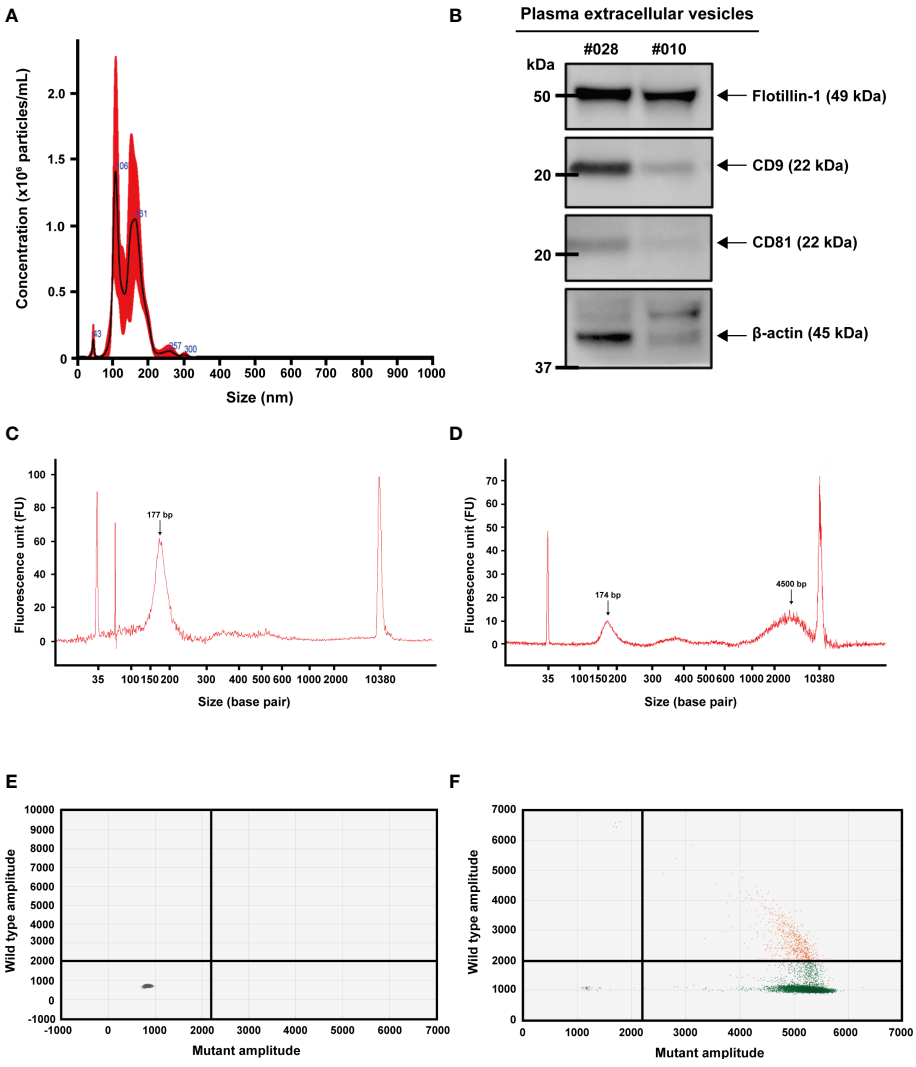

FIGURE 1
Characterization of extracellular vesicles, extracellular vesicle DNA, and cell-free DNA. **(A)** Nanoparticle tracking analysis (NTA) for counting particle number and size distribution of extracellular vesicles isolated from plasma using ultracentrifugation. **(B)** Detection of extracellular vesicle proteins by western blot analysis. Common markers (CD-9, CD-81, and Flotilin-1) were detected in extracellular vesicles isolated from plasma. **(C)** Detection of cell-free DNA (arrow) by Agilent 2100 Bioanalyzer. **(D)** Detection of extracellular vesicle DNA (right arrow) and a sign of minimally remaining cell-free DNA (left arrow). **(E, F)** 2D intensity scatter plot of *KRAS* wild-type and *KRAS* G13D mutant droplets in droplet digital PCR. Plots in each region represent droplets containing wild-type (green; lower right), mutant (blue; upper left), wild-type and mutant (orange; upper right), and no template (gray; lower left).

U test) (Figure 3C). However, FAs of evDNA were higher than cfDNA in the group with CEA less than or equal to 5 ng/mL (P = 0.0244, Mann-Whitney U test), even with lower DNA concentration (Figure 3D). The comparison of FA within the two groups also showed significant differences in conformity with CEA (P = 0.0220 and P = 0.0215, Mann-Whitney U test).

Association of the fractional abundance of cell-free DNA or extracellular vesicle DNA with overall survival

We further evaluated whether the FAs derived from cfDNA and evDNA were associated with the overall survival (OS) of the 30 patients. The median FA of each group (0.3% and 1.2%, respectively) was set as the cutoff value to divide patients into two groups. The cutoff value for cfDNA was able to separate the two groups with significantly different overall rates (P = 0.035) (Figure 4A). For evDNA, the cutoff value was also able to separate the two groups with significantly different overall survival (P = 0.035) (Figure 4B). In contrast, CEA level correlated with OS was not able to significantly separate the patient cohort when evaluated with a cutoff value of 5 ng/mL (P = 0.07) (Figure 4C).

Discussion

Unlike fragmented pieces of cfDNA that originate from apoptotic or necrotic cells, evDNA is safely protected in

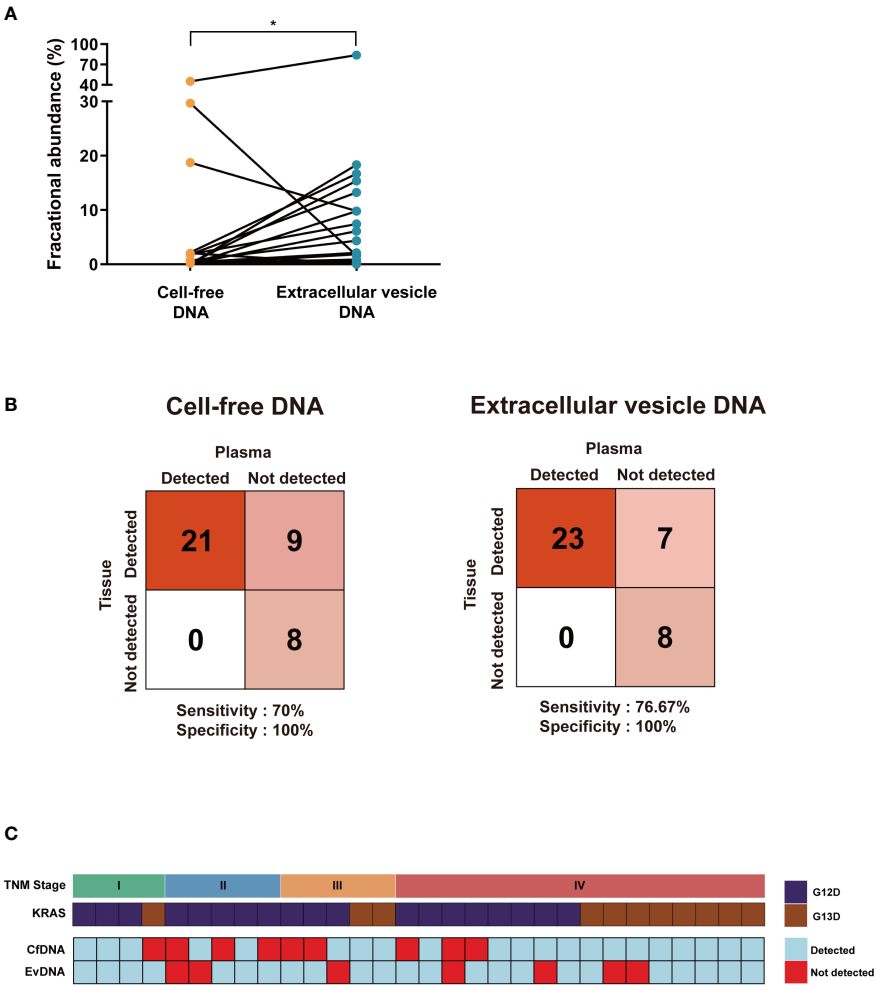

FIGURE 2
Comparing the mutation detection rates of cell-free DNA and extracellular vesicle DNA. **(A)** The mean of extracellular vesicle DNA fractional abundance was relatively higher than cell-free DNA. *P < 0.05. **(B)** A confusion matrix for detecting *KRAS* mutation of cell-free DNA (left) and extracellular vesicle DNA (right) compared with *KRAS* wild-type patient samples. The number of samples identified is noted in each box. **(C)** A detection table of cell-free DNA and extracellular vesicle DNA aligned with TNM stage and *KRAS* mutation status. Samples with fractional abundance greater than 0.1% are considered detected.

extracellular vesicles produced by actively proliferating cells (4, 8). This ensures that evDNA contains a representation of genomic DNA that wholly reflects cancer driver mutations even in the early stages of cancer development (12, 13). Thus, we hypothesized that intact evDNA would be an effective biomarker for the detection of oncogenic mutations in colon cancer. Indeed, droplet digital PCR (ddPCR) using evDNA was able to detect *KRAS* G12D and G13D mutations in colon cancer and demonstrated a comparable association with CEA and OS, which reflects the clinical status of patients. This suggests that evDNA may be valuable as an effective complementary tool for the diagnosis and surveillance of colon cancer.

In our study, an assessment of cfDNA and evDNA of colon cancer patients with *KRAS* mutations and patients with wild-type *KRAS* yielded a sensitivity of 70% and 77%, respectively. This result was consistent with that of other studies that also reported that evDNA had a higher sensitivity than cfDNA in liquid biopsies (14, 25). For instance, Krug et al. reported that evDNA (98%) yielded significantly higher sensitivity than circulating tumor DNA (82%), a tumor-specific type of cfDNA, in the detection of mutant EGFR using a targeted next-generation sequencing assay (15). Moreover, the detection rate of both cfDNA and evDNA was not affected by other patient assessment methods, such as TNM stage, suggesting that evDNA can be used regardless of the grade and stage of tumor progression.

Carcinoembryonic antigen (CEA) has an established role as a biomarker for the evaluation of colon cancer patients, and an elevation in its level is associated with metastasis and poor prognosis (26, 27). We showed that the level of FA derived from the liquid biopsy of evDNA was analogous to that of CEA, and this trend was especially highlighted in early TNM stages. In

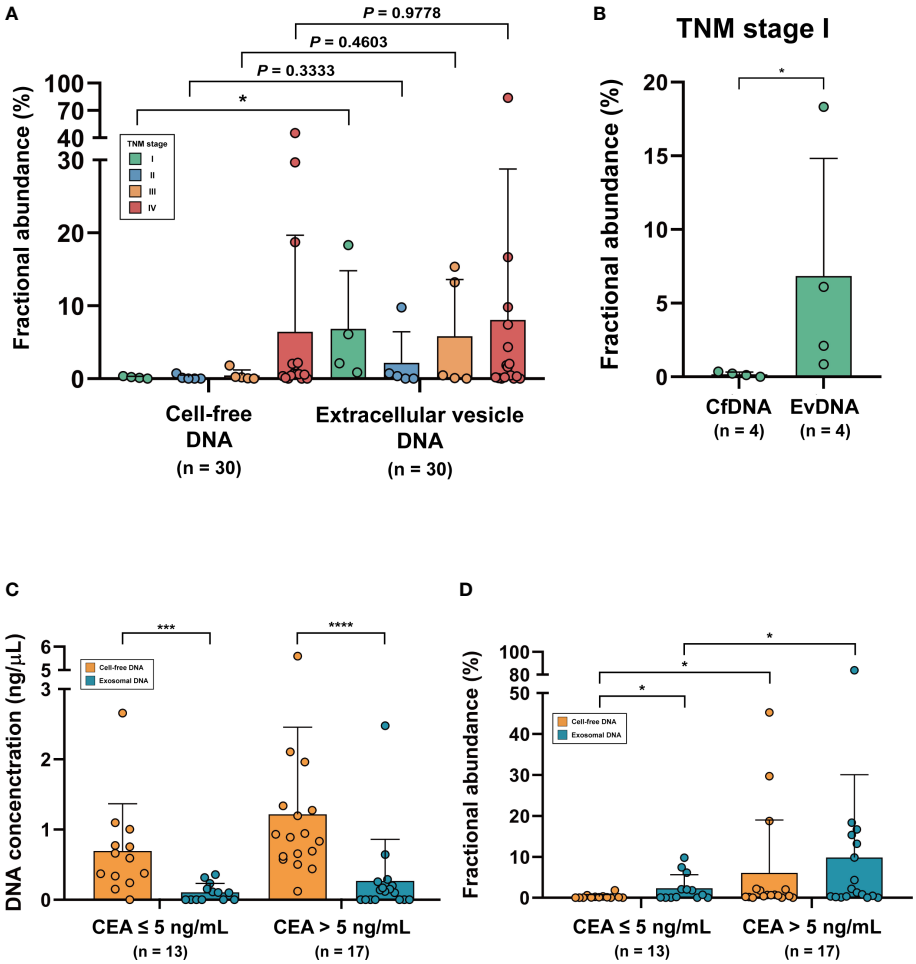

FIGURE 3
Representation of patients' clinical status in cell-free DNA and extracellular vesicle DNA. **(A)** Fractional abundance of cell-free DNA and extracellular vesicle DNA compared with TNM stage. **(B)** Fractional abundance of cell-free DNA and extracellular vesicle DNA in TNM stage (I) *P< 0.05. **(C)** Comparison of DNA concentration of cell-free DNA and extracellular vesicle DNA in CEA-low and CEA-high groups. ***P < 0.001, ****P < 0.0001. **(D)** Comparison of fractional abundance of cell-free DNA and extracellular vesicle DNA in CEA-low and CEA-high groups. *P < 0.05.

addition, the amount of evDNA acquired from the patient's plasma was much lower than that of cfDNA. Mutant *KRAS* was detected in only one of the DNA types in approximately half of the 30 patients, suggesting that liquid biopsy using evDNA can complement the current widely used patient evaluation methods for colon cancer with a minimal amount of DNA fragments.

This study has some limitations. First, although patient-derived extracellular vesicles and extracellular vesicle DNA may serve as a source for cancer driver mutation detection, their extraction may still limit their clinical application. Currently, ultracentrifugation is known as the "gold standard" method for their isolation; however, it is a time-consuming method that requires multiple laborious steps (28). Second, the droplet digital PCR (ddPCR) method used in this study was able to detect KRAS mutants from patient-derived cfDNA and evDNA effectively, but this consistency was not observed in other types of mutations aside from *KRAS* (29, 30). Notably, ddPCR requires careful primer design and enrichment of the *KRAS* region to ensure detection. Next-generation sequencing is often suggested as a novel method to replace ddPCR for the detection of mutations, but its low accessibility and high cost still limit its application (4).

In summary, extracellular vesicle DNA from patients with colon cancer may be a novel source for the detection of cancer driver mutations. The *KRAS* mutation detection rate using evDNA was higher than that using cfDNA. It also showed consistency when compared with the conventional methods of patient evaluation. Thus, we suggest that liquid biopsy using evDNA may have a complementary role in the diagnosis and surveillance of colon cancer, as it can produce consistent results regardless of the patient's clinical status.

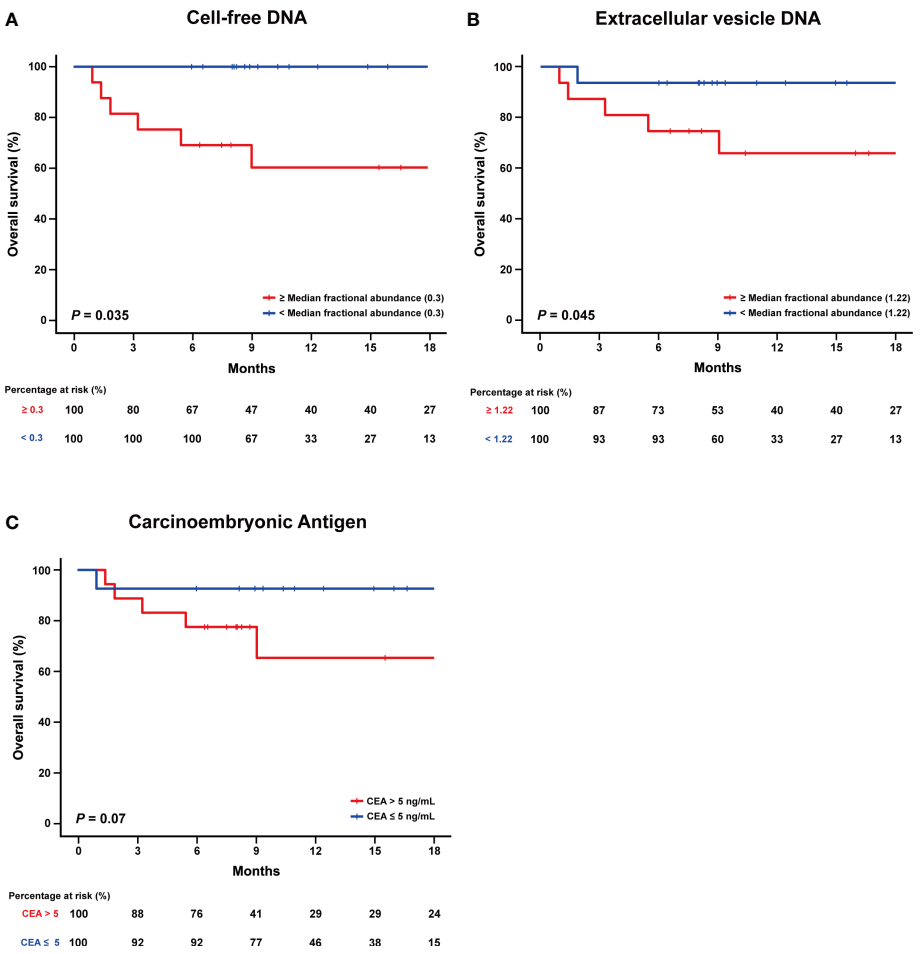

FIGURE 4
Comparing fractional abundance of cell-free DNA and extracellular vesicle DNA with overall survival (OS). **(A)** Kaplan-Meier curve of the overall survival (OS) divided according to low and high fractional abundance derived from cell-free DNA. **(B)** Kaplan-Meier curve of the overall survival (OS) divided according to low and high fractional abundance derived from extracellular vesicle DNA. **(C)** Kaplan-Meier curve of the overall survival (OS) according to CEA level.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by Institutional review board of Severance Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

HSK conceived and designed the study. HYC, JJ, KK, and YDH collected the data. JC, HYC, JJ, and HSK performed statistical analyses. JC, HYC, and HSK wrote the manuscript. JC, HYC, KK, JBA, and HSK discussed the hypotheses and contributed to the data interpretation. DL and IW reviewed the manuscript and provided critical feedback. All authors contributed to the manuscript revision 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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.1067210/full#supplementary-material

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Liquid biopsy approaches and immunotherapy in colorectal cancer for precision medicine: Are we there yet?

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Colorectal cancer (CRC) is the second leading cause of cancer-related deaths globally, with nearly half of patients detected in the advanced stages. This is due to the fact that symptoms associated with CRC often do not appear until the cancer has reached an advanced stage. This suggests that CRC is a cancer with a slow progression, making it curable and preventive if detected in its early stage. Therefore, there is an urgent clinical need to improve CRC early detection and personalize therapy for patients with this cancer. Recently, liquid biopsy as a non-invasive or nominally invasive approach has attracted considerable interest for its real-time disease monitoring capability through repeated sample analysis. Several studies in CRC have revealed the potential for liquid biopsy application in a real clinical setting using circulating RNA/miRNA, circulating tumor cells (CTCs), exosomes, etc. However, Liquid biopsy still remains a challenge since there are currently no promising results with high specificity and specificity that might be employed as optimal circulatory biomarkers. Therefore, in this review, we conferred the plausible role of less explored liquid biopsy components like mitochondrial DNA (mtDNA), organoid model of CTCs, and circulating cancer-associated fibroblasts (cCAFs); which may allow researchers to develop improved strategies to unravel unfulfilled clinical requirements in CRC patients. Moreover, we have also discussed immunotherapy approaches to improve the prognosis of MSI (Microsatellite Instability) CRC patients using neoantigens and immune cells in the tumor microenvironment (TME) as a liquid biopsy approach in detail.

KEYWORDS

liquid biopsy, neoantigen, immune cells, exosomes, colorectal cancer, mitochondrial DNA, circulating cancer associated fibroblasts (cCAFs)

Introduction

Colorectal cancer (CRC) is third in terms of the most common (6.1%) and second in terms of deadly (9.2%) disease worldwide. It is estimated that by the year 2035, the total number of deaths from rectal and colon cancer will increase by 60% and 71.5%, respectively. Overall survival (OS) 5 years after primary diagnosis in stage I–II is 87-90%, decreasing to 68–72% in stage III, and futher lowering to 11–14% in stage IV metastatic CRC (mCRC) (1–5). Today therapeutic algorithms for CRC contain endoscopic and surgical resection, systemic adjuvant chemotherapy, radiation therapy, targeted therapy, and immunotherapy (6, 7). Due to the poor response of numerous colorectal patients to existing therapeutic approaches and since CRC survival is highly dependent on primary diagnosis and early treatment, a known significant biomarker that can predict the beneficial response as early as possible is immediately required. To date, tissue biopsy is one of the best standard options for tumor identification. Though, the main drawback is that it is problematic to screen disease development over frequent biopsies due to recurrent injury and poor patient compliance. Tissue removal also carries hazards, and it is unapproachable for some cancer cases (8, 9). Also, biopsy has some significant boundaries: it is invasive, expensive, painful has technical boundaries related to tumor location, and is not effective in pointing to tumor cells subpopulations (10–12). Indeed, there is a critical need to find a minimally invasive or non-invasive method to screen the high-risk population and detect CRC presence in asymptomatic patients at an earlier and curable stage.

The awareness of liquid biopsy is that of identifying circulating biomarkers to distinguish cancer cells released from the primary tumor and/or metastasis sites (13–15). The meaning of 'liquid biopsy' describes the importance of identifying cancer-derived biomarkers in blood or other body fluids, such as stool, saliva, cerebrospinal fluid or urine (16–22). The very noteworthy targeted constituent studied in liquid biopsy is circulating tumor DNA (ctDNA), circulating tumor cells (CTC), circulating tumor RNAs, and exosomes (23–27). Although they are the most studied component for liquid biopsy, CTCs alone cannot be considered as a clinical diagnostic tool due to the debate over their clinical utility (28). However, it has been reported that tumor cells communicate not only with additional malignant cells, but also with the constituent of the tumor microenvironment (TME), suggesting their stability in circulation is highly reliable on TME (29, 30). So, here we hypothesized that CTC research should be commenced concurrently with other TME components, such as, cancer associated fibroblasts (CAFs), various immune cells, extra-cellular vesicles (EVs) etc. Furthermore, another noninvasive approach being studied is the use of ctDNA, exosomal miRNAs, and proteomics; which though in primary stages, needs to be elucidated in-depth. Additionally, we have highlighted the benefits of immunotherapy treatment for MSI-high (MSI-H) CRC patients and use of neoantigens and immune cells as a liquid biopsy approach for better prognosis.

Overall in this review, we have described the concept of liquid biopsy and its applications in the management of CRC patients (Figure 1). Furthermore, we have highlighted the role of less explored components, such as organoid models of CTC, immune cells in TME, mitochondrial DNA (mtDNA), and neoantigens in the liquid biopsy approach. These approaches could be used noninvasively to gain knowledge about molecular characterization and the mechanism of disease progression in CRC.

Tumor-microenvironment components

The awareness of the tumor microenvironment (TME) has been proposed more than one hundred years ago. In the meantime, cancer research has discovered many several noteworthy roles of TME components not only in cancer metastasis, but also in cancer metabolism and development (31–33). TME consists of a web of cancer cells, stromal cells, immune cells, CAFs, exosomes, and extracellular matrix. In this composition, immune cells and stromal cells are the two major non-tumor cell types in addition to tumor cells (34). Interestingly, TME Web found it possible to achieve immune organization of cancers concerning prognosis, chemotherapy, and prediction of immunotherapy response (35–37). In the current scenario, several studies on cancer have shown that TME meaningfully affects cancer cell proliferation and development and recommends potential worth in the diagnosis and prediction of cancer prognosis (38–40). In addition, it has been suggested that TME is highly affected by the development of CRC (41–43). Furthermore, the TME components play significant roles in defining CRC with poor prediction and immune escape (44, 45). Together, the significant function of TME in the development and metastasis of CRCs and the investigation of the essential molecular mechanism that makes the interaction between the transformation of TME and the progression of CRCs have fascinated important considerations over the past era. But until now, a comprehensive understanding of the TME in CRC development and metastasis has yet to be discovered.

Circulating tumor cells and circulating cancer-associated fibroblasts: Symbiotic siblings and potential drug targets

CTCs are the representative of the cancer cells detached from the primary tumor which enter into the circulatory system (blood, lymphatic system) to cause metastasis (46, 47). Undoubtedly, CTCs have been used as a dynamic component

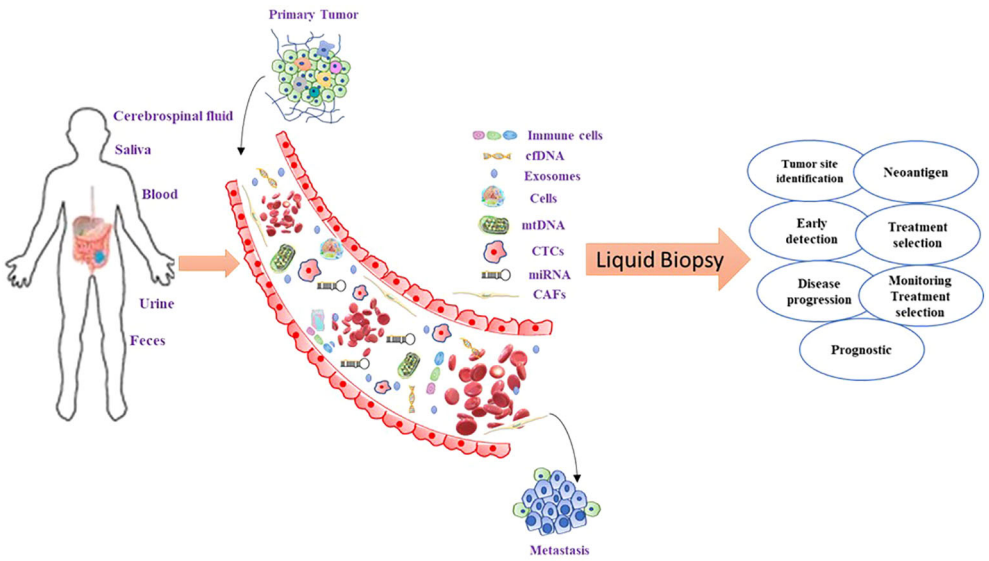

FIGURE 1
Liquid biopsy components in CRC and their clinical utility. CTCs are shedded from the tumor into the blood vessels where they can release their components: nucleic acids and exosomes with tumor-specific cargo material. For the analysis of these molecules, blood can be taken out, and plasma or serum further processed for the extraction of the desired constituents. From the blood circulation, these molecules can be filtered into saliva and urine which can also be collected and further analyzed. Each of these constituents delivers one or more levels of tumor information. The quantity of the concentration of single proteins or panels including numerous tumor proteins is the present gold standard in cancer management.

of liquid biopsies to investigate the presence of residual cancer cells, track treatment response, and prediction of disease recurrence, which is suggestive of the fact that CTCs could play a critical role in the early diagnosis and prognosis of various cancers, including the development of personalized therapeutic options (48–53). Compared to other cancer biomarkers, CTCs are cancer cells that could carry biological and molecular evidence of cancer cells that supports single-cell analysis and directly provide information about ongoing alterations in cancer cells at all different stages of disease progression (54–56). Based on the evidence, CTCs have a favorable role in early prediction, therapeutic observation, and disease progression and would be a significant drug target for various cancers (57–60). The existence of clusters of CTCs has been reported during the last decade, and several groups have described the clinical relevance of CTC clusters. Although the prognostic value of CTC has been well validated, limitations are preventing the use of CTC enumeration in routine clinical practice concerning the use of CTCs either as a clinical marker for early cancer detection, or as a surrogate endpoint in interventional studies (61). These limitations include uncertainty about the specificity of CTC detection assays and justifiable concerns that CTC detection alone may be misleading or inadequate, especially when applied in the early detection of metastases. Additional biomarker assays can enhance the specificity and broaden the application of "liquid biopsies" in early cancer detection, monitoring disease progression, and determining response to therapy (Figure 2). To relate to a single cancer cell, CTC clusters are comparatively low and rare in circulation, but reveal noteworthy, better resistance to apoptosis and additional metastatic potential (62–64). Likewise, research on clusters of CTCs in the peripheral blood of patients with CRC has revealed that the clusters of CTCs are not malignant, but relatively tumor-derived endothelial cells connected to the vascular features; particularly, the separation and counting of these clusters of CTCs can distinguish between healthy individuals and patients with early-stage CRC with a high degree of precision (IIa) (48, 65).

Because CTCs can be detected in the peripheral blood of cancer patients, it follows that a "liquid biopsy" to detect tumor components in blood will not only contain tumor cells but will also contain other cellular components of TME. Cancer-associated fibroblasts (CAFs) – responsible for cancer cell proliferation, migration, invasion, drug resistance, and other important biological processes through secretion of cytokines, chemokines, and growth factors - are a heterogeneous population and an essential component of cells in TME (Figure 3) (66). Various studies have revealed their inevitable role in the regulation of almost all hallmarks of cancer, resulting in tumor progression and metastasis (67–69). According to Dr. Paget's seed and soil theory, the seed has been repeatedly studied as cancer stem cells (CSCs), resident? cancer cells, and more recently as CTCs; whereas soil is represented as the TME (70). It

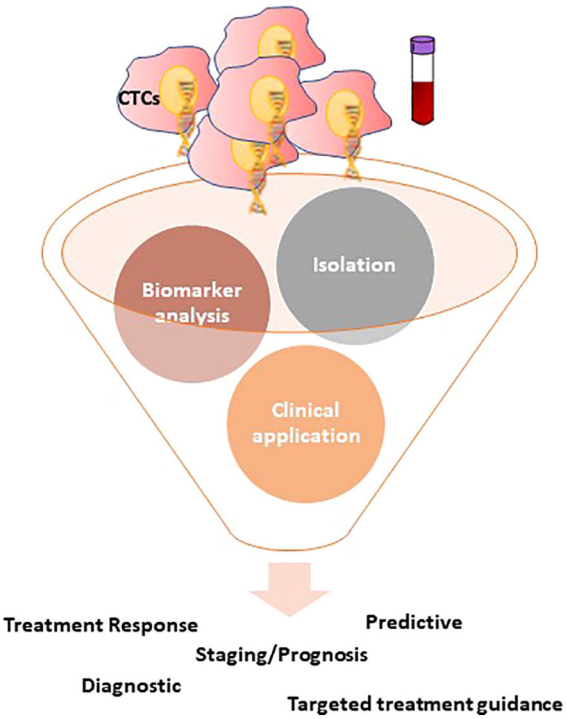

FIGURE 2
Overview of the CTCs detection technologies and the potential clinical applications of CTCs in CRC. CTCs isolation can usually be divided into two groups: physical isolation designed to exploit the differing physical belongings of blood components, such as size, deformity, and charge; and biological isolation, often utilizing antibody-based capture methods to enrich CTCs or deplete various blood cells. Following isolation, CTCs are open to a variety of downstream applications, focusing primarily on one of three categories: enumeration, characterization, and expansion.

is very well known that in the CTC population, most CTCs die at an early stage when they enter the circulation due to the collective effects of environmental and mechanical factors, for example, oxidative and sheer stress and immune response (48, 71). Consequently, only a few drug-resistant cells can escape and spread by undergoing a series of modifications to survive the changing environment. By looking at this theory, it is proposed that caves form clusters with CTCs to provide a suitable TME to CTCs and/or CTSCs (circulating tumor stem cells) for their persistence during metastasis in the circulation.

In CRC, various studies have reported clinical applications of CTC for early diagnosis, prognosis, and treatment monitoring using different techniques (48). In addition to this, recent studies revealed the importance of the CTC cell line in classifying cancer-associated proteins (neoantigens) and pathways connected to cancer cell stemness and metastasis, as well as in assessing anticancer drug sensitivity (72–76). Agarwal et al. identified clusters of cCAFs/CTCs and discovered that the cumulative number of these clusters is associated with cancer growth and metastasis (77). Although these studies have shown the presence of CAFs outside of the primary tumor site or metastatic lesions, there has been little direct evidence showing the presence of CAFs in the circulation of cancer patients in a clinical setting. In addition to this, several biomarkers, genes, and proteins have been extremely highly expressed in CAFs and also have poorer disease progression and overall survival in CRC (Figure 4). To date, the importance and use of CTCs in clinical setting for CRC is increasingly being established (Table 1) (26, 78–88), but the low population and vast heterogeneity of CTCs in addition to the progress of diagnosis and analysis approaches have few common approvals to use CTCs as a new biomarker. Thus, impeding cCAF/CTC complex formation or dismantling them, as well as clusters with other types of cells, may open new frontiers for controlling cancer or preventing metastasis.

Exosomal miRNAs/ctDNA/cfDNA

A stimulating realm of tumor research has advanced over the past decade by concentrating on extracellular vesicles (EVs), known as exosomes, to answer pivotal challenges around therapeutics, diagnosis, and prevention. Exosomes are known as vesicles formed *via* the endocytic pathway and ranging in size from 30-140nm in diameter. As a new significant focus on the enigma of cancer, exosomes signify a noteworthy characteristic of biological signaling between cells and are also used as novel biomarker identification strategies (89–91). In addition, numerous studies have discovered that body fluids harbor abundant quantities of EVs, the constituent's quantity of which varies based on the physiological or pathological state of an individual (92, 93). These diverse populations of extracellular? vehicles transfer detailed cargo such as miRNA, proteins, and lipids from one cell to another to stimulate a specific response. Exosomes can be found in all body fluids and can be detected in liquid biopsies (94). This section focuses primarily on exosomes containing miRNAs, proteins, and mRNA that appear to be consistently altered in patients with CRC. To date, there are only a minority of publications aimed at understanding exosomes in relation to CRC. EVs released from CRC cells can reveal vital evidence about significant molecules and signaling pathways involved in the growth and development of CRC (95, 96). Thus, the existence of tumor-derived EVs in circulating body fluids makes them prospective innovative biomarkers for early prognosis, diagnosis, and prediction of CRC cancer.

Exosomes have a prominent role in cell proliferation, metastasis, and epithelial-to-mesenchymal transition (EMT), as well as by supporting the angiogenic switch and the remodeling of the extracellular matrix (ECM) in CRC (97, 98) (Figure 5). In recent research, it was observed that CRC cells released more exosomes in hypoxic conditions (99, 100).

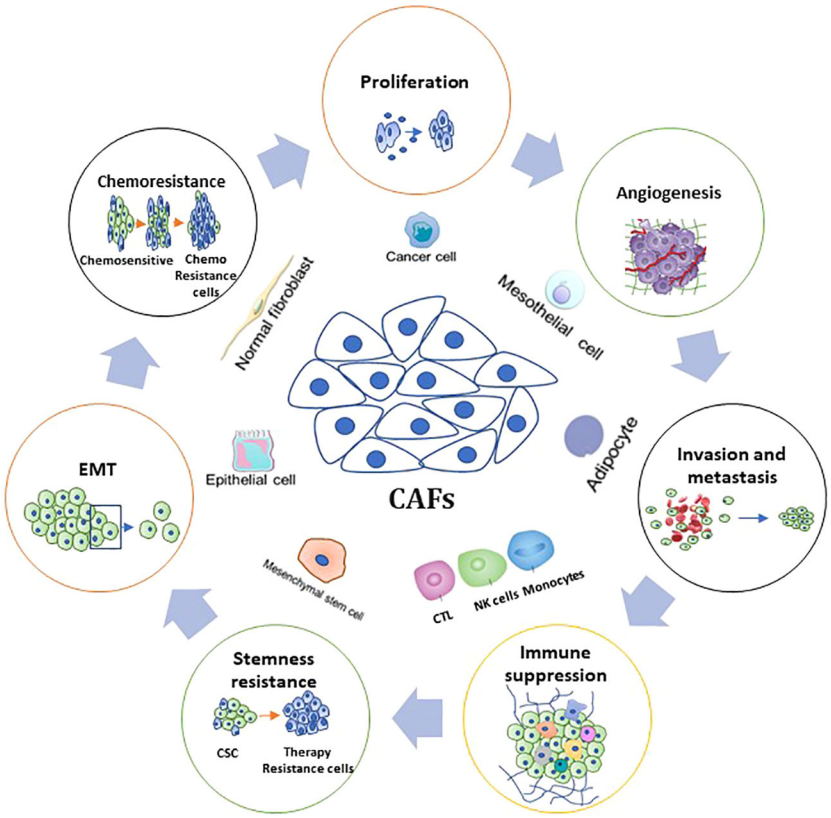

FIGURE 3
Fundamental Functions and associated mechanisms of CAFs in CRC hallmarks. The figure shows the role of CAFs in CRC biology, including tumorigenesis, proliferation, angiogenesis, invasion and metastasis, stemness, therapy resistance, and tumor immunity.

Besides, these exosomes encourage cell proliferation *via* shortening the mitosis period and triggering STAT3 signaling in CRC (95, 101). Furthermore, Mulvey et al. demonstrated that co-culture of the CRC HCT116 cell line exosomes with normal colon cells can increase its clonogenicity (102). Numerous cellular components in exosomes have been reported that could contribute to CRC metastasis through various molecular mechanisms. A recent research report suggested that glycoprotein A repetition-dominant (GARP) knockdown Mesenchymal stroma/stem-like cells prevent the cell proliferation and invasion of mouse colorectal cancer cells through exosomes (103).

Exosomal miRNAs have been significantly concerned in several exosome-mediated biological functions in cell-cell communication in numerous cancers including CRC (104, 105). MiR-21-5p and miR-155-5p have been revealed to be highly expressed in macrophage-derived M2 exosomes, which facilitated the migration and invasion of CRC (106, 107). In addition to this, it also observed that exosomes from bone marrow-derived mesenchymal stem cells (BMSCs) can inspire stem cell-like features of colorectal cancer through miR-142-3p (108). In addition, CAFs, TAM, and MSC exosome proteins are also significant mediators of cancer and TME regulation. Gang, N, and his team used proteomic analysis of CAFs and serum-derived exosomes that have recognized QSOX1 as a biomarker for the early prediction and detection of CRC non-invasively (109). Current research also described novel types of RNAs, such as Piwi-interacting RNA (piRNA) and tRNA-derived small RNA (tsRNA), along with miRNA, lncRNA, and cicrRNA (110, 111). There has only been limited research into the current existence and role of these types of non-coding RNAs in CRC exosomes. Thus, even though the therapeutic approach of exosomes has revealed countless application scenarios in colorectal cancers, many problems remain before we can routinely use exosomes in the clinical treatment of CRC.

In most solid tumors, CAFs are the significant cellular components of the TME (112). CAF-derived exosomes could stimulate neoplastic angiogenesis and cancer cell growth in CRC. Furthermore, these can also activate cancer cell dedifferentiation through the Wnt signaling pathway, therefore increasing the chemical resistance of CRC (95, 113). Compared to RNA and protein, there is little research on exosomal DNA. In previous

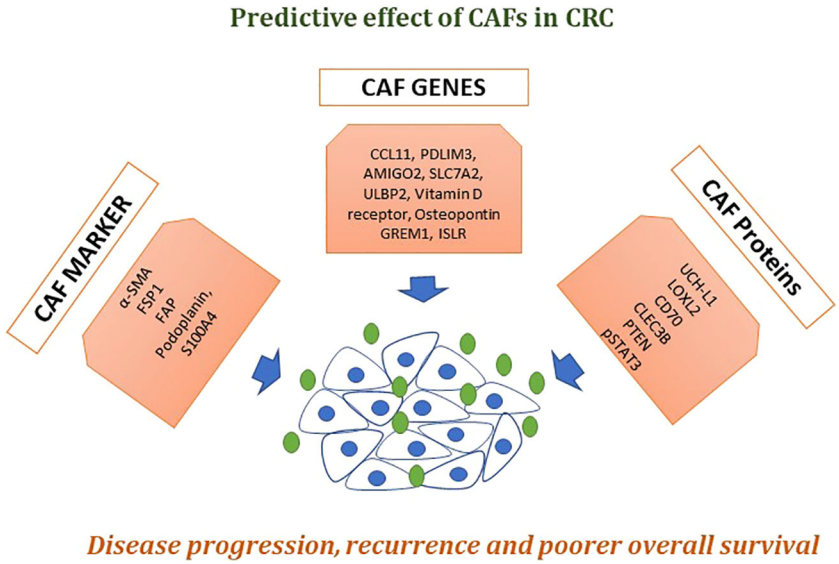

FIGURE 4
Effects of CAFs in CRC. A numeral of biomarkers that are extremely expressed in CAFs, like α-SMA, fibroblast activation protein alpha (FAP), fibroblast-specific protein 1 (FSP-1), platelet-derived growth factor receptor-α (PDGFRα) and PDGFRβ have now been commonly used to classify or isolate CAFs from the pool of fibroblasts present in the whole body. Described genes and proteins showed poorer disease progression, recurrence-free survival, and overall survival. Taken together, these markers could be used as liquid biopsy approach for early detection and treatment prognosis in CRC patients.

TABLE 1 List of CTCs biomarkers and its clinical use in CRC.

Biomarkers	Methods	Clinical use
EpCAM	CellSearchTM, CanPatrolTM	Predictive and prognostic
CEA	RT-PCR	Prognostic
CK19	RT-PCR, CK19-Epispot	Prognostic
CD133	Drug sensitivity analysis of CTC lines	Prognostic
CKs	RT-PCR	Prognostic
VIM	CanPatrolTM	Prognostic
TWIST1	CanPatrolTM	Prognostic
CD26	Drug sensitivity analysis of CTC lines	Prognostic
CD44v6	Drug sensitivity analysis of CTC lines	Prognostic
KRAS	Label-free Vortex technology	Prognostic
BRIEF	Label-free Vortex technology	Prognostic
PI3KCA	Label-free Vortex technology	Prognostic
AKT2	CanPatrolTM	Prognostic
SNAI1	CanPatrolTM	Prognostic

research, it was observed that the gDNA from exosomes is widely used in liquid biopsy, and it has a great impact on tumor immunity and metabolism (114–118). The KRAS and BRAF mutation was identified in serum exosomes of patients with CRC with greater sensitivity and specificity (119, 120). Furthermore, it was also revealed that exosome gDNA plays a significant role in immunity in CRC patients (117). Current research studies have revealed that the number of exosomes in

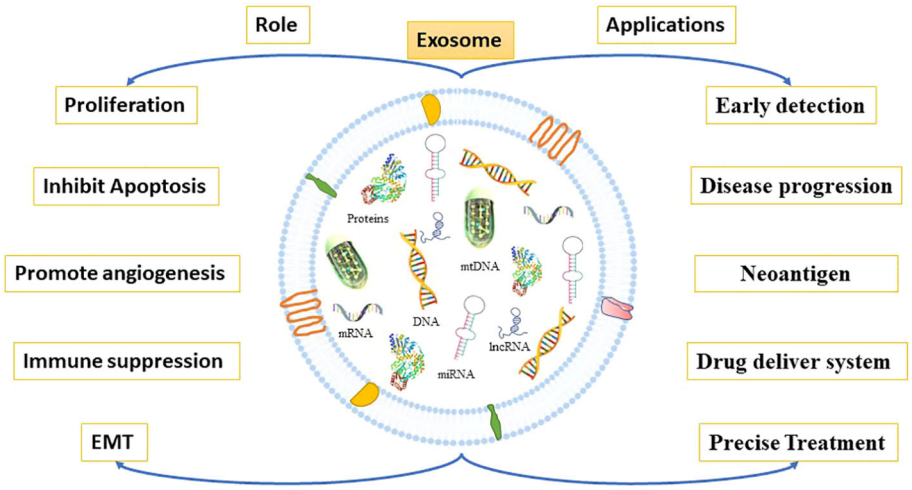

FIGURE 5
Roles and application of exosomes. Tumor-derived exosomes promote cancer growth and metastasis. Through multiple mechanisms, they participate in cancer growth and metastasis by reshaping TME resulting into EMT, cell proliferation, apoptosis inhibition, immunosuppression, and angiogenesis. Exosomes derived from cancer cells are enriched with proteins, mRNA, miRNA, lncRNA, DNA etc. that are more abundant in cancer cells than in normal cells. Thus, exosomes may be used as biomarkers for cancer diagnosis, prediction, and treatment.

the body fluid of CRC patients is markedly higher compared to healthy controls (98). Hence, the studies on CRC exosomes must be encouraged due to this increased presence of CRC exosomes that can likely be used during cancer treatment.

Another promising biomarker that has established noteworthy consideration in the current era is circulating tumor DNA (ctDNA). ctDNA comprises DNA fragments that are released by fragmenting tumor cells into the blood circulation and in principle should have genetic and epigenetic alterations identical to the cancer cells they initiated from (121, 122). Numerous types of DNA modifications have been noticed with adaptable frequency in the ctDNA of patients with CRC. The revealing of mutant DNA in plasma or serum from a CRC patient has been associated with diagnosis, prognosis, and therapeutic response in numerous reports (123). Furthermore, in CRC patients *KRAS* mutations in ctDNA have been identified in different stages, with the highest level found in the more advanced stage (124–126). Furthermore, recent studies found that ctDNA was detected postoperatively in approximately 5% to 30% of patients with stage II to III colon cancer and has established a strong prognostic capacity in numerous observational studies (127). Since the last decade, in CRC, the introduction of next-generation sequencing (NGS) technology has led to the discovery of ctDNA in plasma, which is an encouraging practice (128, 128). Some research reports revealed that ctDNA methylation has a notable sensitivity compared to traditional serum tumor markers in patients with initial-stage CRC and is a significant biomarker for the diagnosis of CRC (129–131). Currently, personalized immunotherapy based on neoantigens requires tissue samples to obtain accurate evidence of somatic genomic modifications in individual cancer patients. Although it is from time to time problematic to obtain many tumor tissues; consequently, the development of ctDNA analysis could be significant in the enlargement of neoantigen-based treatment, even though it is still puzzling. Together, current potential clinical trials with ctDNA focus on the diagnosis, surveillance, and prognosis of CRC. With the rapid progress of research technology, liquid biopsies will play a crucial role in the diagnosis and treatment of CRC. In Tables 2, 3 (132–179), we have listed circulating miRNAs, lncRNAs, circ-RNAs and proteins as diagnostic biomarkers in CRC patients.

Mitochondrial DNA: Unexplored arena

In the recent era, the standard for the molecular profile of colorectal cancer (CRC) is tissue biopsy. However, they are inadequate concerning about sampling rate, illustration of tumor heterogeneity, and sampling can expose patients to antagonistic side effects. To study cell-free DNA (cfDNA) from the various body fluids, this being a component of a liquid biopsy, is relatively invasive, but highly significant to discover all tumor-specific mutations. Furthermore, mitochondria have their circular genome and therefore contribute to the total cfDNA content in the blood. MtDNA plays an essential role in mitochondrial biogenesis and regulates mitochondrial function and the regulation of apoptosis (180–182). A single cell comprises up to several thousand copies of mitochondrial DNA (mtDNA) contrasting to two copies of

TABLE 2 Non-invasive biomarkers (miRNA, Proteins, lnc RNA and circ-RNA) used for CRC detection till date.

Circulating nucleic acids and proteins in CRC				
Sample	miRNA	Protein	lncRNA	Circ-RNA
Plasma	miR-125a-3p, miR-193a-5p, miR-320c, miR-23b, miR-27a, miR-760, miR-130a, miR-29a, miR-210-3p, miR-92a, miRNA-18a, miR-100, miRNA-19a, miR-30e, miRNA-335, miR-16, miRNA-29a, miR-144-5p, miRNA-15b, let-7i, miRNA-19b, miR-486-5p, miR-20a, miR-181a-5p, miR-155, miR-30d-5p, miR-21, miR-24, miR-92, miR-29b, miR-106a, miR-194, miR-200c, miR-320a, miR-372, miR-375, miR-96, miR-423-5p, miR-92a, miR-601, miR-221, miR-760, miR-182, miR-320d, miR-506, miR-7, miR-4316, miR-93, miR-223, miR-31, miR-1290, miR-181b, miR-431, miR-203, miR139-p3, miR-139-3p, miR-409-3p, miR-18a, miR-22, miR-25, miR-29, miR-19a, miR-19b, miR-15b, miR-29a, miR-335, let-7g, miR-15b-5p, miR-18a-5p, miR-29a-3p, miR-335-5p, miR-19a3p, miR-19b3p	CPNE3 CEA Melanotransferrin	LNCV6_116109 LNCV6_98390 LNCV6_38772 LNCV_108266 LNCV6_84003 LNCV6_98602 91H PVT-1 MEG3 ATB CCAT1	circ-133 circPACRGL circ-ABCC1 circ_0000338 ciRS-122 hsa_circ_0004585 circ-FBXW7
Serum	miR-17-92a, miR-99b-5p, miR-19a, miR-150-5p, miR-1229, miR-548c-5p, miR-25-3p, miR-638, miR-17-5p, miR-33a-5p, miR-92a-3p, miR-210-3p, miR-135a-5p, miR-208b, miRNA-21, miR-139-3p, miRNA-31, miR-145, miRNA-92a, mir-92a, let-7g, miR-143, miRNA-181b, miR-21-5p, miRNA-203, miR-21, miR-96, miR-221, miR-139a-5p, miR-196b, miR-338-5p, miR-210, miR-1290, miR-103, miR-720, miR-106a, miR-17-3p, miR-92, miR-125, miR-223, miR-20a, miR-150, let-7a, miR-4516	FOXD2-AS1, QSOX1, NRIR, PKM2, LOC_009459, NNT-AS1, H19, CCAL, UCA1, HOTTIP, PrP(C), CA11-19, MIC-1 (GDF15), IL-6, IL-8, Growth-related gene, product β1, Cyr61, B6-integrin, TIMP-1, RBP4, THBS2, TFF3, COL3A1, COL10A1, AZGP1, Angiopoietin-2 7, Kininogen	CCAT1 UCA1 HOTAIR LOC285194 Nbla12061 RP11-462C24.1 BLACAT1	circ_0004771 circFMN2
Stool	miR-21, miR-29a, miR-135, miR-224, miR-92a, miR-7, miR-938, miR-222, miR-146a, miR-143, miR-138, miR-127-5p, miR-29b, miR-9, iR214, miR-199a-3p, miR-196a, miR-183, miR-17, miR-20a, miR-96, miR-106a, miR-134, miR-135b, miR-221, miR-18a, miR-223, miR-451, miR-144, miR-17-3p, miR-135b-5p, miR-421, miR-27a-3p	Haemoglobin (FIT) M2-PK MMP 9		

TABLE 3 Non-inavsive Protein and miRNA Panel used for CRC detection.

Sample	Protein Panel	miRNA panel
Serum	RBP4 and CEA TFF3 and CEA sDC-SIGN and sDCSIGNR IGFBP-3 and CEA AZGP1, CEA and CA19-9 IGFBP2, DKK3 and PKM2 CEA, hs-CRP, CYFra21-1 and Ferritin	miR-23a-3p, miR-27a-3p, miR-142-5p, miR-376c-3p Let-7a, miR-1229, miR-1246, miR-150, miR-21, miR-223, mir23a miR-19a-3p, miR-21-5p, miR-425-5p miR-301a, miR-23a miR-20a, miR-486 miR-223, miR-92a
Plasma	BAG4, IL6ST, VWF, EGFR and CD44	miR-103a-3p, miR-127-3p, miR-151a-5p, miR-17-5p, miR-181a-5p, miR-18a-5p, miR-18b-5p miR21, miR25, miR18a, miR22 miR-1290, miR-320d
Stool	Complement C3, Lactotransferrin, Haemoglobin subunit α1 and Haptoglobin	(miR-17, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92a miR-144-5p, miR- 451a miR-20b- 5p

nuclear DNA (nDNA). Therefore, investigating hypothetically cell-free mitochondrial DNA (cf-mtDNA) could give an advanced level of understanding, rather than the examination of cell-free nuclear DNA (cf-nDNA). Furthermore, it was reported that mtDNA has a high mutation frequency and in CRC and other cancers fundamental molecular modifications (183, 184). Based on reported research literature, assessment of cf-mtDNA as a significant biomarker is stimulating for liquid biopsies and as a neoantigen due to high copy number might enable discovery of even minor quantities of ctDNA and their molecular modifications. Besides, earlier research has exposed that cf-mtDNA content and fragmentation design distinguish between cancer patients and healthy individuals, therefore also potentially serving as an indicative marker of disease (185–188). Though cf-mtDNA has been not completely categorized yet and an efficient method for comprehensive examination is still missing.

Rigorous research has been done to understand the hereditary risk issues of CRC. Thus far, over 40 nuclear genome alternatives significantly related to CRC risk have been recognized, counting SNP rs10911251, rs1321311, rs1035209, and so on (189, 190). But such loci account for only about 8%–16% of CRC cases, signifying that additional genetic risk factors of CRC still possibly need to be discovered.

Remarkably, numerous somatic mtDNA mutations and copy number alterations have also been commonly recognized in a wide variety of malignancies, including CRC (191). In CRC it was observed that mtDNA copy number is increased during the cancer process. Previous studies by Guo et al. have described that the reduction of mtDNA made by the mitochondrial transcription factor A (TFAM) mutation plays a potential role in cancer progression and resistance to cisplatin in MSI CRC (192). In addition to this, a report from China investigated 104 colorectal cancer patients and found that the percentage of mtDNA deletion of 4977 bp of mtDNA in CRC tissues was significantly reduced (193). Furthermore, recent research revealed that mitochondrial cfDNA had a surprisingly higher plasma copy number in healthy subjects than in CRC patients (188). Though, today the possible contribution of germline mtDNA differences in CRC expansion is a smaller amount of knowledge available including liquid biopsy. Together, we are confident that liquid biopsy is likely to be a substitute standard approach for monitoring the advanced development of genomic changes during cancer progression. Liquid biopsy has revealed remarkable effectiveness in a variety of applications and will contribute to personalized oncology.

Organoids

Tumor organoids were reviewed by Tatullo et al. with 77 references (194). To date, scientific cancer research has been conducted in *in vitro* experiments, performed on tissue culture plates and two-dimensional (2D) samples. In this framework, the development of colonies and spheroids has been determined as morphological indicators of cancer and stemness of cancer cells (195). In the current research scenario, 3D cultures systems have significantly enhanced *in-vitro* tumor models based on new biological mediums that mimic the extracellular environments. Organoids have been described more extensively in many reports in the scientific research literature. The overview of patient-derived organoids (PDO) has allowed for more representative cancer modeling, highlighting their excessive significance in biomedical applications, translational medicine, and personalized therapy approaches (196, 197). Furthermore, patient-derived organoids have certain advantages such as stable morphology, gene expression, and cell signaling, heterogeneity with cancer cells in the tumor, significant drug screening, low cost, and being easily generated “in a dish” (198). The application of the organoid culture method to liquid biopsy is a promising approach that combines the advantages of organoid cultures with the boundless potential of the liquid biopsy component for precision oncology.

Sato and their team first developed an organoid model from mice in the CRC research field and later they also developed an organoid culture protocol that is acceptable and also suitable for colon epithelial cell culture (199). In CRC, PDO developed from metastases taken by serial biopsies at various time points, and various counties of severely pretreated CRC patients were taken as preclinical models in clinical trials studies (200, 201). Those organoids were further treated with anti-cancer drugs, and the outcomes were associated with patients’ responses in clinical trial studies. The outcome suggested the ability of PDO to mimic TME *in vivo*, notable molecular and functional levels, and the most important aspect being to predict patient treatment response (202). Clinically active KRAS signaling suppressors and various drug groupings were observed against non-cancerous colon and CRC organoids (203). In recent research Zhao et al. used the organoid culture approach to identify the metabolic phenotype in cancer stem cells and differentiated cancer cells in CRC (204). To date, only one study has been done on organoids derived from CTCs and it revealed that CTC-derived organoids were more sensitive than Xenograft-derived organoids, to drugs affecting the Survivin pathway, which significantly decreased the levels of Survivin and X-linked inhibitor of apoptosis protein (XIAP), that induce CTC derived organoid death. Based on this first study, future use of the organoid approach to CTCs may open new viewpoints by providing extraordinary visions of the cancer growth and metastatic process, by allowing the discovery of novel CTC markers, beneficial treatment targets, and chemoresistance mechanisms (205).

Notwithstanding organoid significant advantages, patient-derived organoid (PDO) also possesses certain limitations such as abnormalities, noise during drug screening, development and standardization of organoid culture, and lack of major TME

components (206–208). Based on the published literature, PDO is a fascinating *in vitro* model for the development of preclinical drugs in CRC, because of its ability to mimic human physiopathology. Taken together, the potential of the organoid approach for basic and clinical studies of CRC is greater than the treatment of patients with CRC in the new time of personalized medicine. Furthermore, it will open a new door for the liquid biopsy approach using CTCs and/or CTSCs to generate organoid models.

Liquid biopsies and immunotherapy

In the research of CRC treatment, diagnostic and chemotherapy have developed curiously in the last two eras. Still, it is problematic to find minimal residual disease (MRD) essential for primary detection of recurrence of tumors and give suitable drugs timely prior cancer becomes multi-drug-resistant and more aggressive. However, the most thrilling example of change in cancer therapy in the current era has been immunotherapy. Subsequently, with its early approval for the treatment of melanoma, it has become the standard of care for various other tumors. Immunotherapy has also established promising abilities and good tolerance in gastrointestinal (GI)-related cancers (209). All the research conferred so far in CRC are focused either on the association between ctDNA existence and tumor burden or the recognition of molecular modifications that predict response or resistance to targeted agents. The burden of tumor mutations is currently being argued in CRC and various solid tumors were given its association with response to immunotherapy and the current approval of the Food and Drug Administration (FDA) as an agnostic biomarker to access cancer immunotherapy with pembrolizumab or dostarlimab (210, 211). On the other hand, MSI is currently the most applicable potential biomarker for immunotherapy sensitivity in CRC, characteristically measured in solid tissue samples (212). Additional growing manipulation of liquid biopsy in CRC is the examination of methylation biomarkers, which is rapidly developing as an influential approach to early diagnosis and prognosis (213).

MSI colorectal cancer

Microsatellite instability (MSI), also known as a hypermutable phenotype, occurs because of a defective mismatch repair system (dMMR) in approximately 15% of colorectal cancer patients (CRC) (214–216). MSI CRC is most often associated with the proximal colon, increased immunogenicity, and a good prognosis, in contrast to CRC of chromosomal instability (CIN) (also known as stable/low-level microsatellite stable/MSI-low-level [MSS/MSI-L]) CRC which is more commonly found in the distal colon with increased immune tolerance and a poor prognosis (215, 217). Many studies have shown the advantages in detecting MSI status, including prognosis and specific treatment benefits associated with this molecular subtype, with increased survival rates of up to 15% in CRC patients (218, 219). A few studies thus far have illustrated MSI to be a rare occurrence in rectal cancer, and linked to a poorer prognosis with a higher risk of dying (220–222). Better results are observed in locally advanced (stage II/III) MSI CRC compared to CIN CRC, with the recently added benefit of oFDA-approved immunotherapy (i.e. pembrolizumab, nivolumab, and combination nivolumab/ipilimumab) in the treatment of unresectable or metastatic resistant MSI CRC in conventional regimens (223–226). To date, the conventional treatment regimen for rectal cancer continues to be resection surgery, chemoradiation (preoperative), and chemotherapy, with the intolerant response that do not have alternative approved treatment strategies available (227, 228). MSI CRC is known to have a poor response to 5-fluorouracil (5-FU), which is a fluoropyrimidine drug used in the conventional adjuvant treatment regimen of CRC (229). Adverse effects include nausea, diarrhoea, mucositis, neuropathy, neutropenia and more serious complications leading to death have been reported in 1% of patients. Therefore, it is imperative to implement a reliable diagnostic methodology for accurate diagnosis of MSI. Mononucleotide markers have been well described as the most reliable markers for MSI panels, without the need for di-nucleotide markers and matched normal tissue testing (230, 231). Ethnic polymorphisms have also been described in certain markers (eg. African polymorphisms in BAT25 and BAT26) and should therefore be considered when deciding on the implementation of diagnostic markers panel in certain geographical settings (231–233). If instability is required in 30% of markers used in the panel for a diagnosis of MSI, it is important to establish that the markers included are nonpolymorphic in the general population. Additional testing to confirm MSI status is to assess the expression profiles of mismatch repair (MMR) proteins through immunohistochemistry (IHC) (234–236). This is a more cost-effective approach and in addition provides information on the deficient dMMR protein, gaining insight into the possible mechanism of the disease, whether likely sporadic (associated with MLH1 protein loss through MLH1 promoter methylation, and BRAFV600E pathogenic variants) or due to hereditary Lynch syndrome pathogenic variants (MSH2, MSH6, MLH1, or PMS2) (237–239).

MSI CRC is known to have a better response to immunotherapy, and this is due to the active innate inflammatory tumor microenvironment, as a response to the hypermutated phenotype of these tumors (240). The TCGA study revealed that hypermethylated and hypermutated cancers were more commonly associated with the proximal colon and distinct at the genomic level compared to distal colon and rectum cancer (217). This could potentially be due to the difference in the originator cells of the right colon (developed from the midgut) compared to the distal colon (originated from the hindgut) (241,

242). To date, few clinical trials have also begun exploring combination radioimmune therapy, with promising toxicity reports indicating hope for patients with rectal cancer (243). Another remarkable study of a PD-1 blockade (dostarlimab) in the treatment of MSI rectal cancer indicated high sensitivity and 100% complete response rates with no severe adverse events (244). This illustrates the need for more clinical trials in immunotherapy and neoadjuvant therapy with a focus on rectal cancer to be conducted, to provide more effective predictive therapy for the better management and increased survival of these patients. Besides this, the neoantigens currently appear in MSI-H CRC, which is related to a higher tumor mutation burden, so it has potential as neoantigens in the immunotherapeutic strategy for the treatment of various types of CRC. But a liquid biopsy-based examination to assess MSI can successfully assess an extensive subclass of CRC patients, including those with inadequate tissue samples or when protection concerns about invasive surgery arise.

MSS colorectal cancer

Tumors in the distal colon display lower mutational burdens and are less immunologically active, with little to no $CD8^+$ T lymphocyte localization or infiltration. This type is generally referred to as a "cold-tumor" (245). Cold-tumors represent the majority of CRC and mostly do not benefit from immune checkpoint inhibitor (ICI) therapy. Improvement in immune therapeutic strategies includes transitioning "cold-" into immune infiltrated "hot-tumors", and once infiltrated, ensuring an effective inhibitory response on tumor cell activity is attained (245). This is achieved by controlling tumor immunogenicity and the TME by directing the immune system in targeting tumor cells specifically (246). ICIs are designed to inhibit certain receptors such as Programmed-death-1 (PD-1) on T-cells that are controlled by cancer cells to evade immune attack. Monoclonal antibody (mAb) treatment, chimeric antigen receptor (CAR)-T cell therapy, and ICIs are key immunotherapies currently being used against many cancers (247). mAb therapy against the receptor Programmed death ligand-1 (PD-L1) on cancer cells, to block its communication with PD-1 and increase T cell immune response has shown effective in many solid tumors. Adoptive cell transfer (ACT), such as chimeric antigen receptor (CAR) T-cell therapy involving the patient's own T-cells has also gained increased recognition (248). These cells are genetically engineered to include the new CAR, and then re-administered to the patient (247) The CAR increases-affinity and binding of T-cells to target antigens, without the need of the major histocompatibility complex (MHC) receptor. CAR-T therapy has had fewer success rates in solid tumors, mainly due to a suppressive TME (increased cytokine and dense stromal network) (248). Enzymes targeting and degrading stromal matrices (eg. heparanase) have been employed to overcome this hurdle and increase infiltration of CAR T-cells in solid tumors (249, 250). Cancer vaccines have also been introduced as novel immunotherapeutic approaches to target antigens uniquely expressed on tumor cells, thus inducing an anti-tumor immune response in patients (251). In addition, oncolytic viruses destroying cancer cells but non-virulent to normal cells is another immunotherapy strategy (247). Certain virus, have natural tropism to infect certain cells, for example, hepatitis B virus for hepatocytes and parvovirus B19 for human erythroid progenitor cells, and this mechanism has been used to direct virus-mediated cytotoxicity in tumor cells (252). To address effective immunotherapeutic strategies in MSS CRC in future, combination therapy involving two or more approaches would need to be implemented, involving chemotherapy, radiotherapy, mAb, ICI targeted therapy, stromal matrix degradation, oncolytic viral therapy, CAR-T therapy and cancer vaccines (247).

Neoantigen: An emerging concept

Neoantigens have potential high specificity and targeted but are mainly patient-specific and, consequently, are difficult to classify for utility and are mostly remarkable procedures in a cancer patient population. Currently, immunotherapy, inclusive of immune checkpoint inhibitors (ICIs), tumor-specific vaccines, and tumor-infiltrating lymphocytes (TILs) based on neoantigens, has a progressively significant role in cancer treatment (253). The conventional significant cDNA library screening method is labor-intensive, low-throughput, and unable of classifying some altered antigens consequent from GC-rich transcripts and low-expression transcripts (254). However, current scientific developments in next-generation sequencing and a notable improvement in bioinformatics analysis have provided a robust groundwork on which to build these significant efforts. A peptide-based identification method connecting whole-exome sequencing (WES) and MHC-peptide binding prediction algorithms has been effective in identifying neoantigens recognized by TILs in patients with melanoma (255). Neuropeptides are expressed in tumor cells, while healthy cells will not present such antigens. Earlier research on CRC genomics mostly focused on the mechanism of tumor development and progression, with a lower inclusion of neoantigens and neoantigen-based immunotherapy (256). In research, it was observed that certain CRC patients with high microsatellite instability (MSI-H) might benefit from ICI treatment due to the presence of high neoantigens (256). However, not all patients with MSI-H CRC show medical efficacy in ICI treatment. Neoantigen-based immunotherapy is synchronizing with ICI since it does not need a detailed analysis of the patient's MSI status or tumor mutation burden (TMB)

(257). The tumor-specific landscape of neoantigens makes them significant perfect targets for antitumor immunotherapy and has been investigated for the treatment of CRC in a variety of basic and clinical immunotherapy studies. The average TMB of CRC was classified seventh among 30 of the most common categories of tumors. A previous study by Aleksandrov L. B observed that approximately 16% of CRCs have a TMB of >12 mutations per 106 base pairs, which are identified as extremely mutated tumors (258). Patients with higher TMB might have more potent neoantigens that can be used for the clinical approach in CRC. For MSI-H CRC, frameshift mutations generally instigated by INDELs can lead to the creation of novel frameshift peptides (FSP), which are the key cause of neoantigens in CRC. Frameshift mutations can be frequently initiated in DNA segments or genes with a significant biological role in maximum MSI-H CRC. These genes play a vital role in epigenetic regulation, DNA repair, signal transduction, cell apoptosis, and miRNA processing. Besides frameshift mutation currently, it has been described that single-nucleotide variants (SNVs) in genes like KRAS, PIK3CA, PCBP1, and CHEK2, are related to the creation of the 10 most frequent neoantigens. In Table 4, we have listed the mutated antigens that were studied in CRC tissue (259).

One main hurdle for personalized neoantigen-based immunotherapy is the availability of tumor biopsies. To date, neoantigens are usually recognized from genomic profiling of various tumor biopsies (260). Although this predictable approach is time-consuming, invasive, with a low positivity rate, and in the most challenging case where repeated sampling is mandatory or there is an inadequate sample, it is more common with frequent and metastatic cancers. Specifically, at the top immune checkpoint, significant inhibitors can be more effective in the presence of natural neoantigens (261, 262). Based on the current scenario, liquid biopsies can be a good replacement for determining potential neoantigens as budding targets for immunotherapy in numerous cancers. Although there is a certain restriction in the detection of genomic mutations with very low allele occurrence in the plasma sample, the dependability of genetic information has been described concerning the use of liquid biopsy (263). Thus, based on current research on liquid biopsies, valuable visions could be served for making treatment choices using neoantigen.

TABLE 4 List of mutated antigens found in CRC.

Frameshift Mutation Genes	SNVs Genes
OGT	KRAS
TGFβRII	PIK3CA
BAX	PARVA
MSH3	G3BP1
FTO	ACTR10
Caspase 5	RAE1
	PDP1
	QRICH1

Immune cells

The TME generates a potential protective shell in which cancer cells easily and rapidly gather gene alterations and immune escape. Generally, this process occurs in the early stage of cancer, the immune response created by immune cells in the TME has antitumoral properties (264). Collectively, evidence has revealed that TME contains NK cells, $CD8^+$ cytotoxic T cells, M1 macrophages, T helper-1 cells, and antigen-presenting cells (APCs) which act as tumor foes and suppress tumor development. Neutrophils, tumor-associated macrophages (TAMs), $CD4^+$ T helper-2 cells, and regulatory T cells (Tregs) are crucial components for reducing the immune suppression environment, inhibiting cancer cell survival and progression, in addition to helping to avoid immune devastation (265) (Figure 6). In metastatic CRC, it has been confirmed that tumor behavior with the lowest tumoricidal immune infiltrates shows a higher risk of tumor replacement (266). CD8+ T and CD4+ T cells are the utmost powerful cytolytic cell subcategory. Cytotoxic processes are supported by some constituents shaped by CD8+ T cells, such as granzymes, perforin, Fas ligand (FasL), and TNF-α (267). Recent research established that patients with promising CRC regularly have tumor immune cell infiltrates with higher cytolytic events (268). But, the percentage of cytotoxic T cells number decreases as TNM-stage increases in CRC (269).

In humans, Treg cells are the main source of IL10. IL10 has numerous effects on immune cells, including decreasing the cytotoxic activity of CD8+ T cells, down-regulating MHC-II-restricted antigens, preventing the synthesis of IFN-γ or TNF-α, and hindering the effector roles of dendritic cells and other CD4 + T cell subsets (Th1, Th2, or Th17 cells) (270, 271). Based on the results of numerous reviews, there is still support that tumor infiltration of Treg cells possibly extends the survival of CRC patients (272). In concept, Treg cells are susceptible to apoptosis in CRC tumors that could negatively regulate the expression of IFN-γ, TNF-α, and IL-2 by tumoricidal T cells (273). Collectively this suggested that the concentration of Treg cells along with their connected cytokine profiles in cancer should be determined together in a liquid biopsy-based approach to increase the use of Treg cells in predicting CRC prognosis.

B cells consist of diverse subcategories and govern antibody production, antigen appearance, and immunosuppression (274). A currently published study on colon cancer has reported that a high concentration of tumor B cells may provide for promising clinical outcomes only in patients with right-sided colon cancer (275). Furthermore, the higher expression of CXCL9 and CXCL10 in CRC tumors can also attract regulatory B cells (Breg), although

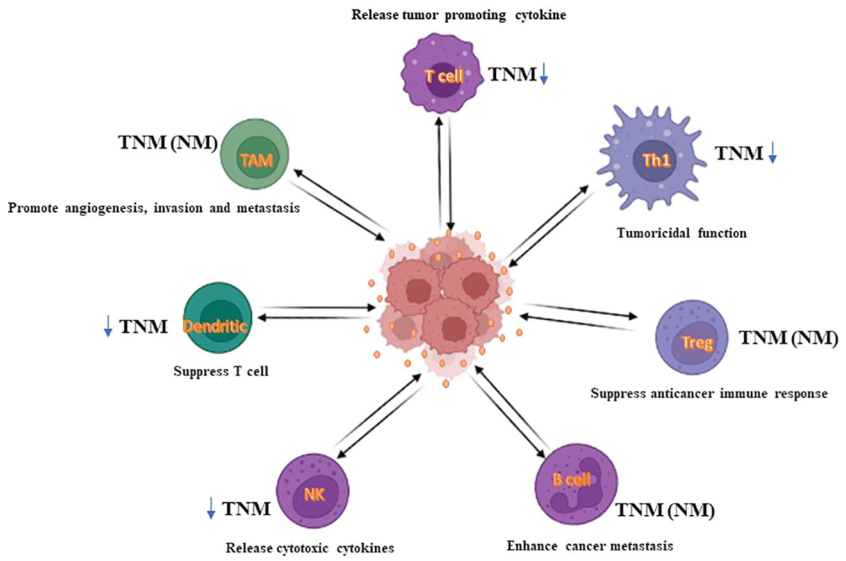

FIGURE 6
The impact of immune infiltrates on CRC. In CRC, immune infiltrates can impact CRC cell death, either directly or *via* tumoricidal T cells (TCT), and consequently affect tumor progression. For example, cytotoxic T cells, macrophages, and NK cells can exert a cytolytic effect on CRC cells. For other populations of cells, such as Treg, B cells, dendritic cells, or M2-like macrophages, generally impact CRC cell death by mediating the tumoricidal activity of TCT cells. Herein, Treg, regulatory B cells, immature dendritic cells, and macrophages enable TCT cells to be exhausted, thus causing substantial progression in CRC tumors. Accordingly, immunoscore system using immune cells could deliver insights into a novel liquid biopsy approach as a diagnostic tool.

such chemoattractants are also effective in employing tumoricidal T cell functions (276). Assessing the concentrations of tumoricidal T cells, Treg cells, and B cells together could significantly improve the prediction of the prognosis of CRC. In addition to this component, natural killer cells (NK) also play a cytolytic role in TME. In CRC, it was found that alteration of MHC-I functions, resulting in NK cells, will reduce its development and decrease the production of IFN-γ, GZMB, and perforin production (277). Surprisingly, in CRC metastasis, it was observed that the number of tumor-formed NKT cells was markedly decreased compared to normal tissue (278). However, it is at minimum knowledge that NK cell infiltration into CRC at progressive disease phases is challenging. In TME, one of the most significant components of dendritic cells (DCs) is specialized antigen-presenting cells in the human body. Previous data suggested that in CRC tumor infiltration of DCs is negatively related to tumor phases because this growth of DC cells with various phenotypes will result in a poor prognosis of CRC (279). Fundamentally, it is indicated that mature or immature DC could have various effects on CRC development. Lastly, the major component of Tumor-associated macrophages (TAMs) are dangerous immune infiltrates in cancer phenotype. In CRC, numerous studies have shown that a high number of CD68+ macrophages in tumor IM expect a promising prognosis (280, 281). Furthermore, Itatani et al. observed that by improving the production of metalloproteinase-9, CCR1+ macrophages support the invasion of CRC (282). Similarly, to CCL2 and CCL15, CCL5 helps as another significant chemokine that controls the development of CRC (283). Besides, in CCL5-deficient mice, xenografted CRC tumors show a high amount of tumoral CD8+ T cells, signifying that CCL5 at minimum influences T cell infiltration (284).

The Immunoscore system delivers insights into a novel approach for consistently predicting CRC diagnosis, particularly since this tool has the potential to screen immunotherapy components. On the other hand, Immunoscore combined with diagnostic tools such as liquid biopsy, and a neoantigen-based approach provides for better CRC treatment, especially for immunotherapy.

Future perspective and conclusion

The prognosis of individuals with CRC has substantially improved in the current era due to the significant improvement and expansion in diagnostic and therapeutic approaches. However, early prediction, diagnosis, and treatment monitoring of CRC have various lacunae; due to this, many patients die each year. In recent years, the field of liquid biopsy has grown rapidly because it is noninvasive, overcomes tumor heterogeneity, and can allow real-time intensive care of tumor development, recurrence, or therapeutic response (285). This is the reason that recently there are numerous ongoing clinical trials from the US National

Laboratory of Medicine (NIH) on liquid biopsy-based approaches to detect CRC. Presently, numerous efforts have been made utilizing CTCs, CAFs, exosomes, immune cells, neoantigens, mtDNA, and ctDNA isolation and characterization-based approaches to detect and treat CRC; and which have shown to be highly sensitive and effective. In addition, genes and proteins expressed by these components can also be used for early CRC detection and therapy. However, a CTC end point value for the clinical evaluation of CRC patients' progression and prognosis is still not adequately developed owing to sampling issues, storage conditions and timing of biopsy; and most importantly enrichment procedures (286, 287). Therefore, it is important to develop a CTC capturing platform that is more precise and effective. Additionally, recent studies on CTCs/cCAFs clusters open a new path for developing an additional personalized and detailed treatment plan for each cancer patient. But there are still several lacunae on the biology of CTCs clusters, and specifically on the heterotypic CTCs-CAFs clusters, that need to be investigated to recognize the mechanism of cellular aggregates and their role in metastasis. Furthermore, it is also important to see which of the CAF-derived signals improve CTC survival and cancer cell growth, besides to govern the efficient alterations between homotypic CTCs clusters and heterotypic CTCs-CAFs clusters. Another significant component of liquid biopsy is the exosome, that has a potential role in tumor initiation, development and metastasis, including EMT, tumor angiogenesis, extracellular matrix remodeling, organ-specific metastasis, and immune evasion. The advantage of exosomes is that they are easier to isolate than CTCs and cfDNA in tumors; a current era is improved and more research will be focused on exosomes in the diagnosis of cancers at an early stage in the future. But there is still uncertainty in clinical approaches due to low effectiveness and informal phagocytosis by the immune system. So, based on evidence, indepth research should be undertaken to solve this hindrance and develop precise clinical applications of exosomes. Furthermore, analysis of ctDNA is a most promising component of liquid biopsy that can play a critical role in numerous characteristics in the clinical management of patients with CRC (288). Furthermore, TMB in ctDNA and immune check point proteins in CTCs show significant roles in tumor immunotherapy. However, due to inadequate and partial knowledge of molecular mechanisms, ctDNA as liquid biopsy has not yet been applied in immune-oncology in the clinic; however, promising available data and advanced noteworthy technologies and methods recommend that this approach certainly has a plausible role in CRC patient therapy. Based on our review, we found that a higher copy number of mtDNA significantly promotes cell proliferation, apoptosis resistance, and CRC metastasis, thus also providing a novel indication for this process as a drug target and prediction of neoantigens in CRC treatment (188). Existing genomic research has revealed that there are many hotspot mutations in significant driver genes; and the neoantigen epitopes made by these mutations are vital "public" immunotherapy targets as a liquid biopsy approach. More recently, liquid biopsy-based neoantigens

TABLE 5 Advantages and disadvantages of liquid biopsy components.

Component	Advantage	Disadvantage
ctDNA	• Well established methods for detection of tumor-specific genetic abnormalities with greater sensitivity • Analyze cancer origin and prediction of drug effectiveness • Detection of acquired resistance and/or minimal residual disease • Cancer progression and metastasis monitoring	• Unsuitable for functional test due to impaired detectability (low ctDNA abundance) • Background noise from typical cell-free DNA • Difficulties in standardizing procedures
mtDNA	• Compared to nuclear DNA, single cell contains several thousand copies of mtDNA • Higher sensitivity • Enable detection of even small amounts of molecular alterations due high mutation rate • Potential prognostic marker due to differential fragmentation pattern between cancer patients and healthy individuals	• Not fully characterized yet • Lack of optimized protocol for cf-mtDNA • Large scale prospective studies are needed
CTCs	• Feasible for molecular and morphological identification • Possible prognostic and/or predictive markers for monitoring cancer progression and metastasis • Potential therapeutic targets • Useful for *in-vitro* culturing to test drug sensitivity • DNA, RNA and protein profiling	• Low specificity- especially in early stage settings • Difficulties with detection method standardization due to EMT and heterogenous biomarkers for identification • Short half-life
cCAFs	• Well established role in cancer progression and metastasis • Advantage survival in circulation by forming clusters with CTCs and/or CSCs • Potential therapeutic target • Detection and monitoring of minimal residual disease • Potential biomarker for early detection and prognosis • Prospective model for better understanding of TME	• Larger confirmatory studies are needed • Lack of robust and standardized methods for detection

are a new immunotherapeutic approach for the treatment of various types of CRC. Though, there are still numerous challenges such as tissue biopsy and identification, which still require further research as explored form of liquid biopsy (Table 5). The worldwide replacement of tumor biopsies with liquid biopsies appears idealistic; however, with a range of approaches using CTCs, CAFs, ctDNA, exosomes, mtDNA and neoantigen, it seems highly likely that useful tools will be developed for CRC with applications in early detection, postoperative monitoring, treatment response and therapeutic resistance. In summary, liquid biopsy is an important part of precision medicine and is held to be a clinical reality soon.

Author contributions

SM and CP conceptualized and designed the manuscript. SM, KB and MM contributed in literature review and drafted manuscript. MJM created all figures. SM, KB, RS and CP contributed to critical review and finalized the manuscript. All authors contributed to manuscript and approved the submission.

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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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Correlation between Metabolite of Prostaglandin E2 and the incidence of colorectal adenomas

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Colorectal cancer is a common malignancy, and the incidence and mortality rates continue to rise. An important factor in the emergence of inflammation-induced colorectal carcinogenesis is elevated cyclooxygenase-2. Prostaglandin E2 (PGE_2) over-production is frequently equated with cyclooxygenase-2 gene over-expression. PGE_2 can be assessed by measuring the level of prostaglandin's main metabolite, PGE-M, in urine. Colorectal adenoma is a precancerous lesion that can lead to colorectal cancer. We conducted research to evaluate the association between urinary levels of the PGE-M and the risk of colorectal adenomas. In a western Chinese population, we identified 152 cases of adenoma and 152 controls patients without polyps. Adenoma cases were categorized into control, low-risk and high-risk groups. There was no significant change in PGE-M levels, between the control group and the low-risk adenoma group. In the high-risk group, the PGE-M levels were 23% higher than the control group. When compared to people with the lowest urine PGE-M levels (first quartile), people with greater urinary PGE-M levels had a higher chance of developing high-risk colorectal adenomas, with an adjusted odds ratio (95% CI) of 1.65 (0.76-3.57) in the fourth quartile group, (p= 0.013). We conclude urinary PGE-M is associated with the risk of developing high-risk adenomas. Urinary PGE-M level may be used as a non-invasive indicator for estimating cancer risk.

KEYWORDS

colorectal adenoma, prostaglandin E2, PGE-M, Colorectal cancer, cancer risk, bio-markers

1 Introduction

Colorectal cancer incidence around the world has increased in tandem with an increase in respective Human Development Indices. This disease has the third highest incidence rate and the second highest fatality rate, globally (1).Similarly, colorectal cancer is becoming more common in China (2). Numerous investigations on the adenoma-carcinoma sequence have conclusively shown that between 60% and 90% of sporadic colorectal cancers result from adenomas that have undergone malignant transformation (3, 4). Therefore, the risk of cancer can be decreased by identifying risk factors for adenomas and preventing their growth (5).

Increasing evidence points to the possibility that inflammation increases the vulnerability of developing colorectal malignancies (6, 7). The enzyme cyclooxygenase-2 (COX-2), mediates the relationship between cancer and inflammation and is 50-85% more abundant in patients with colorectal malignancies (8, 9). COX-2 is a key rate-limiting enzyme for the conversion of arachidonic acid to prostaglandins (PG), and when COX-2 gene expression is elevated, more prostaglandin E2(PGE_2) is produced (10, 11). Clinical studies have demonstrated that non-steroidal anti-inflammatory drugs (NSAIDs) lower the chance of developing adenoma and colorectal tumors, and their effects are linked to the suppression of prostaglandin 2 and cyclooxygenase-2 (12–15). The pro-inflammatory mediator PGE_2 has been shown to be able to support colorectal tumor progression through a variety of mechanisms. PGE_2's primary effects include inhibiting apoptosis, promoting angiogenesis, and encouraging epithelial cell proliferation、 survival、 migration、 invasion、repair and regeneration (16–20).It is the most abundant prostaglandin found in colorectal cancer patients (21).

Multiple lines of research indicate that COX-2-derived PGE_2 has a role in the growth of colorectal adenomas and can predict the risk of developing colorectal cancer (22). However, epidemiological evidence directly linking urinary PGE_2 levels to the risk of colorectal adenoma is lacking in China. Direct measurement of unstable PGE_2, however, is unreliable. Currently the best method for measuring systemic PGE_2 synthesis *in vivo* to assess the quick metabolism of PGE_2 by 15-hydroxyprostaglandin oxidase to form stable 11 alpha-hydroxy-9,15dioxo-2,3,4,5-tetranorprostane-1,20-dioicacid (PGE-M) (23). A nested study design has shown a connection between Chinese women's urinary PGE-M levels and their chance of developing colorectal carcinomatosis (24). By examining samples and data gathered from colorectal adenoma patients and a healthy control population in Guizhou Provence, the present study evaluates the relationship between urinary PGE-M levels and the risk of colorectal adenoma in a in western China population. This study also aims to provide new strategies and tools for implementing interventions in the early stages of tumors development.

2 Materials and methods

2.1 Study population

Study participants were chosen from the Guizhou Cancer Center, Guizhou Provincial People's Hospital, and Songtao Miao Autonomous County People's Hospital in Guizhou province to screen for colorectal adenomas. Patients were initially seen at the endoscopy centers of the aforementioned hospitals. Participants were 18-75 years of age and in generally good health with no vital organ failures. According to WHO guidelines, a colorectal adenoma diagnosis was made, and the degree of neoplasia was assessed. The following criteria was used to diagnosis colorectal adenoma: ①Adenoma was verified by a pathological biopsy, ② villous adenoma or mixed adenoma with more than 25% villous-like features was identified, ③high-grade epithelial neoplasia was identified. Patients with colorectal adenoma who met the aforementioned diagnostic standards had a subsequent colonoscopy adenectomy, and histology was used to confirm the diagnosis. All specimens were examined by two or more experienced pathologists in the hospital's pathology department. Exclusion criteria for our study included those with a history of familial adenomatous polyposis (FAP), inflammatory bowel disease, hereditary non-polyposis colorectal cancer (HNPCC), Turcot syndrome, severe cardiovascular disease, recurrent adenoma with a confirmed diagnosis, colorectal cancer, and tumors in other organs. Because NSAIDs affect PGE-M levels, subjects had used aspirin or any NSAID for at least 48 hours prior to colonoscopy. They were ineligible for analysis. Our study also excluded participants. Because they used any dose of NSAIDs, including aspirin, for 3 days or more in the 3 months prior to enrollment or 3 days per month or used NSAIDs for 36 days in the past year. Finally, a sample of 304 participants' data was kept for analysis. The study protocol was approved by the Ethics Committee of Guizhou Provincial People's Hospital. A signed informed consent was required for all study subjects.

2.2 Sample collection

There were 412 study participants who provided urine samples; however only 304 samples were usable, due to sample damage and inconvenience of follow-up during the novel coronavirus pandemic. There were 152 cases identified as negative controls. This meant they did not have any polyps at the endoscopic screening. Patients with single tubular adenomas with a maximum diameter of less than 1 cm were categorized as low-risk cases, whereas those with a maximum diameter of more than 1 cm and/or histology of tubular villi, villi, and any multiple adenomas were categorized as high-risk cases (22, 25, 26). Table 1 displays the characteristics of the three categories. To stop the oxidation of unstable metabolites, urine samples were taken in sterile cups containing 125 mg of ascorbic acid. Following collection, samples were kept chilled (at about 0 to 4°C) in a portable foam box with an ice pack before being processed within 6 hours for long-term storage at -80 ± 5°C. Each participant had a biospecimen collection form filled out at the time of sample collection, which listed the day and time of sample collection as well as any drug usage within the previous 48 hours of the colonoscopy.

We developed the questionnaire for this study based on the food frequency questionnaire (SQFFQ) used in the 2002 Chinese population dietary survey methodology, and made appropriate adjustments to incorporate the regional dietary habits of Guizhou.

TABLE 1 Baseline characteristics of colorectal adenoma cases and matched controls.

Baseline Characteristics	Control	Case		p^a
		Low-risk adenoma	High-risk adenoma	
n	152	59	93	
Age, mean(SD),years	57.48(12.69)	60.17(10.09)	61.95(8.81)	0.009
Sex, male (%)	65.8	69.5	75.3	0.307
Education, <high school (%)	58.6	64.4	54.8	0.518
Ever smoked regularly (%)	53.3	64.4	61.3	0.249
Ever drank regularly (%)	20.4	25.4	26.9	0.469
History of hypertension (%)	30.3	28.8	45.2	0.035
History of diabetes (%)	9.9	10.2	20.4	0.043
BMI, mean(SD), kg/m^2	25.77(6.21)	23.96(3.34)	25.89(6.69)	0.104
Calcium intake, mean(SD),g/d	664.73(243.32)	657.20(185.95)	707.71(266.01)	0.317

P^a values were obtained by chi-square test for categorical variables, and analysis of variance for age, BMI, and calcium intake.

All participants completed the questionnaire at the time of study enrollment. The first section evaluates the annual average food consumption, including average intake, frequency of consumption, etc.; the second section tracks nutrient supplement usage, including the name of the supplements, dose, and regimen. The Chinese Food Composition Table (2nd edition) and Nutrition Calculator V2.7.3 were used to convert nutrient intakes, to evaluate the overall amount of calcium consumed through diet and dietary supplements. Information on medical history, drug usage, demographics, anthropometrics, daily food habits, physical activity, and other lifestyle factors were also included as added content to this questionnaire.

2.3 Laboratory measurement

PGE-M was measured using liquid chromatography/tandem mass spectrometry to determine the endogenous production of PGE_2 in humans (27). Briefly, urine was placed in a 10 mL polypropylene tube at room temperature, and then a sample of 1.0 mL was acidified to pH=3 by adding 1.0 mol/L HCl. Next, endogenous PGE-M was converted to methoxime derivatives and treated with 1600 mg of methoxamine hydrochloride in 10 mL of 1.5 mol/L sodium acetate solution (pH=5). The methoxylated PGE-M was dissolved in 8 ml of water after 1 hour of greenhouse incubation, and the aqueous sample was then transferred to C-18 Sep-Pak that had been prepared with 5 ml of methanol and 5 ml of water (pH 3). Sep-Pak was then eluted with ethyl acetate. Thermo SCIENTIFIC Hypersil GOLD (1.9 μm, 2.1×50 mm) column linked to a TSQ-Altis, and Thermo Fisher mass spectrometry pump was then used for liquid chromatography. Heated electrospray ion source was used as the ionization technique. The mass to charge ratios (m/z) monitored were 385.3 ~ 336 and m/z 385.3 ~ 367 for endogenous PGE-M, in the selected response monitoring (SRM) mode. The ratio of the mass spectral peak regions of the m/z 336 and m/z 367 ions was used to calculate the amount of endogenous PGE-M. With a coefficient of variation of 4.1% between batches and 8.7% within batches, the lower limit of detection for PGE-M was set at 2.00 ng/ml. There were no incidents that compromised data integrity or quality throughout the experiment. The quality control samples' identities and the status of the urine samples used in the study were both unknown to the laboratory staff. Additionally, Urinary creatinine was measured using a Sigma kit (Sigma Co., Inc., St. Louis, MO, USA). The levels of urinary creatinine were determined and reported as standardized PGE-M values, PGE-M (ng)/creatinine (mg).

2.4 Statistical methods

Selected baseline characteristics for cases and controls were computed as means, standard deviations, and percentages. We compared the means of age, body mass index (BMI), and calcium intake data between case and control participants using analysis of variance. To compare categorical variables, we employed the chi-square test. Urinary PGE-M levels for each sample were normalized using the urinary creatinine level of the sample and expressed as ng/ml creatinine. The PGE-M data in urine were skewed to the right; therefore, the median, interquartile range, and geometric mean were estimated for descriptive statistics. After adjusting for age, sex, smoking status, alcohol use, education, and prior hypertensive diabetes mellitus, Wilcoxon rank-sum tests and log-transformed linear regression models were used to analyze differences in PGE-M levels between groups. The PGE-M concentrations in the control group's quartile distribution served as the basis for establishing cut points for categorical variables. The odds ratio (OR) and 95% confidence interval (95% CI) between urine PGE-M levels and the risk of colorectal adenoma were calculated using logistic regression models. Trend p-values were derived by using categorical variables as continuous parameters of the model and passing the linear trend test. We also stratified associations by subgroups, such as BMI, sex and calcium intake level, in order to focus on these factors' influence

on the association of urinary PGE-M levels with the incidence of high-risk colorectal adenomas. We performed all analyses using SPSS 26.0 (SPSS Inc, Chicago, IL, USA), and P values ≤0.05 (two-sided probability) were interpreted as statistically significant for all analyses.

3 Results

3.1 Baseline characteristics

In this investigation, 304 patient urine samples were examined. We found that the sample group had 153 controls, 59 low-risk, and 93 high-risk cases. Table 1 displays the characteristics of the cases and the controls. The high-risk adenoma group had a greater prevalence of diabetes mellitus, hypertension, and a higher BMI when compared to the control group. Low-risk adenomas were more prevalent in people with lower education levels and low calcium intake. In contrast to controls, patients with adenomas were more likely to be male, smokers, and drinkers, although the difference was not statistically significant.

3.2 Baseline levels of urinary PGE-M

Table 2 shows the baseline urinary PGE-M levels. Urinary PGE-M levels in the low-risk adenoma patients' group did not differ statistically from those in the control group. However, urinary PGE-M levels were higher in individuals with high-risk adenomas. patients with high-risk adenomas had urinary PGE-M levels 7% and 23.56% higher than patients with low-risk adenomas or control group patients, respectively (p=0.04).

3.3 Association between urinary PGE-M and incidence of colorectal adenoma

The Spearman correlation coefficients between urine PGE-M level and several lifestyle factors are shown in Table 3. The results showed a direct correlation between PGE-M and age, gender, and smoking status. We further analyzed urinary PGE-M levels and the risk of developing colorectal adenomas (Table 4). High-risk adenomas were more likely to occur in patients with higher urine PGE-M concentrations (p =0.013-0.016). The highest, fourth quartile, urine PGE-M levels were associated with a 1.65-fold higher incidence of high-risk colorectal adenoma compared to the lowest urinary PGE-M levels. Results did not indicate an association between urine PGE-M levels and an increased incidence of low-risk adenomas.

3.4 Association of urinary PGE-M level with incidence of high-risk colorectal adenoma, stratified by BMI, gender, and calcium intake

Urinary PGE-M levels were tested in relation to the incidence of high-risk colorectal adenoma, stratified by BMI, gender, and calcium intake (Table 5). Within the highest PGE level, fourth quartile, PGE level subgroup, women had a stronger association with the incidence of high-risk colorectal adenomas (adjusted OR, 3.72, 95% confidence interval, 0.54-25.45) than did males (adjusted OR, 1.75; 95% confidence range, 0.66-4.61). Although not statistically significant, the patient subgroup with BMI ≥25 (kg/m^2) had OR changing from 1.0 to 3.46, to 1.50, to 2.30 for each increasing PGE-M quartiles respectively. Body weight may modify the association between urinary PGE-M and incidence of high-risk colorectal adenoma. The present study did not find a significant interaction between BMI, gender, calcium intake and PGE-M in the high-risk adenoma group (P for interaction >0.05).

4 Discussion

In the current investigation, we discovered a positive correlation between high urine PGE-M levels and the incidence of high-risk colorectal adenomas, but no such correlation was found for single tubular adenomas smaller than 1 cm. These findings imply that urine PGE-M level may be a helpful non-invasive indicator of adenoma risk. Additionally, we discovered that individuals with above-average or low body weight had a strong correlation with PGE-M.

Numerous pieces of evidence point to colorectal adenoma as a prominent precancerous condition of the colorectum. Colorectal adenoma has a complicated etiology, involving a number of biological pathways, one of which has been demonstrated to be

TABLE 2 Baseline levels of urinary PGE-M (ng/mg creatinine) in cases and controls.

Study Group	n	Median (Q1, Q3)	Difference (%)	p[a]	Geometric mean(95%CI)	Difference (%)	p[b]
Controls	152	10.54(5.73,16.66)			9.76(11.25-14.76)		
Cases							
High-risk adenoma	93	11.28(6.07,25.41)	7.02	0.11	12.06(13.71-21.84)	23.56	0.04
Low-risk adenoma	59	8.70(6.11,15.79)	17.46	0.51	9.08(9.27-12.97)	6.97	0.68

P[a] values were calculated by Wilcoxon signed rank test.
P[b] Linear regression models from log-transformed PGE-M levels, adjusted for age, sex, smoking status, alcohol consumption, education, and previous hypertensive diabetes.
Difference= (geometric mean or median of risk groups - geometric mean or median of control groups)/(geometric mean or median of control groups).

TABLE 3 Spearman's correlation coefficient between urinary PGE-M and several lifestyle factors.

Variable	PGE-M	p
PGE-M (ng/mg Cr.)	1.00	
Age	-0.126	0.029
Sex, male	0.215	<0.001
BMI (kg/m^2)	0.085	0.142
Ever smoked regularly	0.115	0.045
Ever drank regularly	0.046	0.423
History of hypertension	0.027	0.642
History of diabetes	0.068	0.234
Calcium intake(mg/d)	0.066	0.250

COX-2 (8, 9). Arachidonic acid is one of the fatty acid substrates that are converted by COX-2, an inducible isoform of COX, into pro-inflammatory prostaglandins (7). PGE_2 is a crucial mediator of the proto-oncogenic actions of COX (28).Therefore, COX-2 inhibitor use lowers urine PGE_2 levels, which lowers the incidence of colorectal cancers and adenomas (12, 13). The primary urinary PGE_2 metabolite is PGE-M. Additionally, earlier research by others has demonstrated an association between the down-regulation of 15-Hydroxyprostaglandin dehydrogenase (15-PGDH) expression and activity in colorectal cancer and the generation of the urinary PGE_2 metabolite PGE-M (29–31).

It was found in a prospective trial of Chinese women that baseline urine PGE-M was most likely a urinary marker for early prediction of CRC and was linked to a high likelihood of advanced colorectal cancer diagnosis (24). In several earlier investigations, it was discovered that patients with advanced adenomas and numerous tubular adenomas had much greater PGE-M levels than the controls group patients (26, 32, 33). Therefore, based on the quantity, size, and complexity of the adenomas, we categorized the cases in this study into high-risk, low-risk and control groups. Our results confirmed this association between PGE-M and high-risk adenomas. We observed higher levels of urinary PGE-M in the western Chinese population in the high-risk of adenoma group, compared to the low-risk group. Calcium may control the inflammatory response affecting colorectal adenomas by affecting a variety of mechanisms including bile acid catabolism, immune regulation, and fatty acid metabolism (34). The present study also considered the influence of calcium intake. However, only the potential effect of calcium intake on correlation among US registered female nurses was assessed in the relevant study ($p>0.05$) (22). Our results are in line with those of earlier research, although the associations between PGE-M level and the low and high-risk groups was not significantly changed by calcium intake levels. This is the first epidemiological study of calcium intake levels, urinary PGE-M levels, and risk of colorectal adenoma in western China. Currently, calcium is still one of the most deficient nutrients among Chinese residents, and this situation may be even more serious in western China (35). We hypothesize that high levels of PGE_2 may be determined by a combination of calcium deficiency and individual genetic susceptibility. Therefore, further studies are needed to explore and reveal the mechanisms and significance of calcium intake levels related to PGE_2 and colorectal adenoma, and identify key genes for calcium re-absorption.

In this study, we had several advantages. These included the random recruitment of participants in a large sample database at the Guizhou Cancer Center. This allowed us to collect patient urine samples before diagnosis and avoid selectivity bias. All participants received an endoscopy, and all adenomas excised from the case group underwent a pathological evaluation, which contributed to the accuracy of the groups. The Tennessee Colorectal Polyp Study in

TABLE 4 The relationship between baseline urinary PGE-M levels and incidence of colorectal adenoma.

Study Group	PGE-M(quartile)				p for trend
	Q1(low)	Q2	Q3	Q4	
Controls					
n	38	38	38	38	
High-risk					
n	19	27	14	33	
OR (95%CI) [a]	1.00(reference)	1.42(0.69-2.98)	0.74(0.32-1.68)	1.74(0.85-3.58)	0.016
OR (95%CI) [b]	1.00(reference)	1.16(0.53-2.52)	0.75(0.31-1.80)	1.65(0.76-3.57)	0.013
Low-risk					
n	12	22	12	13	
OR (95%CI) [a]	1.00(reference)	1.83(0.80-4.23)	1.00(0.40-2.50)	1.08(0.44-2.68)	0.222
OR (95%CI) [b]	1.00(reference)	1.80(0.76-4.28)	1.00(0.38-2.63)	1.09(0.42-2.63)	0.321

a: OR and 95 CIs from conditional logistic models.
b: OR and 95 CIs from conditional logistic models corrected for BMI, smoking, alcohol consumption, education, hypertension, and diabetes

TABLE 5 Association of urinary PGE-M levels with incidence of high-risk colorectal adenoma stratified by BMI, gender, and calcium intake.

	PGE-M(quartile)				p for trend	P for interaction
	Q1(low)	Q2	Q3	Q4		
BMI<25(kg/m^2)						
Case/Controls	14/18	12/24	7/18	21/22		
OR (95%CI) [a]	1.00(reference)	0.49(0.17-1.39)	0.44(0.13-1.41)	1.25(0.48-3.26)		
					0.82	0.11
BMI≥25(kg/m^2)						
Case/Controls	5/20	15/14	7/20	12/16		
OR (95%CI) [a]	1.00(reference)	3.46(0.91-13.16)	1.50(0.33-6.76)	2.30(0.58-9.20)	0.57	
Males						
Case/Controls	11/19	23/21	9/30	27/30		
OR (95%CI) [a]	1.00(reference)	1.90(0.70-5.16)	0.52(0.17-1.63)	1.75(0.66-4.61)	0.12	
						0.05
Females						
Case/Controls	8/19	4/17	5/8	6/8		
OR (95%CI) [a]	1.00(reference)	0.19(0.02-1.38)	0.56(0.06-5.25)	3.72(0.54-25.45)	0.12	
Calcium intake<613(mg/d)[b]						
Case/Controls	8/18	15/18	2/18	15/22	0.09	
OR (95%CI) [a]	1.00(reference)	2.10(0.63-7.02)	0.21(0.03-1.43)	1.38(0.42-4.60)		
Calcium intake>613(mg/d)[b]						0.12
Case/Controls	11/20	12/20	12/20	18/16		
OR (95%CI) [a]	1.00(reference)	0.92(0.30-2.80)	1.32(0.43-4.01)	2.01(0.68-5.94)	0.77	

a: OR and 95% CI from conditional logistic models corrected for BMI, smoking status, alcohol consumption, education, hypertension, and diabetes.
b: Median calcium intake of the control group was taken

the United States has conducted a number of studies on PGE-M and the risk of acquiring colorectal adenomas (26, 32). To further these earlier findings, the present study was carried out in a population in western China. For the first time in China, we used liquid chromatography/tandem mass spectrometry to detect PGE-M levels. It must be acknowledged that our study also has many limitations. For example, our sample size was constrained after relevant cases were excluded due to individual differences or other factors like the use of NSAIDs. Second, the long-term association between urinary PGE-M levels and the risk of developing colorectal adenoma needs to be further studied because, further studied only measured the PGE-M levels from urine samples at one time point. Although we studied calcium intake level and PGE-M levels' influence on the incidence of colorectal adenoma, we could not assess how calcium deficiency alters urinary PGE-M level and colorectal adenoma incidence. A more comprehensive understanding of colorectal adenoma development at the molecular and genetic level is needed.

In conclusion, this study evaluated the association of urinary PGE-M levels with increased incidence of high-risk colorectal adenomas in a western Chinese population. Because high-risk adenomas have the greatest likelihood of malignant development, it may be possible to intervene early and prevent cancer by using urine PGE-M levels as a non-invasive indicator for estimating cancer risk.

Data availability statement

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of Guizhou Provincial People's Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

(I) Conception and design: YoL, SL. (II) Administrative support: YoL, AL, HZ. (III) Provision of study materials or patients: HW, CW, LL. (IV) Collection and assembly of data: XL, JJ, YuL. (V) Data analysis and interpretation: XL, JJ, XL, CY, CZ. (VI) Manuscript writing: All authors. (VII) Final approval of manuscript: All authors. 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.

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Postoperative circulating tumor DNA testing based on tumor naïve strategy after liver metastasis surgery in colorectal cancer patients

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Objective: There is still a lack of highly sensitive methods for monitoring recurrence of colorectal cancer patients after liver metastasis surgery. The aim of this study was to evaluate the prognostic value of tumor-naive ctDNA detection after resection of colorectal liver metastases (CRLM).

Methods: Patients with resectable CRLM were prospectively enrolled. Based on the tumor-naive strategy, NGS panels containing 15 colorectal cancer hotspot mutated genes were used to detect ctDNA 3-6 weeks after surgery.

Results: A total of 67 patients were included in the study, and the positive rate of postoperative ctDNA was 77.6% (52/67). Patients with positive ctDNA had a significantly higher risk of recurrence after surgery (HR 3.596, 95% CI 1.479 to 8.744, P = 0.005), and a higher proportion relapsed within 3 months after surgery (46.7% *vs* 3.8%). The C-index of postoperative ctDNA in predicting recurrence was higher than that of CRS and postoperative CEA. The nomogram combining CRS and postoperative ctDNA can improve the accuracy of recurrence prediction.

Conclusion: Tumor-naive ctDNA detection can detect molecular residual lesions in patients with colorectal cancer after liver metastasis, and its prognostic value is superior to conventional clinical factors.

KEYWORDS

minimal residual disease (MRD), circulating tumor DNA (ctDNA), colorectal cancer liver metastases (CRLM), next-generation sequencing – NGS, recurrence

1 Introduction

Colorectal cancer (CRC) is one of the most common malignancies, accounting for 10% of all cancers, and is the second leading cause of cancer death, with approximately 1.9 million new cases and 90,000 deaths worldwide in 2020 (1). The liver is one of the most common metastatic sites of CRC, with an incidence as high as 25% – 30% (2). With the continuous improvement of treatment methods, including surgery of liver metastasis and novel anticancer drugs, the prognosis of CRC patients with liver metastases (CRLM) has been significantly improved (3). However, it has been shown that radical resection of liver metastases improves survival by 40% in patients with CRLM (4), and approximately 50% relapse postresection (5). Repeated surgical treatment has been proven effective for liver and lung recurrence after CRLM resection when the recurrent lesion remains curatively resectable (6). Therefore, early detection of postoperative recurrence is the key to improving the prognosis of these patients.

The traditional means of postoperative follow-up mainly include imaging (B ultrasound, CT, magnetic resonance imaging, etc.) and tumor markers (CEA). However, studies have shown that the sensitivity of CEA in detecting recurrence is limited, ranging from only 68% to 82% (7). Imaging can only detect overt lesions and has shown limited sensitivity in detecting recurrent metastatic disease (8). Assessing the prognosis of patients with solid tumors based on postoperative pathological parameters is the most commonly used strategy. The clinical risk score (CRS) scoring system, established in 1999, is a recognized prognostic indicator after resectioning CRC liver metastases and has also been included in clinical guidelines. CRS score consists of five parameters: (1) positive lymph node metastasis from the primary tumor, (2) >1 liver metastasis, (3) largest diameter of liver metastasis >50mm, (4) preoperative CEA level >200ng/ml, and (5) disease-free interval <12 months between resection of the primary tumor and diagnosis of liver metastasis (9). Each item is 1 point, 0 to 2 points for low CRS scores, and 3 to 5 points for high CRS scores. The higher the CRS score, the greater the risk of postoperative recurrence and the more beneficial perioperative chemotherapy. However, this predictive model based on preoperative clinical and postoperative pathological parameters remains somewhat imprecise, and we need a more precise way that directly reflects postoperative residual disease.

Since circulating tumor DNA (ctDNA) has a short half-life (ranging from minutes to a few hours) and allows for a more accurate, real-time, and dynamic measure of tumor burden, it has emerged as an ideal biomarker (10). Previous studies in various solid tumors have shown that postoperative ctDNA detection of molecular residual disease (MRD) can better identify patients with early recurrence (11–13). Several studies of early colorectal cancer have yielded similar results (13–18). However, there are still many controversies about ctDNA-based MRD detection strategies, including panel selection, the cutoff value of MRD positivity, and the timing of ctDNA testing. In previous studies, the tumor-informed strategy was mainly used to detect MRD; that is, tumor-specific genetic alterations were identified by whole-exome sequencing or targeted sequencing of the primary tumor (e.g., SignateraTM, SafeSeqS) from each patient to track them in ctDNA samples (13, 19). However, this strategy increases turnaround time (TAT) and has a certain failure rate. It has been shown that the QC failure rate for sequencing is as high as 16.9% due to poor-quality tumor tissue samples (20). In contrast, the tumor-naïve strategy performed without prior knowledge of the patient's tumor mutational profile using a fixed panel, which has several advantages, including fast TAT, logistical simplicity, and low cost.

In this study, we investigate the clinical validity of postoperative ctDNA testing by using a tumor-naive NGS panel containing colorectal cancer hotspot mutations.

2 Methods

2.1 Patient enrollment and sample collection

Patients with resectable CRC liver metastases (CRLM) were recruited for this prospective study. The main inclusion criteria included: pathologically confirmed colorectal cancer, primary lesions resected, and underwent liver metastasectomy with curative intent. The main exclusion criteria included: extrahepatic metastasis and a history of other cancers. All patients underwent standard preoperative staging investigations to assess liver lesions and the presence of metastases at other sites, including liver MRI and chest CT, or PET-CT. To assess the resectability of liver lesions, all patients underwent a multidisciplinary discussion (MDT) with our hospital's team of experts. The MDT team consisted of colorectal surgeons, liver surgeons, imaging physicians, radiotherapy physicians, medical oncologists, and pathologists. Blood samples for ctDNA analysis were collected 3-6 weeks after liver resection before commencing chemotherapy. At least 10 mL of blood was drawn into EDTA tubes, centrifuged twice at 3000rpm and 14000rpm, and plasma was then stored at −80°CC for ctDNA analysis. The study complied with the ethical standards of the Declaration of Helsinki and was reviewed and approved by the institutional ethics committee (Zhongshan Hospital Fudan University; B2018-099). Written informed consent was obtained from all participants.

2.2 ctDNA analysis

A tumor-naive strategy was used to detect postoperative ctDNA (Oncomine ™ Colon cfDNA), covering 14 colorectal cancer hotspot genes: AKT1, APC, BRAF, CTNB1, EGFR, ERBB2, FBXW7, GNAS, KRAS, MAP2K1, NRAS, PIK3CA, SMAD4, and TP53, which were detected at Illumina MiSeq (Illumina, USA) next-generation sequencing platform. Using tag sequencing technology, a limit of detection (LOD) as low as 0.1% can be achieved. Plasma samples with at least one mutation detected above a predefined confidence threshold were deemed ctDNA positive.

2.3 Clinicopathological data and follow-up

Clinicopathological characteristics were collected based on medical history records, including age, gender, primary tumor location, time to metastases, size and number of metastases, serum CEA levels, and clinical risk score (CRS). All patients received standard-of-care postoperative treatment and surveillance, according to the investigator's choice, per protocol follow-up after liver resection included clinical review, CEA evaluation, and imaging exam every three months.

2.4 Statistical analyses

The primary objective was to measure the recurrence-free survival (RFS) from the time of surgery to the first radiologic evidence of disease progression or a CRC-caused death. Patients were censored by the end of follow-up or by a non-CRC- caused death. Survival analyses were performed using the Kaplan-Meier method. The performance of ctDNA as a marker of RFS outcome was evaluated using the concordance index (C-index). C-index of 0.5–0.7, 0.7–0.85, and 0.85–0.95 were defined as low, middle, and high credibility, respectively. Group comparisons were performed using chi-square tests or Fisher exact test, as appropriate. SPSS23.0 (SPSS Inc., Chicago, IL) and R (R version 3.2.1, http://www.r-project.org) were used for statistical analysis. All P values were based on two-sided testing, and differences were considered significant at $P \leq 0.05$.

3 Results

3.1 Associations between postoperative ctDNA status and clinicopathologic factors

A total of 67 patients were included in this study, and the median time from the date of liver surgery to postoperative blood collection was 28 days (inter-quartile range (IQR), 23.5 to 34 days). Of these, 22.4% (15/67) of patients were positive for ctDNA after surgery, and the remaining 77.6% (52/67) were negative. The differences in clinicopathologic characteristics between the postoperative ctDNA positive and negative groups were analyzed. The results showed that CRS score, primary tumor location, whether synchronous liver metastasis, number of liver metastases, the maximum diameter of liver metastases, RAS/RAF status, and preoperative and postoperative CEA levels were not significantly correlated with postoperative ctDNA status (Table 1).

3.2 Postoperative ctDNA status predicts early recurrence

At the time of data cutoff, 41 of 67 patients had a recurrence, of which 9 had a recurrence within three months after surgery, with a median follow-up time of 9.67 months. Early recurrence was only associated with postoperative ctDNA status but not with age, gender, primary tumor location, preoperative and postoperative CEA, and CRS scores. Of the postoperative ctDNA-positive patients, 46.7% (7/15) developed recurrence within three months after surgery; however, only 3.8% (2/52) developed early recurrence in the postoperative ctDNA-negative group (Table 2).

Univariate survival analysis was performed to assess the ability of postoperative ctDNA status to predict recurrence compared with other clinicopathological variables. The results showed that RFS was significantly associated with postoperative ctDNA status and CRS score (Table 3). The postoperative ctDNA-positive group had a considerably shorter RFS than the ctDNA-negative group (5.93 *vs.* 14.30, P = 0.005, HR 3.596, 95% CI 1.479 to 8.744); the high CRS group had a significantly shorter RFS than the low CRS group (8.27 *vs.* 17.00, P = 0.005, HR 2.517, 95% CI 1.317 to 4.810) (Figures 1A, B).

Out of 67 patients, 28 (42%) received a combination of targeted therapy (cetuximab or bevacizumab) and fluoropyrimidine-containing doublet chemotherapy (FOLFOX/FOLFIRI), while 39 (58%) received only fluoropyrimidine-containing doublet chemotherapy post-surgery. The duration of chemotherapy did not exceed six months for any patient. Statistical analysis revealed no significant difference in progression-free survival (PFS) between patients who underwent targeted combination chemotherapy and those who only received chemotherapy (P = 0.546). Additionally, our study found that the postoperative ctDNA status was significantly associated with PFS in both the targeted combined chemotherapy and chemotherapy groups (P = 0.002, P = 0.050) (Figure S1). Therefore, the results suggest that the predictive ability of postoperative ctDNA status for recurrence is not influenced by the type of postoperative treatment regimen administered.

3.3 Development of a predictive nomogram

The previous survival analysis showed that CRS and postoperative ctDNA status were prognostic factors, so we developed a nomogram recurrence prediction model. As mentioned earlier, the CRS score contained five clinical variables, namely, preoperative CEA level, number of liver metastases, the maximum diameter of liver metastases, lymph node metastasis status of the primary tumor, and the time interval between the primary tumor and liver metastases, combined with postoperative ctDNA, a total of six variables were included in the nomogram model (Figure 2).

Subsequently, The C-index was used to evaluate the discrimination power of postoperative CEA, CRS, postoperative ctDNA, and the nomogram. The accuracy of postoperative ctDNA in predicting recurrence was higher than that of CRS and postoperative CEA (C-index 0.619 *vs.* 0.583 *vs.* 0.542), and the nomogram model had the highest C-index of 0.702, indicating that the multi-parameter model combining CRS and postoperative ctDNA can improve the accuracy of recurrence prediction. (Table 4)

TABLE 1 Relationship between clinic-pathological variables and postoperative ctDNA status.

	Postoperative ctDNA negative	Postoperative ctDNA positive	*P* value
CRS			0.281
Low (score 0-2)	23 (44.2%)	9 (60.0%)	
High (score 3-5)	29 (55.8%)	6 (40.0%)	
Location of primary tumor			0.344
Left colon	17 (32.7%)	8 (53.3%)	
Right colon	14 (26.9%)	3 (20.0%)	
Rectum	21 (40.4%)	4 (26.7%)	
Synchronous liver metastases			0.742
Yes	37 (71.2%)	12 (80.0%)	
No	15 (28.8%)	3 (20.0%)	
Number of liver metastasis			1.000
Single	37 (71.2%)	11 (73.3%)	
Multiple	15 (28.8%)	4 (26.7%)	
Diameter of largest liver metastasis			1.000
≥5cm	9 (17.3%)	2 (13.3%)	
<5cm	43 (82.7%)	13 (86.7%)	
KRAS/NRAS/BRAF			0.321
WT	16 (30.8%)	2 (13.3%)	
MT	36 (69.2%)	13 (86.7%)	
Preoperative CEA			0.568
≥200ng/mL	4 (7.7%)	0 (0.0%)	
<200ng/mL	48 (92.3%)	15 (100.0%)	
Postoperative CEA			0.542
≥5ng/mL	16 (30.8%)	6 (40.0%)	
<5ng/mL	36 (69.2%)	9 (60.0%)	

CRS, clinical risk score; WT, wild type; MT, mutant type; CEA, carcinoembryonic antigen.

3.4 ctDNA MAF and RFS

Of the 15 patients with positive postoperative ctDNA, 10 had MAF ≥ 0.5%, and 5 had MAF < 0.5%, the median MAF was 0.93% (IQR 0.35% to 2.09%). Patients in the MAF ≥ 0.5% group had significantly shorter RFS than those in the MAF < 0.5% group and those in the ctDNA-negative group (2.5 *vs.* 7.5 *vs.* 14.37, P = 0.002) (Figure 1C).

3.5 Postoperative ctDNA Status and site of recurrence

Previous studies have shown that metastatic sites are associated with detection rates of ctDNA (21). Therefore, we analyzed the relationship between the site of recurrence and postoperative ctDNA. Among the 41 patients with recurrence, the detection rate of ctDNA was 29.3% (12/41). Postoperative ctDNA was detected in 37% (7/19) of patients with liver recurrence, 36% (4/11) patients with multiple sites of recurrence, 0% (0/8) of patients with lung recurrences and 33% (1/3) of patients with recurrence to other sites (peritoneal or lymph nodes) (Figure 1D).

4 Discussion

This study used a tumor-naïve strategy to detect postoperative ctDNA status in mCRC who underwent resection of liver metastases. The results showed that the positive rate was 22.4% (15/67). Patients with positive ctDNA had significantly shorter RFS (5.93 *vs.* 14.30, P = 0.005, HR 3.596, 95% CI 1.479 to 8.744) and a considerably higher proportion of recurrence within three months after surgery (46.7% *vs.* 3.8%) compared with negative patients. Two previous studies based on tumor-informed strategies showed that

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TABLE 2 Analysis of factors associated with short-term recurrence.

	Total	Recurrence within 3 months	No recurrence within 3 months	*P* value
All patients, n	67	9	58	
Age				0.714
≥ 65y	20	2 (22.7%)	18 (31.0%)	
<65y	47	7 (77.8%)	40 (69.0%)	
Gender				0.721
Male	38	6 (66.7%)	32 (55.2%)	
Female	29	3 (33.3%)	26 (44.8%)	
Location of primary tumor				0.449
Left colon	25	5 (55.6%)	20 (34.5%)	
Right colon	17	2 (22.2%)	15 (25.9%)	
Rectum	25	2 (22.2%)	23 (39.7%)	
LN from primary tumor				0.671
Positive	52	8 (88.9%)	44 (75.9%)	
Negative	15	1 (11.1%)	14 (24.1%)	
Time interval from diagnosis of primary tumor to liver metastases				0.186
≥12 months	14	0 (0.0%)	14 (24.1%)	
<12 months	53	9 (100.0%)	44 (75.9%)	
Synchronous liver metastases				0.426
Yes	49	8 (88.9%)	41 (70.7%)	
No	18	1 (11.1%)	17 (29.3%)	
Number of liver metastasis				0.706
Single	48	6 (66.7%)	42 (72.4%)	
Multiple	19	3 (33.3%)	16 (27.6%)	
Diameter of largest liver metastasis				0.336
≥5cm	11	0 (0.0%)	11 (19.0%)	
<5cm	56	9 (100.0%)	47 (81.0%)	
KRAS/NRAS/BRAF				0.426
WT	18	1 (11.1%)	17 (29.3%)	
MT	49	8 (88.9%)	41 (70.7%)	
Preoperative CEA				1.000
≥200ng/mL	4	0 (0.0%)	4 (6.9%)	
<200ng/mL	63	9 (100.0%)	54 (93.1%)	
Postoperative CEA				0.461
≥5ng/mL	22	4 (44.4%)	18 (31.0%)	
<5ng/mL	45	5 (55.6%)	40 (69.0%)	
CRS				1.000
Score 0-2	32	4 (44.4%)	28 (48.3%)	
Score 3-5	35	5 (55.6%)	30 (51.7%)	

(Continued)

TABLE 2 Continued

	Total	Recurrence within 3 months	No recurrence within 3 months	*P* value
Postoperative ctDNA (day 30)				**0.000**
Positive	15	7 (77.8%)	8 (13.8%)	
Negative	52	2 (22.2%)	50 (86.2%)	

LN, lymph node; WT, wild type; MT, mutant type; CEA, carcinoembryonic antigen; CRS, clinical risk score. Bold values indicates that the P value have statistically significant differences.

TABLE 3 Recurrence-free survival analysis by clinic-pathological variables.

	Median RFS (95% CI) months	*P* value
Age		0.597
<65y	9.030 (7.412-10.648)	
≥ 65y	16.000 (0.000-33.412)	
Gender		0.165
Female	8.200 (5.888-10.512)	
Male	14.500 (7.177-21.823)	
Location of primary tumor		0.335
Left colon	12.700 (5.890-19.510)	
Right colon	8.230 (4.845-11.615)	
Rectum	15.000 (5.461-24.539)	
LN from primary tumor		0.063
Positive	8.500 (7.481-9.519)	
Negative	18.800 (6.277-31.323)	
Time interval from diagnosis of primary tumor to liver metastases		0.061
≥12 months	15.000 (2.549-27.451)	
<12 months	8.630 (7.622-9.638)	
Synchronous liver metastases		0.227
Yes	9.030 (7.401-10.659)	
No	14.300 (3.893-24.707)	
Number of liver metastasis		0.491
Single	9.800 (3.827-15.773)	
Multiple	7.630 (0.071-15.189)	
Diameter of largest liver metastasis		0.146
≥5cm	7.500 (1.328-13.672)	
<5cm	9.800 (5.147-14.453)	
KRAS/NRAS/BRAF		0.377
WT	15.200 (undetermined)	
MT	8.500 (6.446-10.554)	
Preoperative CEA		0.344
≥200ng/mL	6.230 (0.781-11.679)	
<200ng/mL	9.800 (4.611-14.989)	

(Continued)

TABLE 3 Continued

	Median RFS (95% CI) months	*P* value
Postoperative CEA		0.371
<5ng/mL	9.800 (4.330-15.270)	
≥5ng/mL	8.470 (5.964-10.976)	
CRS		**0.005**
Low risk (score 0-2)	17.000 (2.377-31.623)	
High risk (score 3-5)	8.270 (7.844-8.696)	
Postoperative ctDNA (day 30)		**0.005**
Negative	14.300 (7.743-20.857)	
Positive	5.930 (0.000-13.309)	

LN, lymph node; WT, wild type; MT, mutant type; CEA, carcinoembryonic antigen; CRS, clinical risk score. Bold values indicates that the P value have statistically significant differences.

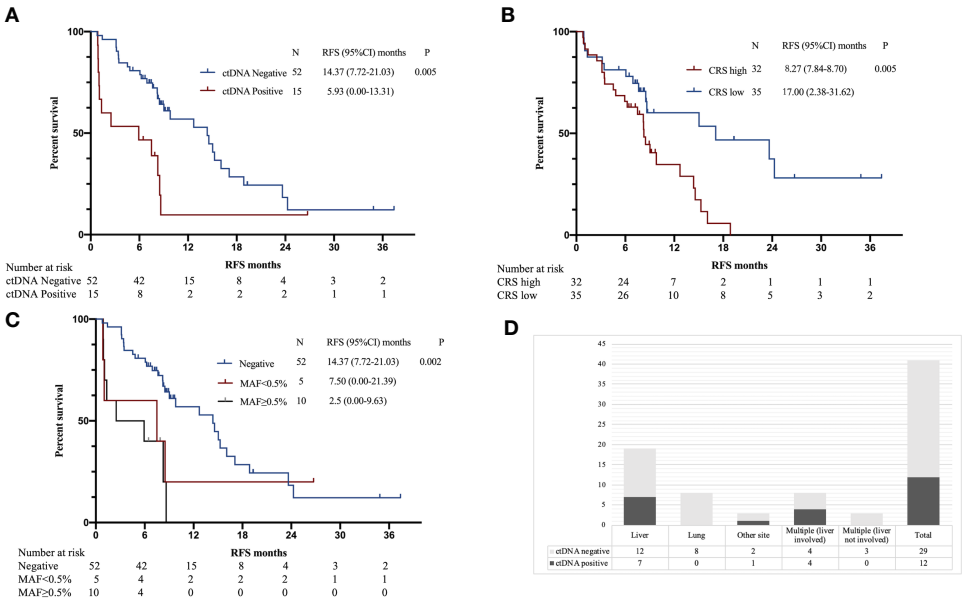

FIGURE 1
(A) Kaplan–Meier estimates of recurrence-free survival (RFS) for postoperative ctDNA. **(B)** Kaplan–Meier estimates of RFS for CRS. **(C)** Kaplan–Meier estimates of RFS for postoperative ctDNA MAF. **(D)** Postoperative ctDNA positivity according to the site of recurrence.

postoperative ctDNA positivity in patients undergoing curative resection of CRLM was 24% and 54.4%, and positive ctDNA both indicated shorter RFS (HR 6.3, 95% CI 2.58 – 15.2, P < 0.001; HR 5.78, 95% CI 3.34 – 10.0, P 0.001) (20, 22). Compared with the previous two studies using a tumor-informed strategy, our study using a tumor-naïve strategy showed slightly lower ctDNA positivity as well as a lower hazard ratio for recurrence. In this study, the sensitivity of the tumor-naïve strategy for detecting postoperative MRD was 37%. Previous studies on early-stage colorectal cancer reported a sensitivity of approximately 40%-50% for MRD detection using a tumor-informed strategy one month after surgery[18,19]. However, these studies focused on patients with stage II/III colorectal cancer, with a median follow-up time of 1-2 years and a postoperative recurrence rate of 15% to 18%. In contrast, our study focused on colorectal cancer patients with liver metastasis, with a median follow-up time of 9.67 months and a significantly higher postoperative recurrence rate of 61%. The sensitivity of ctDNA detection is influenced by disease stage and tumor burden, with a higher ctDNA positivity rate in patients with advanced disease stages or higher tumor burdens (23). Therefore, the relatively high sensitivity of MRD detection in our study is largely attributed to the advanced stage of disease in the study population.

It is generally accepted that the sensitivity of the tumor-naïve strategy is lower than that of the tumor-informed strategy. Combining the detection of multiple types of markers, such as methylation markers, can improve the sensitivity of MRD detection using the tumor-naïve strategy (16). For example, a study in

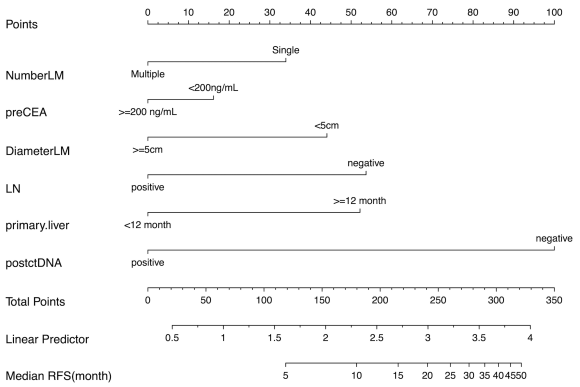

FIGURE 2
The Nomogram for predicting recurrence-free survival (RFS) in patients undergoing curative resection of colorectal cancer liver metastases. Number of liver metastasis (NumberLM), Preoperative CEA level (preCEA), Largest diameter of liver metastasis (DiameterLM), lymph node metastasis from the primary tumor, Postoperative ctDNA (postctDNA).

colorectal cancer found that integrating methylation signatures increased sensitivity by 25%–36% versus genomic alterations alone (16). Therefore, we believe that the future trend in applying the tumor-naïve strategy to MRD detection is to combine methylation and mutation markers. It is worth noting that in our study, we only analyzed MRD at a single "Landmark" time point and did not perform longitudinal monitoring of ctDNA. However, MRD results at the "Landmark" time point (usually about one month after curative treatment) have important clinical significance for predicting patient prognosis and making treatment decisions. Studies have shown that continuous monitoring can improve the sensitivity of recurrence monitoring (18). Nevertheless, challenges in MRD detection remain, such as the need for a large amount of blood collection and high costs. Therefore, the clinical utility of longitudinal continuous monitoring needs to be further verified. Nevertheless, our study is able to show that tumor-naïve ctDNA assay is effective in identifying patients with relapse. In addition, since the tumor-naïve strategy has a fixed panel and short TAT, it has certain advantages in clinical applications in the future.

Currently, most studies categorized ctDNA results as either positive or negative, and few investigated the relationship between ctDNA MAF and recurrence risk. This study found that higher MAF was associated with shorter RFS (mRFS, 2.5, 7.5, and 14.37 months for MAF of ≥ 0.5%, < 0.5%, and 0%, P = 0.002). In other words, Recurrence risk increased with increasing ctDNA MAF.

TABLE 4 C-index for the nomogram, postoperative CEA, CRS and postoperative ctDNA.

Variable	C-Index	95CI%
Postoperative CEA	0.542	0.454-0.630
CRS	0.583	0.491-0.675
Postoperative ctDNA	0.619	0.537-0.701
Nomogram	0.702	0.604-0.800

CEA, carcinoembryonic antigen; CRS, clinical risk score.

Then, we need to consider whether ctDNA predicts recurrence no longer accurately when MAF is reduced to a certain extent. This question also determines what limit we pursue the sensitivity of ctDNA detection. A study in early colorectal cancer showed the HR for recurrence was only 1.2 for postoperative ctDNA MAF of 0.1% (24). In addition, as the detection sensitivity increases, the specificity decreases, especially the interference of clonal hematopoiesis. Therefore, in real-world clinical applications, we need to balance multiple factors such as sensitivity, specificity, TAT, and cost rather than just pursuing the limit of a single parameter.

Previous studies have found that ctDNA detection can vary by the site of metastases. Kagawa et al. observed that CRC patients with liver metastases were associated with increased ctDNA detection rates compared to patients with lung and peritoneal metastases (21). Therefore, we further analyzed the relationship between sites of recurrence and postoperative ctDNA positivity. We also found that the site of recurrence affected the ability to detect ctDNA prior to radiological diagnosis of recurrence. The results showed that the rate of positive ctDNA was higher in patients with liver metastasis than those with other sites of metastasis (such as lung and peritoneal) after CRLM surgery. This difference may be associated with physiological barriers or tumor burden, but the specific mechanisms remain to be explored.

It is important to emphasize that ctDNA outperforms conventional clinical parameters (CEA, CRS) in predicting cancer relapse in patients with resected CRLM. The accuracy of postoperative ctDNA in predicting recurrence was higher than that of CRS and postoperative CEA (C-index 0.619 *vs.* 0.583 *vs.* 0.542). In this study, we combined postoperative ctDNA status with traditional prognostic markers to construct a nomogram predicting recurrence after CRLM surgery to assist in distinguishing patients at high risk of recurrence who may require more aggressive treatment as well as closer follow-up strategies. The nomogram provides better predictive accuracy for postoperative recurrence than traditional prognostic markers (CEA, CRS) in CRLM patients.

There are several limitations to our study. These include a small sample size, a lack of a validation cohort, and a relatively short follow-up period. In addition, this study did not test the preoperative ctDNA level and it is unclear what percentage of patients had negative preoperative ctDNA. Theoretically, patients with negative preoperative ctDNA may not have their disease recurrence effectively reflected by postoperative ctDNA status. However, the present study demonstrated the potential use of tumor-naïve ctDNA analysis as a prognostic tool. The results also revealed the correlation between preoperative ctDNA detection and the site of recurrence, as well as the impact of ctDNA MAF on RFS. All these factors are essential to consider in future MRD testing.

5 Conclusion

In summary, we have confirmed the prognostic significance of detecting ctDNA by tumor-naïve strategy in patients undergoing resection for CRLM. Nomogram based on postoperative ctDNA and CRS might be promising biomarkers in future trials to select high-risk CRLM patients for personalized therapy.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by Zhongshan Hospital Fudan University(B2018-099). The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conception and design: WG, BW. Provision of study materials or patients: HJ, FH, YY, XC, MS, CZ, BP. Collection and assembly of data: HJ, FH, YY. Data analysis and interpretation: HJ, FH, YY. HJ, FH, and YY contributed equally to this work. WG and BW contributed equally to this work as corresponding authors. 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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2023.1153685/full#supplementary-material

SUPPLEMENTARY FIGURE 1
(A) Kaplan–Meier estimates of recurrence-free survival (RFS) for postoperative therapy. **(B)** Kaplan–Meier estimates of RFS for postoperative ctDNA in targeted combined chemotherapy group. **(C)** Kaplan–Meier estimates of RFS for postoperative ctDNA in chemotherapy group.

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Liquid biopsy can cure early colorectal cancer recurrence – Case Report

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In the context of colorectal cancer (CRC), circulating tumor DNA (ctDNA) is frequently used to monitor the minimal residual disease (MRD). ctDNA has become an excellent biomarker to predict which patients with CRC are likely to relapse due to the persistence of micrometastases. MRD diagnosis *via* analysis of ctDNA may allow much earlier detection of relapse compared with conventional diagnosis during follow-up. It should lead to an increased rate of curative-intended complete resection of an asymptomatic relapse. Besides, ctDNA can provide crucial information on whether and how intensively adjuvant or additive therapy should be administered. In the present case, analysis of ctDNA gave us a crucial hint to the use of more intensive diagnostics (MRI and Positron emission tomography–computed tomography PET-CT) which led to earlier detection of CRC relapse. Metastasis detected early are more likely to be completely resectable with curative intent.

KEYWORDS

liquid biopsy, ddPCR, colorectal cancer, follow-up, Ras, tumor promotor P53 TP53, mutation detection, PET-CT

Introduction

Despite advances in diagnostic imaging, surgery, and chemotherapy, the 5-year mortality rate of colorectal cancer (CRC) remains high with nearly 40% (1). The key for successful CRC treatment is early detection, as the 5-year survival rate at stages I and II is above 80%, but after the development of distant metastases, it decreases to approximately 10% (2). The primary goal of curative CRC therapy is complete resection of the tumor tissues combined with adjuvant chemotherapy in advanced situations. Identifying patients with minimal residual disease (MRD), i.e. clinically hidden micrometastases remaining after initial therapy, and treating with additional or intensified therapy could potentially increase the rate of cured patients.

One limiting factor in MRD diagnostics of CRC is the low sensitivity of standard diagnostical tools including imaging (e.g., Magnetic Resonance Imaging (MRI), Computed

Tomography (CT), or Positron emission tomography–computed tomography (PET/CT) and serum tumor markers, e.g. carcinoembryonic antigen (CEA) or cancer antigen 19-9 (CA 19-9) (3). Serial CEA analyses may recognize relapse with a sensitivity of 69% and specificity of 64% (4). Furthermore, detection of ctDNA after precise and complete surgical removal of the tumor can be used to manage the chemotherapy options for patients with CRC stage II and III (5, 6). Only patients with MRD should receive chemotherapy, while 13% of patients could be spared the unnecessary chemotherapy (6, 7). The reduction of the number of chemotherapies results in the same 83% rate of cure as the standard treatment management.

Stage IV CRC is associated with higher likelihood of relapse and poorer survival outcomes. Oligometastatic CRC is eligible for surgery with curative intent, but 60%–70% of patients will go on to relapse postresection (8).

Thus, there is an unmet need for the development of better tools to facilitate physician's decision-making in identifying and stratifying resected patients by risk of relapse with still curative intent. Strikingly, patients with asymptomatic recurrences reveal a more than 5-fold higher 5-year overall survival compared to symptomatic recurrences (9). It is remarkable that nowadays, over 60% are still diagnosed with recurrence secondary to symptoms. Thus, early detection of higher risk of metastasis after tumor resection is crucial for improving clinical outcomes of patients.

In the past few years, blood-based liquid biopsies - especially the analysis of cell-free tumor DNA (ctDNA) - have received widespread attention due to increased sensitivity of modern polymerase chain reaction (PCR)-based technologies. The detection of plasma ctDNA is prognostic in CRC and has the potential to serve as a highly specific and low-invasive test for early prediction of disease recurrence in clinical routine may enable a locoregional approach (5, 6, 10).

In the here reported CRC case, we present the significance of liquid biopsy for MRD diagnostics in advanced CRC.

Case description

A 47-year-old man without any significant pre-existing conditions was diagnosed in September 2019 with a rectum adenocarcinoma (upper rectal) with synchronous liver metastases (limited to right liver lobe) (see the magnet resonance imaging (MRI in Figure 1). The molecular diagnostics of tumor tissue revealed mutations in *neuroblastoma RAS viral oncogene homolog* (NRAS2 G12D c.35G>A) and in *tumor protein p53* (TP53 C141Y c.422G>A). A neoadjuvant therapy with four cycles of FOLFOX4 (Folic acid, fluorouracil, and oxiliplatin 24h) and bevacizumab was administered and was well tolerated. In December 2019, the patient underwent exploratory laparoscopy and right hemihepatectomy. Five metastases were removed. By the tumor regression grading (TRG) of 2, the histological response to chemotherapy of hepatic colorectal metastases (HCRM) was grade 4 with 90% of necrosis of surface.

One month later, laparoscopically assisted anterior rectum resection, sigmoid resection and descendorectostomy followed. The TNM stage was ypT2 ypN0(0/17) ypM1 (hep). Thereupon, additive therapy with eight cycles of FOLFOX4 was given and was well tolerated again.

Starting in September 2020, liquid biopsies to detect the known *NRAS* mutation were carried every six months. In July 2021, *NRAS* mutations were not yet detectable.

A routine examination for relapse in November 2021 included computed tomography of chest and abdomen, MRI of the liver, sigmoidoscopy and analyses of tumor markers CEA and CA19-9. The MRI showed an annular contrast medium enhancement at the resection margin in liver segment IV. To further classification of the contrast medium enhancement, subsequently conducted 2-Deoxy-2-[18F]fluoro-d-glucose emission tomography (FDG-PET-CT) showed diffusion restriction at the resection margin with albeit minimal FDG activity (white arrow; Standardized uptake value (SUV) max 4.3), which fell within the range of the liver parenchyma (SUV 1.9-4.8) (See PET-CT scan in Figure 2A). Overall, the examinations were assessed as not malignancy-suspect.

However, five weeks later (January 2022), both, the original *NRAS* and *TP53* mutations were detectable with mutant allele fractions (MAF) of 0.37% and 0.9% respectively (Figure 3).

An intensified search for a relapse was undertaken since January 2022. CT scans, MRI of the liver and the tumor markers showed unchanged findings and an assessment that no metastasis exist. The next liquid biopsy analysis in March 2022 and April 2022 continued to show increasing MAF for both mutations. Based on liquid biopsy result, a premature PET-CT was performed in May 2022. This PET-CT revealed a clear increase of FDG activity in the resection margin in liver segment IV (See PET-CT scan in Figure 2B). The curative-

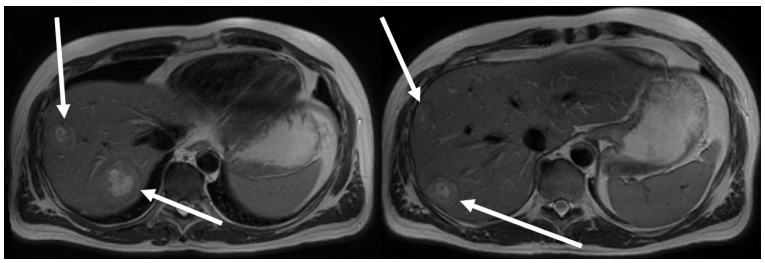

FIGURE 1
Multiple liver metastases limited to right liver lobe in MRI at diagnosis in September 2019 (white arrows).

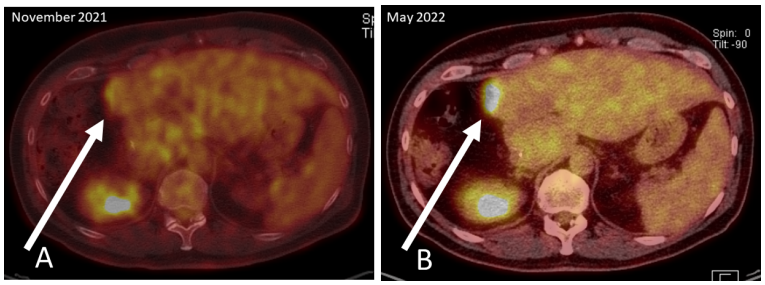

FIGURE 2
PET-CT scans: **(A)** November 2021; diffusion restriction at the resection margin with albeit minimal FDG activity (white arrow), **(B)** May 2022; clear increase of FDG activity in the resection margin in liver segment IV.

intent complete resection of asymptomatic relapse followed. The tissue analysis of resected material confirmed the *NRAS* and *TP53* mutations detected in liquid biopsy and measured the *NRAS2* mutation with an allele frequency of 47.4% and the *TP53* mutation with 68.4%. The metastasis tissue was graded as G2 with 50% necrotic parts. No hint for microsatellite instability was found. The postoperative liquid biopsy samples no longer contained ctDNA with *NRAS* or *TP53* mutations. The tumor marker carcinoembryonic antigene CEA was not substantially elevated until March 2022 and decreased to normal after surgery in July 2022 (Figure 3). We have no signs of a recurrence so far (till February 2023). The patient is symptom-free, works full-time and leads a normal family life.

Discussion

We report the clinical course of a patient in follow-up after resection of rectum carcinoma and hepatic metastases. Longitudinal liquid biopsies revealed MRD with detection of *NRAS* and *TP53* mutations 4 month earlier as detected by PET-CT. Only by intensifying the search for disease recurrence on the basis of ctDNA detection, the location of CRC relapse could be unraveled.

MRD-positivity in liquid biopsy displays without ifs and buts an overwhelming suspicion of a persistent disease. Several studies have demonstrated that patients with detectable ctDNA postsurgery will finally develop a relapse (4, 5, 11). In patients diagnosed with resected stage I-III CRC, MRD-positivity at the end-of-treatment (surgery with/without adjuvant chemotherapy) was accompanied by an over 40 times higher probability of disease recurrence as compared to MRD-negative patients (4, 11). In a study by Tie et al., patients who underwent curative intended resection of colorectal cancer liver metastases were further analyzed. MRD-positivity at the end-of-treatment was associated with a 5-year RFS (recurrence free survival) of only 0% and a 14.9 times higher recurrence probability. In line with our clinical report, longitudinal ctDNA analyses identified disease recurrence up to 16.5 months and in mean 8.7 months earlier than standard radiologic imaging (4).

In our clinical report, the knowledge of the MRD-positivity influenced the intensity and manner of follow up. Based on this finding, the decision to carry out the PET-CT examination was made. This approach may have enabled the curative-intent complete resection of the relapse.

Methods

Molecular analysis of the tissue samples was routinely performed by next generation sequencing (Institute of Pathology, University Hospital Essen, Germany). Two mutations were found at a 60% cellularity with a proportion of 68.4% MAF of *TP53 C141Y c.422G>A* mutation and 47.4% MAF% of *NRAS2 G12D c.35G>A* mutation.

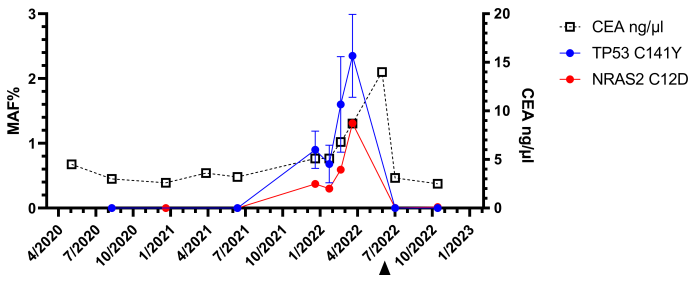

FIGURE 3
Longitudinal liquid biopsy results: The first three liquid biopsy analyses until the 15th month of the follow up were negative. The fourth sample revealed *NRAS* (red)- and *TP53*-(circles blue) mutations. Only after the resection (arrowhead), the ctDNA was no longer detectable. Three months after the reappearance of the *NRAS2 C12D* mutations, the CEA level also increased substantially.

Blood samples were obtained in cell free DNA collection tubes (Cell-Free DNA BCT; Streck™) and sent to our lab (Ruhr University Bochum, Knappschaftskrankenhaus). Plasma was isolated latest three days after blood collection.

The ctDNA was isolated from 3 ml plasma using the circAMP circulating nucleic acid isolation procedure (Qiagen™, Hilden, Germany) as described earlier (12). NRAS2-12 mutation detections were performed using the IVD certified all RAS mutation kit with ONCOBEAM technology (Sysmex Inostics, Hamburg, Germany).

The assay to detect the mutation *TP53 C141Y c.422G>A p.*C141Y, NM_002524.4 COSM43708 was produced by Bio-RAD (Hercules, California, USA). The context sequence was given in supplemented file (Supplementary material). The ddPCR was performed as described in (12).

To validate the ddPCR-based assay to detect the *TP53 C141C* mutation, genomic DNA from the patient´s liver metastasis was used (Supplementary material).

The tumor marker carcinoembryonic antigene was measured by routine procedure in clinical labs.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

Author contributions

AB and SK-S contributed equally to the writing of the manuscript and designed the figures. AB was involved in the treatment of the patient. AB, DT, HB, RS, SK-S, and TM reviewed and approved the final version of this work. All authors contributed to the article.

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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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2023.1141833/full#supplementary-material

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