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Cancer-associated fibroblasts of colorectal cancer: Translational prospects in liquid biopsy and targeted therapy

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Abstract: Colorectal cancer (CRC) is a major global health concern. Accumulation of cancer-associated fibroblasts (CAFs) in CRC is associated with poor prognosis and disease recurrence. CAFs are the main cellular component of the tumor microenvironment. CAF-tumor cell interplay, which is facilitated by various secretomes, drives colorectal carcinogenesis. The complexity of CAF populations contributes to the heterogeneity of CRC and influences patient survival and treatment response. Due to their significant roles in colorectal carcinogenesis, different clinical applications utilizing or targeting CAFs have been suggested. Circulating CAFs (cCAFs) which can be detected in blood samples, have been proposed to help in determining patient prognosis and enables the detection of cancer through liquid biopsy. Liquid biopsy is gaining traction as it is non-invasive, allows frequent and easy sampling, and shows concordance to tissue biopsy analysis. In addition, CAF-targeted therapy is currently being studied extensively to be used as one of the treatment avenues for CRC. Various mechanisms of CAF-targeted therapy have been reported, including blocking the signaling pathways involving CAFs and cancer cells, thus abolishing the CAF-tumor cell crosstalk and subsequently hindering tumorigenesis. These translational applications of cCAFs and utilization of CAFs as key targets for CRC therapy, although still in the early phases of development, will potentially improve CRC patient management in the future.

Introduction

Colorectal cancer (CRC) is one of the deadliest and commonly diagnosed malignancies, affecting men and women worldwide. It is predicted that the global new CRC cases will increase to 3.2 million in 2040. CRC incidence is higher in highly developed countries, although the cases are increasing steadily in middle- and low-income countries (Xi and Xu, 2021). In addition, there is an alarming global trend of early-onset CRC reported in individuals below 50 years of age (Akimoto *et al.*, 2021). It is estimated that roughly 60% of CRC patients perished from their cancer (Riihimäki *et al.*, 2012). Despite this, CRC mortality is steadily decreasing in developed countries, mainly due to efficient national screening initiatives, adaptation to a healthier lifestyle and diet, and significant uptake of colonoscopy, which is a gold standard procedure to screen for CRC (Ait Ouakrim *et al.*, 2015).

CRC develops as the result of the accumulation of genetic and epigenetic changes involving oncogenes and tumor suppressor genes. The multistep process of classical adenoma-carcinoma transition in colorectal carcinogenesis occurs over a period of approximately 10–15 years (Fearon and Vogelstein, 1990; Binefa *et al.*, 2014). CRC can spread systemically and metastasize to distant sites, most commonly to the liver and peritoneum (Riihimäki *et al.*, 2016). Various risk factors ranging from genetics to environment, contributes to the onset and progression of CRC. The majority of CRC cases arise sporadically, without a family history of the disease or inherited genomic changes (Keum and Giovannucci, 2019; Tian *et al.*, 2019). Despite extensive studies on CRC pathogenesis and the discovery of advanced cancer treatment, the survival rates of CRC

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patients are still poor. Multiple factors, such as late detection and low compliance in CRC screening, lead to high morbidity and mortality due to CRC.

CRC is a heterogeneous disease, and this influences patient prognosis and treatment efficacy (Xi and Xu, 2021). Genomic profiling on CRC has revealed the complex nature of this disease (Hinoi, 2021), which likely contributes to the patient's lack of response to therapy.

A major factor that promotes CRC evolution and heterogeneity is the tumor microenvironment (TME). The accumulation of cancer-associated fibroblasts (CAFs), which are the main stromal cells of the TME, is associated with a worse prognosis and recurrence of CRC (Anderson and Simon, 2020; Musa and Ali, 2020).

Rather than focusing on cancer cells alone, scientists are shifting their focus to TME components, including CAFs. The CAF-cancer cell crosstalk has been reported widely as a key driver of CRC progression and metastasis (Bussard *et al.*, 2016). Considering their vital role in colorectal carcinogenesis, CAFs can be potentially utilized in clinical setting, as a basis for liquid biopsy and targeted therapy. In this review, we discuss the biology of CAFs in CRC and unravel the translational prospects of CAFs, which could overcome present limitations in CRC management and thus may greatly improve the prognosis of CRC patients in the future.

The biology of colorectal cancer and tumor microenvironment

CRC is a heterogeneous entity and constantly evolving, which promotes its complex nature. Phenotypic plasticity exists in cancers, including CRC. Intra-tumor heterogeneity is a result of genetic and epigenetic variations as well as transcriptional plasticity (Black and McGranahan, 2021). The roles of these biological processes in tumor evolution remain unknown and is also yet to be fully explored in colorectal carcinogenesis. Dissecting this process will provide insight into CRC progression and the roles of the TME in promoting tumorigenesis (Ahmad Zawawi and Musa, 2022).

Over the past two decades, scientists have focused on the different components of the tumor, namely the TME, rather than studying the epithelial cancer cells alone. Previous reports have shown that the neoplastic cells can be reverted to a normal proliferation state despite having a highly mutated genome by blocking signaling pathways activated by the TME (Bizzarri *et al.*, 2011; Northey *et al.*, 2017). This highlights the essential role of the TME in dictating the fate of a tumor.

Rather than a silent bystander, the TME is considered an active driver of cancer progression. The TME is highly complex, heterogeneous, and continuously evolving. Its components vary between different tumors. Hallmark features of the TME include stromal, immune, and endothelial cells and extracellular matrix (ECM). The stromal cells have pro- and antitumorigenic properties, where cancer progression can be delayed or blocked by normal stroma, whereas aberrant stroma promotes carcinogenesis (Bissell and Radisky, 2001). However, evidence has suggested that stromal cells produce various factors that promote angiogenesis, tumor growth, invasion, and metastasis through reciprocal communication with cancer cells (Bussard *et al.*, 2016; Anderson and Simon, 2020). Particularly, genetic mutation of cells can promote tumorigenesis, but nonmutant cells in the TME, such as CAFs, significantly influence the progression of cancer and treatment sensitivity (Bochet *et al.*, 2013; Sahai *et al.*, 2020).

Cancer-associated fibroblasts in the tumor microenvironment

CAFs are the most abundant cellular components of the TME. These cells are also known as activated fibroblasts or myofibroblasts in the tumor stroma and are reported to promote tumor development. The origin of CAFs is heavily debatable. Various cell types, including fibroblasts, endothelial cells, vascular mural cells, adipocytes pericytes, and mesenchymal stem cells, may give rise to CAFs (Kalluri, 2016; Nair *et al.*, 2017; Öhlund *et al.*, 2017; LeBleu and Kalluri, 2018; Miyazaki *et al.*, 2020).

A build-up of stromal cells and activated fibroblasts in CRC is associated with poor prognosis and disease recurrence (Anderson and Simon, 2020; Musa and Ali, 2020). This corroborates previous report stating that the mesenchymal or stromal CRC subtype (consensus molecular subtype 4-CMS4) are associated with the worst survival outcomes compared to other CMSs (Guinney *et al.*, 2015; Varga *et al.*, 2020; Kasashima *et al.*, 2021).

Interestingly, CAF-derived gene signatures also demonstrated an association with poor survival in CRC patients with advanced stage of the disease than in earlier stages (Herrera *et al.*, 2021). Additionally, colorectal CAF activation is linked to metastasis, treatment outcome, and patient prognosis, solidifying their vital roles in CRC carcinogenesis (Calon *et al.*, 2012; Tauriello *et al.*, 2018; Khare *et al.*, 2021).

Dienstmann *et al.* (2019) reported that the patterns of TME infiltration indicate the risk for distant dissemination in the early stages of CRC. Subsequent studies need to be conducted to examine the role of CMS groups and CAF and immune cell infiltration patterns in predicting survival over TNM staging and microsatellite status (MSI) status.

There is increasing evidence on the effects of CAFs on the immune response towards CRC. CAFs in the TME influence the prognosis of CRC patients by inhibiting immune response, subsequently driving tumor progression, and contributing to treatment failure (Chen *et al.*, 2021; Maia *et al.*, 2023). CAFs secrete a variety of cytokines and chemokines, including interleukin-6 (IL-6) and CC chemokine ligand 2 (CCL2), which promote tumor invasion and metastasis and lead to immunosuppressive TME (Kalluri, 2016). Additionally, CAFs also express checkpoint ligands such as PD-L1 and PD-L2, which inhibit T-cell activation and hinder autoimmunity (Latchman *et al.*, 2001; Gorchs *et al.*, 2019). In addition, in the CRC mice model, CXCL5 produced by CAFs was found to regulate the PD-L1 in tumor via PI3K/AKT signaling (Li *et al.*, 2019). There are a number of clinical trials in different tumors involving the combination of immunotherapies with CAF-targeting for cancer treatment. Despite encouraging data from pre-clinical models, the effect of this mode of therapy is yet to be confirmed in human subjects (Maia *et al.*, 2023).

TABLE 1

Cancer-associated fibroblast markers and their prognostic values for colorectal cancer

Markers	Type of analysis	Expression	Prognosis	Ref.
OPN	Meta-analysis	Upregulated	Poor	Zhao *et al.* (2015)
VDR	Single-cell multi-omics sequencing	Downregulated	Good	Ferrer-Mayorga *et al.* (2017)
BGN	Transcriptome sequencing, IHC	Upregulated	Poor	Zhou *et al.* (2020), Liang *et al.* (2023)
RCN3				
TAGLN				
MYL9				
TPM2				
ZNF532	Single-cell RNA sequencing analysis, qPCR	Upregulated	Poor	Wang *et al.* (2023)
COLEC12				

Note: BGN, biglycan; COLEC12, collectin subfamily member 12; IHC, immunohistochemistry; MYL9, myosin light chain 9; OPN, osteopontin; RCN3, reticulocalbin 3; TAGLN transgelin; TPM2, tropomyosin 2; VDR, vitamin D receptor; ZNF532, zinc finger protein 532.

Tumor cell-CAF crosstalk occurs through paracrine and autocrine signals (Fiori *et al.*, 2019). This involves different molecular pathways such as transforming growth factor-beta (TGF-β) signaling and C-X-C motif chemokine 12-chemokine receptors C-X-C chemokine receptor type 4 (CXCL12-CXCR4) axis (Li *et al.*, 2013; Khare *et al.*, 2021).

CAFs are identified using various biomarkers. Depending on cancer type and staging, these CAF markers exhibit differential expression patterns, which corroborates the idea that different CAF subpopulations exist in the cancer tissue. The presence of heterogeneous populations of fibroblasts in a tumor can be partly attributed to the variations in the activation process of resident fibroblasts with organ-specific features (Miyashita and Saito, 2021).

Classical biomarkers of CAFs include the alpha-smooth muscle actin (α-SMA), fibroblast specific protein 1 (FSP-1), vimentin and fibroblast activation protein (FAP) (Herrera *et al.*, 2013; Togo *et al.*, 2013). Additionally, there are several CAF biomarkers with prognostic value and functional relevance, such as caveolin-1 (CAV1) and CD90 (Thy-1) (Huynh *et al.*, 2016). CAV1 expression in the stroma is associated with poor survival (Goetz *et al.*, 2011). A recent report has shown the association of multiple CAF markers, namely α-SMA, collagen I, and platelet-derived growth factor receptor-beta (PDGFR-β), which are widely distributed in the stroma, with high venous invasion in advanced CRC (Nishishita *et al.*, 2018). This supports a previous observation of the correlation of α-SMA positive and desmin negative myofibroblasts in advanced CRC with malignancy potential (Takatsuna *et al.*, 2016). On the contrary, the expression of podoplanin in CAFs indicates a favorable prognosis of CRC (Choi *et al.*, 2013), although other reports have shown podoplanin expression as a marker for an unfavorable prognosis (Kitano *et al.*, 2010; Schoppmann *et al.*, 2013).

The gene set expressions associated with worse prognosis in CRC were found to increase in CAFs. Additionally, elevated levels of expression in genes upregulated in CAFs such as α-SMA and FAP indicate poor disease-free or lower survival for individuals with CRC (Herrera *et al.*, 2013; Calon *et al.*, 2015). Another potential marker is secreted glycoprotein stanniocalcin-1 (STC1), which is expressed in CAFs and acts as a mediator through platelet-derived growth factor (PDGF)-mediated signaling and drives the metastasis in CRC (Peña *et al.*, 2013). Other recently discovered CAF-related prognostic markers for CRC are listed in Table 1.

Despite being used widely for CAF identification, conventional CAF biomarkers such as α-SMA are found to be heterogeneous in expression and have a lower specificity, whereby they are also expressed by other cell types besides CAFs, namely mesenchymal and hematopoietic cells (Nurmik *et al.*, 2020). Therefore, it is impertinent to discover robust and specific markers, which can be applied in concordance with morphological evaluation for better identification and classification of heterogeneous CAF subpopulations.

CAFs have a complex nature and are thus poorly defined. Depending on the cancer type, phenotypic variation of CAFs may be found. Simon and Salhia (2022) broadly classified CAFs in the TME into four groups: immune, desmoplastic, contractile, and aggressive, where the immune and desmoplastic CAF subtypes have tumor-suppressive properties, while the contractile and aggressive are protumorigenic. Another study by Lavie *et al.* (2022) reported three major CAF subsets, identified using single-cell RNA sequencing analysis: (a) inflammatory CAFs, (b) antigen-presenting CAFs, and (c) myofibroblastic CAFs (myCAFs). Central characteristics of different CAF subgroups are found to be conserved across different organs and cancer types. Several hallmark genes which signify specific CAF features were identified. Nevertheless, the CAF signatures differ according to the organ. For example, specialized stromal cell subgroups (e.g., pericryptal Ptgs2-expressing fibroblasts and crypt-bottom fibroblasts) were reported to support epithelial stemness in the colon (Roulis *et al.*, 2020).

TABLE 2

Cancer-associated fibroblast subpopulations in colorectal cancer and their respective biomarkers

Type of analysis	CAF subset	Biomarker	Ref.
Single-cell transcriptomes	CAF-A	*MMP2, DCN, COL1A2, PDPN, FAP*	Li *et al.* (2017)
	CAF-B	*ACTA2, TAGLN, PDGFA, LUM*	
Multiplex staining IHC, IF	$CD73^{hi}$ population	CD73	Yu *et al.* (2020)
Single-cell transcriptomics	*SOX2/SFRP2*$^{+}$ CAFs	*SOX2, SFRP2*	Jackstadt and Norman (2021), Kasashima *et al.* (2021)
Single-cell RNA sequencing	CAF-S1 (ecm-myCAF, IL-Icaf, detox-iCAF, wound-myCAF, TGFß-myCAF)	Positive for: *PDGFRA, PDPN, CXCL1, CXCL2, CXCL12, CXCL14, TNFRSF12A, LOXL1, LOX*	Khaliq *et al.* (2022)
	CAF-S4 (Immature phenotype, differentiated myogenic subtype)	Positive for: *RGS5, MCAM, CSPG4, PDGFRA, TAGLN, MYH11, CD248, EPAS1*	
Single-cell transcriptomics, bioinformatics analysis	Contractile CAFs	*MYH11, RGS5, MCAM*	Giguelay *et al.* (2022)
	ECM remodeling CAFs (collagen-producing CAFs, CAFs complement-secreting CAFs)	*MMP2, LTBP2*	
Imaging, IF techniques	COL1+ CAFs (COL1+ COL6 +, COL1+ COL6-) COL6+ CAFs	*COL1, COL6*	Heichler *et al.* (2022)
single-cell RNA sequencing	inflammatory CAFs, antigen-presenting CAFs, myCAFs	Inflammatory CAFs: IL-6, Ly6C and PDGFRα Antigen-presenting CAFs: HLA-DRA, HLA-DPA1, HLA-DQA, CD74 marking myCAFs: ACTA2, TAGLN and POSTN	Lavie *et al.* (2022)
Single-cell RNA sequencing, IF staining, spatial transcriptomics	*FAP*$^{+}$ fibroblasts	*FAP, MMP1, MMP3*	Qi *et al.* (2022)
Single-cell sequencing and transcriptomics	CAF_1, CAF_2, CAF_3, CAF_4	*TCF7LI, FLNA, GPX3, MMP11*	Zhao and Chen (2022)

Note: IFC, immunohistochemistry; IF, immunofluorescence.

As stated by various reports, different CAF populations in CRC have been identified using various markers. Table 2 summarizes these CAF subtypes, identified using different biomarkers, in the TME of animal and human CRC models.

Translational outlook of cancer-associated fibroblasts in colorectal cancer

CAFs are proven to be an active player in colorectal carcinogenesis, and studies surrounding this cell population have been extensively explored over the past two decades. This emphasizes several promising clinical applications of CAFs in CRC management.

Future CRC treatment will evolve to be more tailored to patients, which fulfills the need for personalized medicine. Recent discoveries in oncology have presented different strategies for CRC therapy, including targeting CAFs (Sahai *et al.*, 2020), which potentially serve as a complement to conventional therapy. Conventional CRC therapy involves chemotherapeutic drugs targeting tumors more broadly, which renders it less effective and causes multiple adverse effects. Combining CAF-targeted therapy with a conventional mode of treatment hypothetically will increase the effectiveness of treatment and abolish cancer progression, growth, and metastasis.

Although the notion of CAF- or TME-targeted therapy, in general, is intriguing, existing treatments targeting the TME approved by the United States Food and Drug Administration (FDA) have limited efficacy. However, pre-clinical studies have shown promises results in employing the TME components, including chimeric antigen receptor-transduced natural killer (CAR-NK) cells, stellate cells, and fibroblasts as potential new targets for cancer treatment (Anderson and Simon, 2020; Zhang *et al.*, 2022).

Designing an effective treatment against CAF populations would require robust biomarkers for identifying cells of the target. To fully characterize CAFs in CRC, multiple markers are suggested for their identification. Although various CAF markers have been established, their relationship and correlation with expression in CRC are still under investigation due to their complexity.

To improve patient management, further prognostic and/or predictive CRC markers are needed. Current diagnosis involves tumor, node, and metastasis (TNM) classification is limited as it relies on anatomical evaluation. Other lower-cost, simpler, and quick assessments, such as tumor-stroma ratio (TSR), have been suggested for patient stratification (Gao *et al.*, 2021). However, this pathological marker may provide limited information regarding the tumor phenotype. Although other biomarkers or biomolecules based on molecular tumor profiling, such as genomic and transcriptomics analyses, acquire comprehensive and massive data, these are costly compared to conventional histopathological examination.

Circulating biomolecules of CRC have been reported to possess clinical utility. These biomolecules include carcinoembryonic antigen (CEA), calretinin, CDH17, MOC-31 (Ep-CAM), CK20, CK7, CA19-9, and MUC2 (enzyme) (Petrik *et al.*, 2022). Mutational profiling of KRAS, BRAF, and PIK3CA also are being investigated due to their high prevalence in CRC. These biomarkers represent the epithelial origin of CRC instead of the fibroblast or CAF population of this cancer. Therefore, it is paramount to identify robust and specific biomarkers for CAFs for application in liquid biopsy for CRC.

Proposed translational prospects of CAF in CRC are elaborated further in the following section and depicted in Fig. 1.

Cancer-associated fibroblasts in liquid biopsy application for colorectal cancer

A significant number of CRC cases are diagnosed at the later stages (Meester *et al.*, 2019; Siegel *et al.*, 2020), largely due to a lack of screening and awareness of cancer prevention among the public. A high recurrence rate was found in CRC, and this may differ according to disease staging. Approximately 80% of stage IV CRC patients relapse within the first 2 years following surgery with curative intent (de Jong *et al.*, 2009), whereas recurrence is lower (30%) for stages I–III patients (van der Stok *et al.*, 2017). Existing clinical procedures to screen and diagnose CRC, such as colonoscopy, can be considered invasive, present a risk of complications, and cost ineffective and inconvenient, thus rendering CRC screening underused. The non-invasive option to screen for CRC is predicted be increasingly important in the future (Shaukat and Levin, 2022).

Liquid biopsy, which is the most current interest in oncology, allows a less invasive, more convenient, and frequent sampling compared to tissue biopsy and complements molecular imaging assessments (Cherry *et al.*, 2018). The collected specimens include peripheral blood, urine, saliva, and other bodily fluid. The advancement in liquid biopsy aims to dissect spatial and temporal heterogeneity in tumor biology and can be achieved through serial blood test, rather than biopsy of the primary tumor, which is performed years prior on a single metastatic site when multiple metastases are potentially present before any drug treatment. This may improve patient management and treatment outcomes as well as enable early cancer detection (Pantel and Alix-Panabières, 2010; Wan *et al.*, 2017). Despite great advances in liquid biopsy, the application of this approach in clinics has been slow (Ignatiadis *et al.*, 2015).

Liquid biopsy involves the isolation and detection of tumor-derived analytes, including (a) circulating tumor cells (CTCs), (b) circulating nucleic acids such as circulating tumor DNA (ctDNA), cell-free DNA (cfDNA), cell-free RNAs (mRNAs, long non-coding RNAs, and microRNAs), (c) tumor extracellular vesicles, and (d) proteins and metabolites in the body fluids of individuals with cancer, followed by genomic and proteomic analyses (Ramalingam and Jeffrey, 2018; Lone *et al.*, 2022). To date, a very little study has been conducted to explore circulating TME cellular components, such as CAFs in liquid biopsy, despite its promising clinical prospects.

CTCs, one of liquid biopsy analytes, refer to subsets of tumor- or metastasis-derived cells that circulate in the bloodstream. CTC isolation is less invasive and safer compared to conventional tissue biopsy. CTCs can be applied to monitor cancer progression and assess response to therapy in real-time. Detection of CTCs in peripheral blood serves as valuable tumor markers, including for CRC (Yap *et al.*, 2014). CTCs in CRC include a vast range of cells, including epithelial tumor cells, tumor cells undergoing epithelial-mesenchymal transition (EMT), and cancerous stem cells (Hardingham *et al.*, 2015). It is proposed that epithelial cancer cells migrate and invade the blood vessel after EMT, which is the main cause of metastasis *in vivo* (Masuda *et al.*, 2016; Jia *et al.*, 2017). Upon reaching a secondary organ, CTCs undergo a mesenchymal-epithelial transition, reacquire stemness properties and form a new metastatic site (Suarez-Carmona *et al.*, 2017). Contrary to other cancer biomarkers, CTCs are viable tumor cells, carrying overall molecular and biological profiling of the tumor, promoting single-cell analysis, and directly indicates the ongoing alteration in tumor mass at various stages (Li *et al.*, 2020).

Over the past 20 years, great advancements have been made in the detection methods of heterogeneous cells in colorectal tumors. Currently, tumor staging is performed by histology and radiological imaging. These techniques lack sensitivity for the detection of micrometastases or early tumor dissemination, which are vital for metastatic progression of the disease. The presence of CTCs indicates micrometastasis events and potentially have prognostic values independently of established staging factors (e.g., lymph node involvement) (Hardingham *et al.*, 2015).

Vast arrays of advances in science, such as high-throughput sequencing (Jones *et al.*, 2008), CRISPR/Cas9 editing tools (Chen *et al.*, 2015), and single-cell technologies (Navin *et al.*, 2011), have helped to gradually uncover biological phenomenon including mechanisms underlying metastasis as well as involvement of key driver genes, EMT of tumor cells, the role of exosomes in tumorigenesis, CTCs and complex interplay between tumor cells and the TME

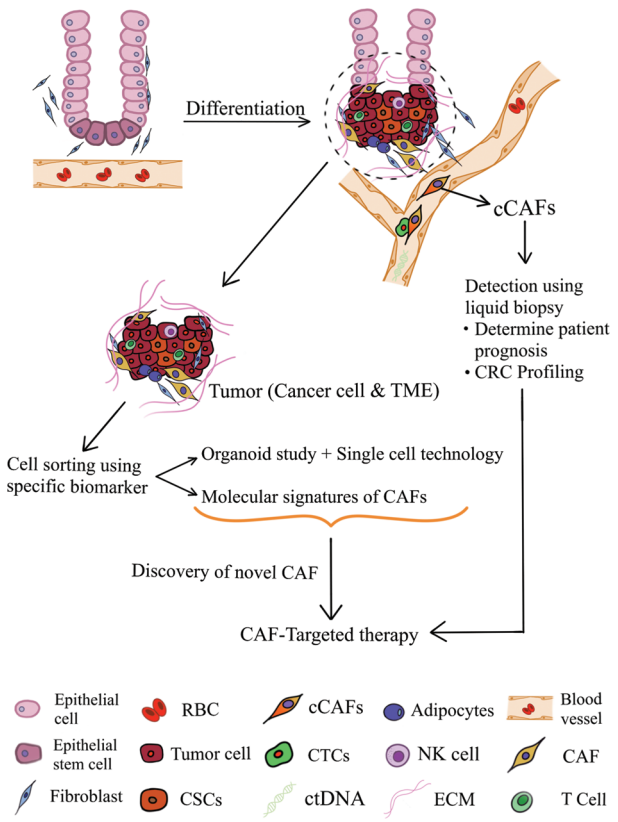

FIGURE 1. Translational prospects of cancer-associated fibroblasts in colorectal cancer (TME: tumor microenvironment; CAF: cancer-associated fibroblast; cCAF, circulating CAF; CTCs, circulating tumor cells; RBC, red blood cell; ctDNA, circulating tumor DNA; NK cell, natural killer cell; ECM, extracellular matrix; CSC, cancer stem cell).

(Peinado *et al.*, 2012; Quail and Joyce, 2013; Yu *et al.*, 2013; Aceto *et al.*, 2014). CTC detection is usually highly dependent on molecular markers, the most commonly used being the epithelial cell adhesion molecule markers. These molecular markers vary according to cancer types (Lin *et al.*, 2021).

To date, there is very little knowledge of CAF-CTC interplay. The earliest report by Duda *et al.* (2010) on animal models of lung cancer showed that CTCs could interact and carry CAFs from the primary tumor to the metastatic site (Ortiz-Otero *et al.*, 2020a). Molecular changes such as decreased expression of epithelial markers, including E-cadherin and claudins, as well as elevated levels of mesenchymal markers (vimentin, N-cadherin, FSP-1, and fibronectin) are observed during the EMT process (Mittal, 2018). A small fraction of CTCs interacts with different TME components, such as neutrophils, macrophages, and CAFs, to escape the immune system, thus driving their survival (Rejniak, 2016; Garrido-Navas *et al.*, 2019).

In a study on human CRC samples, CAFs have been proposed to promote metastasis by creating gaps in the basement membrane that facilitates cancer cell invasion independently of matrix metalloproteinase (MMP) (Glentis *et al.*, 2017). Additionally, during the dissemination process, CAFs are able to protect CTCs from the fluid shear force via stable intracellular contact and secretomes such as CXCL5, CCL2, and CCL7, which are linked to cell survival, invasion, and EMT (Ortiz-Otero *et al.*, 2020a). A high proportion of mature CAFs was reported in intratumoral stroma (57.6%) and invasive front of CRC (60.3%). Interestingly, overexpression of epidermal growth factor receptor (EGFR) was found in the mature CAFs in the invasive front in comparison to immature CAFs. Increased lymphatic invasion directly correlates with the number of mature fibroblasts in the intratumoral stroma (Shin *et al.*, 2019).

Currently, there is a lack of studies on circulating CAFs (cCAFs) of CRC, although reports have been previously published on cCAF detection in breast cancer. The isolation of cCAFs has been reported in the peripheral blood of metastatic breast cancer patients but not in individuals in their early stages (Ao *et al.*, 2015). A recent report by Jiang *et al.* (2021) has demonstrated a novel, effective, label-free, and high throughput microstreaming platform for the isolation of cCAFs, CTCs, and immune cells from breast cancer patients. As stated in the previous section, CTCs represent the precursor of metastasis, where they can migrate as individual CTCs or in clusters (CTCs with stromal cells such as CAFs). However, until now, there is little direct evidence indicating CAF presence in the circulation of cancer patients in the clinical setting.

Ishii *et al.* (2007) have reported the presence of fibroblast progenitor cells in individuals with lung cancer, while Jones *et al.* (2013) demonstrated the detection of fibroblast-like cells in blood samples of metastatic prostate cancer but not in patients with localized prostate cancer or no known malignancy. Both studies employed vimentin as the marker for fibroblast population profiling despite this is not specific to fibroblast cells considering that it is also expressed by lymphocytes (Nieminen *et al.*, 2006), as well as breast cancer cells and CTCs that have undergone EMT (Aktas *et al.*, 2009). A more recent finding by Ao *et al.* (2015) used widely accepted CAF biomarkers, namely $FAP^+/\alpha\text{-}SMA^+/Cytokeratin^-/CD45^-$ for the detection of cCAFs in the peripheral blood of patients with metastatic breast cancer. In addition, their technique of evaluating the co-expression of FAP/α-SMA on the top of novel cell-size-based microfilter technology, which was initially used to capture CTCs (Zheng *et al.*, 2007), has enabled the separation of CAFs from CTCs from blood samples of patients with breast, prostate, and colon cancer.

Using blood samples from various metastatic cancer patients, including CRC, followed by an assessment of CTC and CAF numbers, Ortiz-Otero *et al.* (2020b) established the correlation between cCAF levels with poor prognosis and lower survival rate in the selected samples. This report highly suggests CAF level as a predictive biomarker for CRC prognosis.

One of the challenges in studying CRC metastasis *in vivo* is the transient nature of carcinoma *in situ*, which rendered the onset of cancer invasion and basement membrane breakage laborious to study. Nevertheless, previous *in vitro* studies indicated that the metastasis process could be inhibited by hindering CAF formation and functions (Cazet *et al.*, 2018; Li *et al.*, 2019). Yang *et al.* (2021) reported different possible mechanisms of CAF metastasis in CRC. They further confirmed that the inhibition of CAF migration and invasion abolished the metastasis of both

tumor cells and CAFs through an *in vivo* study. Worth noting that this study presents several limitations, including a lack of confirmation on the correlation between cCAF levels and the prognosis of CRC patients and the mechanism of CAF colonization and survival in the distant niche. This may be achieved by collecting peripheral blood from patients with CRC and isolating cCAFs for further analysis.

Based on the potential of cCAFs in liquid biopsy, we proposed that cCAF utilization in the clinical management of CRC can be potentially expanded to:

i) stratify patients more effectively
ii) indicate the staging and/or subtype of the disease
iii) be used as complementary to established genetic/genomic testing and tissue biopsy analyses

Cancer-associated fibroblasts-targeted therapy

Over the past decade, there is a great enthusiasm surrounding the idea of designing a targeted treatment against TME components in various cancers. Targeted therapies, which embody the concept of "precision medicine," are preferred compared to traditional cancer treatment due to their higher efficacy and less adverse effect. Considering the major role of CAFs in colorectal carcinogenesis, it is not surprising that many are focusing on CAF-related treatment to eradicate CRC. Although studying cCAFs seems to be a good direction to pursue, there are still valid concerns on the lack of biomarkers for the detection of specific populations of CAFs. It is an established notion that CAFs are heterogeneous; thus, having robust and specific CAF biomarkers will ensure optimum specificity and sensitivity in the detection and isolation of cCAFs. This subsequently will drive the discovery of CAF-targeted therapy for CRC.

Taking into account that CRC has a molecular subset characterized by accumulation of stromal cells (CMS4), this serves as a great target for targeted treatment against CAFs. Moreover, as stated previously, this CRC subset is characterized by the worst prognosis among the four CMSs (Varga *et al.*, 2020; Kasashima *et al.*, 2021). In a recent report by Zhong *et al.* (2022) on transcription factor (TF) signatures of CMS4 has revealed nine TF-related genes as main regulators for the CMS4 subtype. These genes are *MEIS2*, *MEIS3*, *SNAI1*, *KLF17*, *BARX1*, *ZNF532*, *HEYL*, *FOXL2* and *LHX6*. Interestingly, these genes are implicated as prognostic factors in CRC in both analyses using The Cancer Genome Atlas (TCGA) and International Cancer Genome Consortium (ICGC)-ARGO data. Additionally, these TF-related genes are able to discriminate patient survival after adjustment to clinical factors linked to prognosis, such as gender, TNM classification, MSI status, and the location of primary tumors (left *versus* right-sided). Microenvironment components in high-risk CRC patients with highly immunosuppressed profiles (overexpression of TIM3, CD39, and CD40) indicated that these individuals might not benefit from conventional immune checkpoint inhibitors (ICIs) such as anti-PD1 therapies. Stratification of CRC patients through gene expression profiling of CAFs, as reported, will drive the exploration of targeted drugs. Additionally, this information also can explain the behavior of high-risk CRC patients and predict drug response.

The interplay between CAF and cancer cells is facilitated by intricate autocrine and paracrine signaling pathways. These include TGF-β, phosphoinositide 3-kinase/AKT/mammalian target of rapamycin (PI3K/AKT/mTOR), mitogen-activated protein kinases (MAPK), Wnt, EGFR, Hippo, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathways (Wu *et al.*, 2021). These elaborate signaling pathways induce the transition of CAFs from stromal fibroblasts, which subsequently lead to CAF heterogeneity (Erez *et al.*, 2010; Ringuette Goulet *et al.*, 2018). Blocking these signaling pathways may impair the activation of CAFs and affect the CAF-tumor cell crosstalk. Thus, targeting the essential signal cascades involved in the bidirectional communication between cancer cells and CAFs may serve as an excellent strategy to explore treatment avenues for CRC.

The cancer-stromal interactions also promote CAF activation, which leads to CRC progression. It is an established notion that a tumor is a wound that never heal; thus, activated fibroblasts behave in a similar manner as they would to injury. This response involves CAF recruitment to tumor sites that mimic the fibrosis secondary to chronic inflammation (Dimmeler and Zeiher, 2017). Recruitment and activation of CAF are mainly facilitated by various growth factors, including TGF-β, chemokines, and cytokines. Despite many studies conducted on CAF-tumor cell crosstalk, the exact nature of CAF trans-differentiation from non-activated fibroblasts is yet to be fully described.

However, due to the complexity of molecular signaling in CRC, extensive studies must be conducted to further dissect CAFs' role in tumor progression, their activation process, and their potential in antitumor therapy. Wu *et al.* (2021) have proposed three alternative mechanisms of CAF-targeted therapies, namely:

Epithelial-mesenchymal common target (EMCT)–targeting both tumor cells and adjacent stromal cells.

Sequential target perturbation (STP)–blocking protumorigenic process prior to treating cancers cells to acquire anticancer effects.

Crosstalk-directed signaling target (CDST)–targeting two different components of a signaling pathway in CAFs and tumor cells simultaneously.

This report by Wu *et al.* (2021) corroborated a recent publication by Wang *et al.* (2022). They suggested four main strategies for anticancer therapy against CAFs through:

i) CAF deletion (by targeting specific markers of CAFs such as α-SMA and FAP)
ii) CAF normalization (shift from pro-carcinogenic to tumor-suppressive state using all-trans retinoic acid (ATRA) or VDR ligands)
iii) blocking CAF activation (targeting cytokines and growth factor signaling)
iv) ECM remodeling

Although the prospect of targeting CAFs for CRC treatment is intriguing, the clinical data have not yet fully supported the benefit of these therapies. A pre-clinical study

employing the co-culture method on CRC patient-derived organoids would be extremely helpful in discovering the activity of drugs against CAFs (Barnett and Vilar, 2017). An example of this approach, as reported by Biffi *et al.* (2019) using organoid and mouse model of pancreatic ductal adenocarcinoma (PDAC), demonstrated that IL-1-induced leukemia inhibitory factor (LIF) expression and downstream JAK/STAT activation resulted in inflammatory CAF (iCAFs) production. TGF-β is found to inhibit this signaling by suppressing IL-1 receptor type 1 (IL1R1) expression and suggesting a transition from iCAFs into myofibroblasts.

Organoid study on human or animal models must be accompanied by the advanced technique of flow cytometry that allows cell separation and identification of CAFs based on established molecular markers. Subsequent analysis at the single-cell level will provide insight into more accurate profiling of CAF populations in CRC. This step is essential, considering that various CAF subgroups with different molecular signatures were detected in CRC (Li *et al.*, 2017; Joanito *et al.*, 2022; Zhao *et al.*, 2022), which highlights their functional diversity that may influence carcinogenesis differently.

Conclusion

CAFs in the TME of CRC represent highly complex and heterogeneous populations. The diversity in the CAF subtypes in the colorectal tumor niche and their interplay with malignant cells drive the evolution and progression of cancer, thus contributing to the heterogeneity of CRC. A repertoire of biomarkers is applied to identify various subgroups of CAF and to aid further understanding of molecular pathways involving CAF-tumor cell crosstalk in colorectal tumor progression. Deciphering the complexity of CAFs will provide insight into more accurate CRC patient profiling through a liquid biopsy approach and analysis of cCAFs. In addition, CAFs also potentially serve as targets for CRC therapy. Despite encouraging pre-clinical data and great promises of the translational prospects of CAFs, there are scientific challenges that need to be addressed in order to implement effective clinical applications utilizing CAFs in the future.

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