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REVIEW ARTICLE

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5-Fluorouracil resistance mechanisms in colorectal cancer: From classical pathways to promising processes

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Abstract

Colorectal cancer (CRC) is a public health problem. It is the third most common cancer in the world, with nearly 1.8 million new cases diagnosed in 2018. The only curative treatment is surgery, especially for early tumor stages. When there is locoregional or distant invasion, chemotherapy can be introduced, in particular 5-fluorouracil (5-FU). However, the disease can become tolerant to these pharmaceutical treatments: resistance emerges, leading to early tumor recurrence. Different mechanisms can explain this 5-FU resistance. Some are disease-specific, whereas others, such as drug efflux, are evolutionarily conserved. These mechanisms are numerous and complex and can occur simultaneously in cells exposed to 5-FU. In this review, we construct a global outline of different mechanisms from disruption of 5-FU-metabolic enzymes and classic cellular processes (apoptosis, autophagy, glucose metabolism, oxidative stress, respiration, and cell cycle perturbation) to drug transporters and epithelial-mesenchymal transition induction. Particular interest is directed to tumor microenvironment function as well as epigenetic alterations and miRNA dysregulation, which are the more promising processes that will be the subject of much research in the future.

KEYWORDS

5-fluorouracil, colorectal cancer, resistance mechanism

1 | INTRODUCTION

Worldwide, colorectal cancer (CRC) is the third most frequent cancer.[1] Colon cancers led to approximately 551 000 deaths whereas it approached 310 000 for rectal cancers.[1] For patients suffering from node invasion, or with metastatic CRC (lung or liver), or more widely all patients with risk of recurrence and when uncontrolled disease is superior to chemotherapy-induced risk, neoadjuvant or adjuvant treatments can be proposed. They are composed of cytotoxic chemotherapies used alone or in combination with targeted therapies.[2] The most important chemotherapeutic molecule currently used in CRC treatment is the 5-fluorouracil (5-FU), a synthetic fluorinated pyrimidine analog administered i.v. that requires intracellular conversion into active metabolites.[3] Despite advances in systemic therapy, the 5-year survival remains too low.[4] One essential reason that treatment fails is the presence of innate or acquired resistance (90%

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of patients with metastatic cancer[5]), which remains one of the greatest challenges in long-term management of incurable metastatic disease.

The fundamental enzyme in 5-FU metabolism is thymidylate synthase (TS), which belongs to 1 of the most important of the 3 pathways of 5-FU anabolism. Like other enzymes involved either in 5-FU anabolism or catabolism, they are often altered, promoting 5-FU resistance. In addition, the main cellular functions (apoptosis, autophagy, respiration, glucose metabolism, and cell cycle) can also be altered in CRC cells exposed to 5-FU. Drug transporters are key factors involved in multidrug resistance (MDR). Epithelial-mesenchymal transition (EMT) induction as well as 5-FU-mediated epigenetic changes and microRNA (miR) dysregulations, constitute crucial 5-FU resistance mechanisms. In this review, we focused on these mechanisms set up by CRC cells to resist 5-FU-induced cytotoxic effects.

2 | ALTERATION OF TS AND OTHER ENZYMES INVOLVED IN 5-FU METABOLISM AND ACTIVATION

5-Fluorouracil is an analogue of uracil with a fluorine atom at the C5 position in place of hydrogen. This heterocyclic aromatic organic compound shares a common structure with pyrimidine and is incorporated in DNA and RNA in the same ways. The 5-FU enzymatic reaction is well known and described (Figure 1).

In 5-FU-resistant cells, TS mRNA levels were found to be increased, leading to enhanced TS catalytic activity[6] and impaired TS transport and/or inhibition (without change in its enzymatic activity). High intrinsic levels of TS were related to 5-FU resistance in vitro, in vivo, and in patients.[7] Moreover, following 5-FU treatment, the overall survival of patients with high TS levels, observed in primary

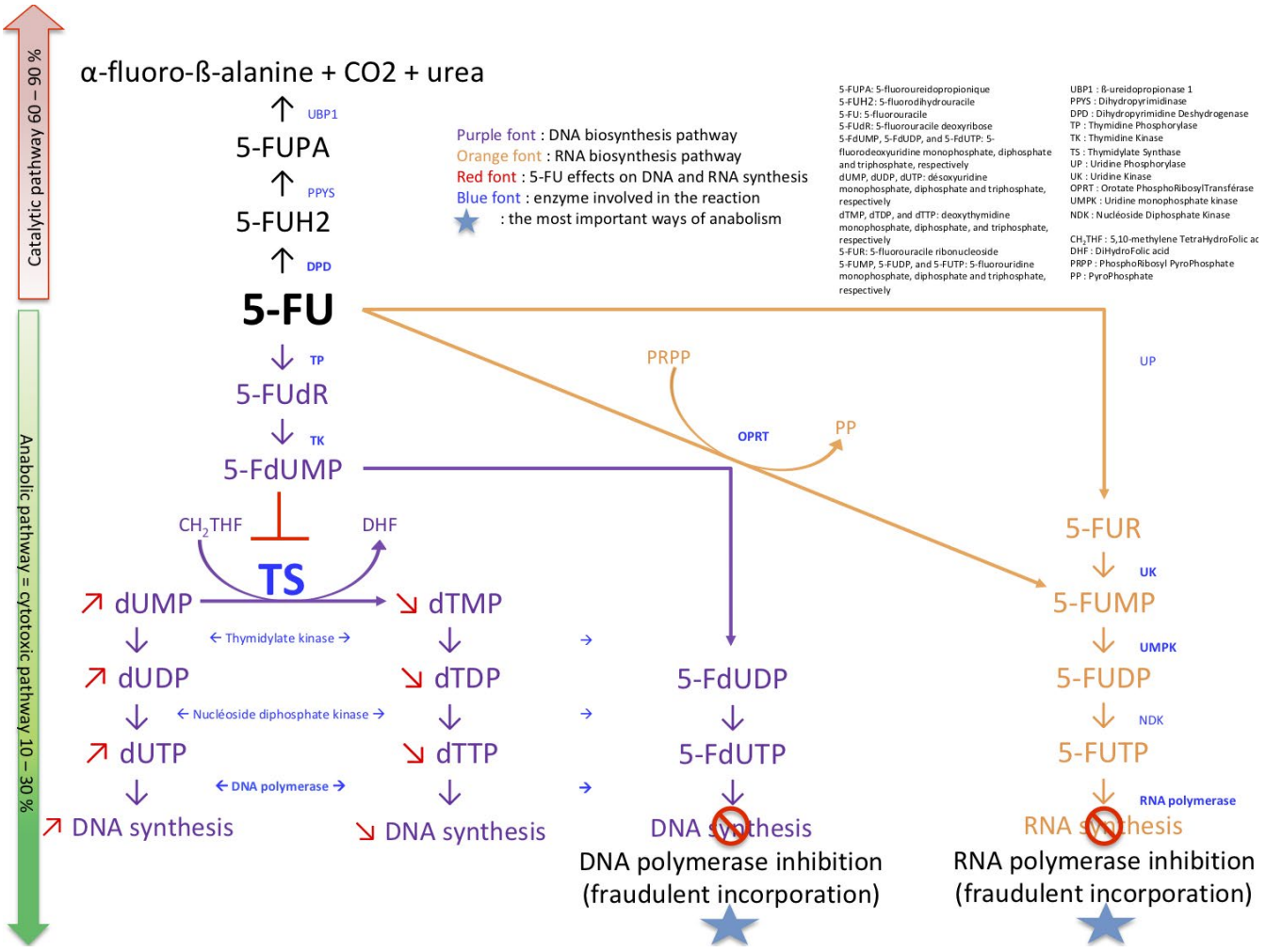

FIGURE 1 5-Fluorouracil (5-FU) metabolism. 5-FUPA, fluoroureidopropionic acid; 5-FUH2, 5-fluorodihydrouracil; 5-FdUDP, 5-fluorodeoxyuridine diphosphate; 5-FdUMP, 5-fluorodeoxyuridine monophosphate; 5-FdUTP, 5-fluorodeoxyuridine triphosphate; 5-FUdR, 5-fluorouracil deoxyribose; 5-FUDP, 5-fluorouridine diphosphate; 5-FUMP, 5-fluorouridine monophosphate; 5-FUR, 5-fluorouracil ribonucleoside; 5-FUTP, 5-fluorouridine triphosphate; CH2THF, 5,10-methylene tetrahydrofolic acid; DHF, dihydrofolic acid; DPD, dihydropyrimidine dehydrogenase; DPYS, dihydropyrimidinase; dTDP, deoxythymidine diphosphate; dTMP, deoxythymidine monophosphate; dTTP, deoxythymidine triphosphate; dUDP, deoxyuridine diphosphate; dUMP, deoxyuridine monophosphate; dUTP, deoxyuridine triphosphate; NDK, nucleoside diphosphate kinase; OPRT, orotate phosphoribosyltransferase; PRPP, phosphoribosyl pyrophosphate; PP, pyrophosphate; TK, thymidine kinase; TP, thymidine phosphorylase; TS, thymidylate synthase; UP, uridine phosphorylase; UBP1, β-ureidopropionase 1; UK, uridine kinase; UMPK, uridine monophosphate kinase.

tumors with metastasis and in those with lymph node metastasis, is shorter than in patients with low TS levels.[8] One of the leading causes of increased levels of TS in colorectal tumors is *TS* polymorphism, with notably a triple tandem repeat (TSER *3) detected in the 5′-UTR of the *TS* gene.[9] Moreover, *TS* copy number and different genomic instability statuses (chromosome instability, CpG island methylator phenotype, and microsatellite instability [MSI]) should also be considered in 5-FU responsiveness. High levels of TS before 5-FU-based treatments are associated with disturbed folate pools and lead to intrinsic resistance. In contrast, acquired resistance is related to *TS* gene amplification and mutations.[10] These results suggest new and better approaches to treatment: patients with tumors displaying *TS* amplification should not be treated with 5-FU.[11]

Uridine kinase, orotate phosphoribosyltransferase (a key enzyme involved in de novo pyrimidine biosynthesis), and thymidine kinase activities are lower in resistant cells.[12] A lower expression of UMP kinase, an enzyme involved in 5-FU activation in 5-fluorouridine triphosphate (5-FUTP) as well as in its incorporation into RNA, has also been observed.[13] Alterations in the uridine monophosphate synthetase (*UMPS*) gene (responsible for 5-FU conversion into active anticancer metabolites in tumor cells), such as heterozygous splice site mutations and aberrant exon splicing and downregulations, leading to a decrease of its activity, are recently described mechanisms explaining 5-FU resistance in CRC.[14]

3 | CELLULAR FUNCTIONS DISRUPTED BY 5-FU

Cancer cells are able to develop resistance mechanisms in response to anticancer drugs. This section describes how CRC cells are able to modulate cell cycle, glucose metabolism, oxidative stress, mitochondrial activity, and EMT in order to survive inhibiting 5-FU-induced cell death.

3.1 | Apoptosis and autophagy disruptions

Apoptosis and autophagy are 2 regulatory events that can lead to cancer cell death. However, cancer cells frequently counteract these processes to become drug-resistant.

Among others, chemotherapies promote apoptosis through induction of the tumor suppressor gene p53 (*TP53*). It has been described that some CRC cells become resistant to 5-FU due to a modification of binding of cytoplasmic p53. Thus serine hydroxymethyltransferase 2 can bind cytoplasmic p53 and not HDM2, preventing cytoplasmic p53 degradation.[15] However, it was described that *TP53* is mutated in 50% of cancers with a loss of its functionality.[16] Similarly, P53 regulators, such as caspase-9 and its cofactor, apoptotic protease activating factor 1, can be inactivated, also leading to drug resistance. Moreover, several studies have reported the role of glycogen synthase kinase 3β (GSK3β) in 5-FU-mediated CRC cell resistance. It was observed that the inhibition of GSK3β mediates TS and β-catenin upregulation and Bcl2 and E2F1 downregulation, hence promoting CRC cell survival to 5-FU through the inhibition of 5-FU-induced apoptosis and cell cycle progression in S and G_2/M phases.[17] In addition to *TP53* and GSK3β roles in 5-FU-mediated resistance, the possible functions of Rho GDP dissociation inhibitor 2 (RhoGDI2) and Maspin (pro-apoptotic protein), which are upregulated and downregulated, respectively, in resistant cells, were also highlighted. Indeed, knockdown of RhoGDI2 is able to resensitize 5-FU-resistant CRC cells to this drug.[17]

Autophagy is a complex process by which cells can survive through the formation of an independent process of nutrition when the stress induced is too high. It promotes tumor growth and resistance to treatment. Activation of the p38MAPK pathway constitutes a key determinant in this process but also in cellular responses to 5-FU. Indeed, it was reported that the inhibition of this pathway correlates with a decrease in 5-FU-mediated apoptosis, promoting CRC cell resistance. This 5-FU resistance mediated by p38MAPK pathway inhibition is associated with an autophagic response as it induces a decrease in p53-driven apoptosis without effect on p53-dependent autophagy. Consequently, the p38MAPK signaling pathway plays a critical role in CRC cell 5-FU resistance by controlling the balance between apoptosis and autophagy.[18]

The induction of autophagy by 5-FU has also been linked to the p53-AMPK-mTOR pathway: by activating AMPK, p53 thus inhibits mTOR and can trigger autophagy.[19] It was reported that receptor associated-coactivator 3 (RAC3), a member of the SRC/p160 coactivator family highly expressed in cancers with key functions in tumor initiation, progression, metastasis, and survival, promotes 5-FU resistance. Under 5-FU treatment, RAC3 is overexpressed in CRC cells. It inhibits both apoptosis and autophagy.[20] Activation of p38MAPK and PI3/Akt pathways is concomitant with an inhibition of caspase-8 and -9 and a blockade of apoptosis-inducing factor-1 translocation from mitochondria to the nucleus.

The CD44 antigen isoform containing variant exon v6 (CD44v6) corresponding to an intercellular adhesion cell[21] contributes to resistance when overexpressed by modulating autophagy with activation of both PI3K-AKT and MAPK-Ras-Erk pathways.

3.2 | Disruption of cellular functions participating in EMT

Transforming growth factor-β (TGF-β) is a key actor of EMT known to play paradoxical roles in carcinogenesis. Studies have reported that it also seems to be an important regulator of 5-FU resistance; it is upregulated in 5-FU-resistant CRC cells and its inhibition restores 5-FU CRC cell sensitivity through modulation of some gene expression profiles,[22] including the transcription factor TWIST1. Moreover, it was observed that TWIST1 suppression (*TWIST 1*, well known to be an oncogene) in CRC cells sensitizes them to 5-FU-induced apoptosis.[23]

Integrins are cell surface adhesion protein family members that might participate in chemotherapeutic drug-induced apoptosis of

cancer cells.[24] It was observed that β-6 integrin plays important roles in invasion, metastasis, and degradation of ECM of CRC cells. Moreover, β-6 integrin is overexpressed following 5-FU treatment, protecting CRC cells from 5-FU cytotoxic effects on cell growth inhibition and apoptosis, through Bcl2 upregulation, Bax downregulation, and ERK/MAPK pathway activation.[25] Among the cell surface adhesion proteins, Connexins (also called gap junctions) were also described to decrease the efficiency of 5-FU toxicity in CRC cells.[26] Expression of E-cadherin, a calcium-dependent intercellular adhesion molecule, decreases with CRC stage[27] after 5-FU chemotherapy. This molecule linked to the EMT process has also been found downregulated by overexpression of HES1 (Hairy enhancer of split-1, transcriptional factor of Notch signaling pathway) in stage II-III CRC treated with 5-FU, inducing higher CRC recurrence rates.[28] E-cadherin expression can be affected by nuclear factor-κB (NF-κB), β-catenin, and ZEB1 transcription factors. Recently, a study found that the activation of the cascade NF-κB/AKT/β-catenin/ZEB1 inducing 5-FU chemoresistance was linked to the induction of PSMD4 expression by cytoplasmic nuclear factor erythroid-2-related factor 2 (Nrf2).[29]

Cell surface adhesion molecules are not the only actors of EMT and cancer progression. It was recently suggested that cell fusion would be strongly involved during the initiation, progression, and phenotypic diversification of cancers. ADAM10, GTP-binding protein a13, radixin, myosin regulatory light chain, and RhoA proteins were described to promote cell fusion in CRC,[30] resulting in the acquisition of a resistant phenotype to 5-FU.

Finally, some pathways such as Hedgehog signaling, initially described as important regulators of embryonic development, tissue polarity, and cell differentiation (EMT), were also described to be involved in CRC development and 5-FU resistance through an increase of GLI1 expression in 5-FU-resistant cells. Indeed, it was observed that *GLI1* knockdown sensitized CRC cells to 5-FU treatment, reducing tumor invasiveness.[31]

3.3 | Glucose metabolism, oxidative stress, and mitochondrial respiration

The pyruvate dehydrogenase (PDH) enzyme, which converts pyruvate to acetyl-CoA, is a key mediator of glucose oxidative metabolism. Its activity is inhibited by PDH kinase (PDHK) proteins. It was observed that the expression of PDHK4 is positively correlated with 5-FU resistance of CRC cells. Indeed, 5-FU induces PDHK4 expression in a TGF-β signaling-dependent manner with the phosphorylation of Smad2, and PDH4 knockdown or inhibition significantly increases 5-FU-inhibitory effects on Bcl2 and Survivin expression.[32] Prolyl hydroxylase domain proteins are oxygen-sensitive enzymes initially known for their ability to regulate cellular adaptation to hypoxic conditions through targeting the hypoxia-inducible transcriptional factors HIF-1α and HIF-2α to their proteasomal degradation. Pyruvate dehydrogenases are also involved in cell damage and metabolic stress and mediate 5-FU CRC resistance through p53 phosphorylation induction.[33] This p53 posttranslational modification allows the interaction between p53 and XPB, a component of the nucleotide excision repair machinery, leading to 5-FU-induced DNA damage repair and consequently to CRC cell survival and proliferation.

Many anticancer agents, such as 5-FU, generate reactive oxygen species (ROS), which induce oxidative damage leading to cancer cell death. However, a small subpopulation of cancer cells called cancer stem cells (CSCs) are able to develop cellular adaptive responses to ROS to survive.[18] Some proteins were highlighted as new markers of these CRC subpopulations able to counteract the 5-FU-induced ROS: 5-FU induces nuclear translocation and activation of Nrf2, known to be linked with drug resistance through its constitutive activation,[34] which in turn leads to upregulation of antioxidant enzymes, increasing cell resistance to the cytotoxic effects mediated by this drug.

Mitochondria provide energy for cells and mediate apoptosis. Downregulation of ATP synthase inhibits the electron flux to the respiratory chain, which leads to increased levels of superoxide radical, causing DNA damage and mitochondrial-mediated cell death, but also transformed phenotype.[35] Consequently, mitochondrial ATP synthase defect could constitute a bioenergetic feature of cancers as well as a key determinant of chemotherapy-induced resistance.[36] In this way, a lower expression of the subunits of the mitochondrial F1F0-ATP synthase was reported in 5-FU-resistant cells.[37] Consistently, oligomycin A, an ATP synthase inhibitor,[37] strongly antagonizes 5-FU cytotoxic effects. Mitochondrial division is a mechanism that leads to the production of fragments with increased ROS levels. A recent study focused on SW480, a CRC cell line corresponding to an early stage of CRC, and showed that 5-FU resistance was induced by a reduction of mitochondrial division in relation with decreased expression of both mitochondrial elongation factor 1 and the large tumor suppressor kinase 2 (LATS2)-Hippo pathway.[38]

3.4 | Cell cycle perturbation

5-Fluorouracil-resistant CRC cells showed significant cell cycle delay in G_1 and G_1/S and prolonged DNA synthesis time.[39] Moreover, protein expression levels of cyclin-dependent kinase 2 (CDK2) and its phosphorylated form, cyclin D3, and cyclin A, involved in G_1 and S phase transition as well as pRB phosphorylation rates, were reduced in 5-FU-resistant cells. These results suggest a cell cycle slowdown, hence preventing incorporation of 5-FU metabolites into DNA and providing cancer cells sufficient time for DNA repair.[40] Moreover, a clonal subpopulation was observed with typical CSC features and resistant to 5-FU, able to enter into a reversible quiescent G0 state following reexposure to higher 5-FU concentrations. These quiescent 5-FU-resistant CRC stem cells overexpressed both the activated and membrane-bound tyrosine kinase c-Yes. In addition, YES1 and Yes-associated protein (YAP) transcript levels were found at higher levels in liver metastases of patients with CRC after 5-FU-based neoadjuvant chemotherapy. Consistently, YES1 and YAP transcript levels positively correlate with CRC relapse and shorter patient survival.[40]

A study published in 2015 that focused on Smad4 null-SW620 CRC cell line reported the activation of the PI3K/Akt/CDC2/survivin cascade by induction of low expression of Smad4, resulting in activation of G_2-M cell cycle transition showing indirect 5-FU chemoresistance.[41]

4 | MULTIDRUG RESISTANCE AND MEMBRANE DRUG TRANSPORTERS

Resistance to cytotoxic agents like 5-FU could be due to MDR[5] through increases in the expression and activities of some membrane drug transporters including the following.

The MDR-associated protein (*MRP*) superfamily is composed of 9 members, MRP1/ATP binding cassettes (ABC) subtype C1 to MRP6/ABCC6, MRP7/ABCC10, MRP8/ABCC11, and MRP9/ABCC12. These MRP/ABCCs reportedly mediate ATP-dependent transport and efflux of anticancer agents out of cells, promoting drug resistance.[42]

Both MRP8/ABCC11 and ABCC5 have been described to promote resistance to therapies based on 5-FU and its active derivative metabolites in CRC.[43] Additionally, MRP7/ABCC10 is indirectly involved in 5-FU resistance; 5-FU induces upregulation of Forkhead box M1 (FOXM1) transcription factor, which in turn upregulates ABCC10 in 5-FU-resistant cells. Consistent with these data, inhibition of FOXM1 and/or ABCC10 sensitizes resistant CRC cells to 5-FU. Thus, the FOXM1/ABCC10 axis might be a new potential therapeutic target to counteract 5-FU resistance in CRC.[44]

The ABC subtype B1 (ABCB1), a phosphoglycoprotein encoded by the *ABCB1/MDR1* gene, is expressed in many cancer cells and represents one of the major causes of MDR. The ABCB mechanisms of action were linked with those of TrpC.[45]

5 | TUMOR MICROENVIRONMENT

Some 5-FU resistance mechanisms occur in carcinoma cells but others are slightly regulated by the TME, which plays crucial roles in the bioavailability of chemotherapeutic molecules and in the general biological behavior of tumors.[46] The TME is composed of tumor cells, cancer-associated fibroblasts (CAFs), endothelial cells, and immune cells with key roles played by tumor-associated macrophages (TAMs) and CSCs.

Extracellular soluble molecules (growth factors, cytokines, and chemokines) and vesicles (exosomes) play important roles in carcinogenesis initiation, as well as in TME-associated resistance. In this section, we discuss the involvement of both immune cells (TAMs) and CAFs and the effect of their released soluble factors on CSC maintenance and subsequent 5-FU resistance (Figure 2).

5.1 | Subtypes macrophages, TAMs, and released soluble factors

In solid tumors, including CRC, immune cells and especially macrophages have emerged as key components of the tumor stroma, playing crucial roles in cancer progression.[47] Two macrophage subtypes (M1, proinflammatory macrophages, and M2, antiinflammatory ones) have been well established for several decades and a third has been recently described, the TAMs.

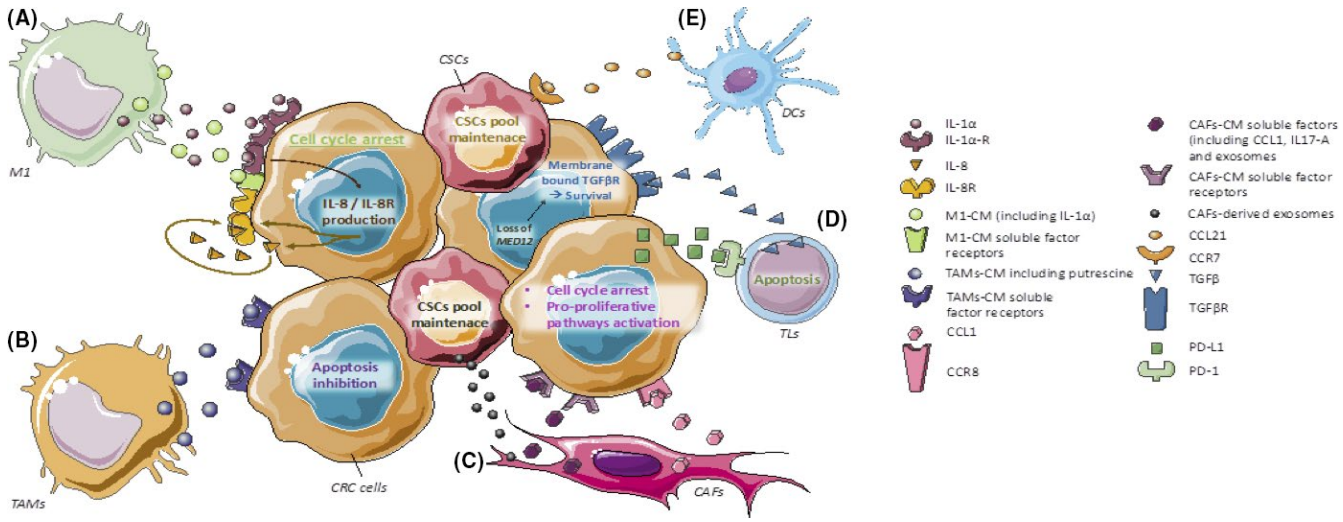

FIGURE 2 Tumor microenvironment (TME) implication in 5-fluorouracil (5-FU) resistance. Colorectal cancer (CRC) tumors are mainly composed of CRC cells and cancer stem cells (CSCs), which interact with other cellular partners like immunity cells M1 (macrophage type 1), tumor-associated macrophages (TAMs), T lymphocytes (TLs), and dendritic cells and also with cancer-associated fibroblasts (CAFs). Each association is responsible of a reaction playing a key function in 5-FU resistance driving to apoptosis inhibition, cell cycle arrest, proliferative induction, immune escape and CSC maintenance. A, M1 response. B, Activation of TAMs and released soluble factors. C, CAFs, CAF-released soluble factors, and CSCs. D, E, Implication of other players like cytokines (chemokine [C-C motif] ligand 21 [CCL21], transforming growth factor-β [TGF-β]) and chemokines (programmed cell death-ligand 1 [PD-L1]). CCR, chemokine (C-C motif) receptor; CM, conditioned medium; IL, interleukin; PD-1, programmed cell death-1;

M1 culture medium (M1-CM) attenuates 5-FU-induced cytotoxic effects by reducing cell proliferation and promoting cell cycle arrest[48]; M1-CM abolishes the 5-FU-induced S phase CRC cell blockade that accumulates in G_0/G_1 and G_2/M, protecting them from drug-mediated cytotoxic effects. In addition, M1-CM upregulates mRNA and protein levels of the transcription factor Forkhead box protein O1 and 3 (FOXO1/3) which regulates the expression of p21 also found overexpressed at mRNA and protein levels in CRC cells exposed to M1-CM. p21 is an inducer of cell cycle arrest in G_0/G_1 and G_2/M but is also an inhibitor of the complex CDK2/cyclin E1 (CCNE1) initially required for G_1/S transition, both having been found downregulated by M1-CM. Moreover, p21 expression increase has been associated with 5-FU resistance in cell lines from colon and other origins.

M2 promotes tumor development through enhancing cell proliferation, angiogenesis/lymphangiogenesis, metastasis, and immunosuppression.[49] Therefore, TAMs modulate not only cancer progression but also treatment-mediated cytotoxic responses and chemoresistance.[50] Indeed, there is a negative correlation between TAM infiltration and CRC progression.[51] 5-Fluorouracil increases TAM infiltration in CRC tumors and the clearance of myeloid cells, which include TAMs, potentiates 5-FU efficiency on tumor growth inhibition. Moreover, culture medium from 5-FU-primed TAMs (TAM-CM) counteracts 5-FU-induced tumor growth, size, and weight inhibition, suggesting that 5-FU might stimulate production of TAM factors that antagonize 5-FU-induced cytotoxicity. Only the metabolic fraction of TAM-CM enriched in putrescine (a polyamine) abolishes 5-FU cytotoxic effects through decreasing cleaved caspase-3 and JNK pathway activation. Consequently, putrescine might be a key component released by 5-FU-primed TAMs in 5-FU resistance. In that way, depletion of TAM-released putrescine, through ornithine decarboxylase (ODC) inhibition, abolishes CRC cell 5-FU resistance and potentiates 5-FU efficiency on tumor growth inhibition.[52]

5.2 | Cancer-associated fibroblasts, CAF-released soluble factors, and CSCs

Cancer-associated fibroblasts originate from heterogeneous cell types. The most abundant cells in the matrix, they constitute key components of stroma in cancer. They support tumorigenesis by stimulating angiogenesis and cancer cell proliferation and invasion, playing crucial roles in resistance to conventional therapies of many cancers, including CRC. Cancer-associated fibroblasts express several specific markers like α-smooth muscle actin as well as others shared with fibroblasts, such as the fibroblast growth factor receptor 4 (FGFR4). In CRC, FGFR4 plays roles in tumor-stroma interaction, promotes EMT, and might also constitute a novel important determinant of 5-FU resistance in CRC.[53] Its inhibition enhances cytotoxic effects of 5-FU through downregulation of signal transducer and activator of transcription 3 (STAT3) and subsequent inhibition of cFLIP (an antiapoptotic protein that normally inhibits caspase-8 processing), altogether leading to caspase-8 and -9 and BAX activations, cytochrome c release, and caspase-9 and -3 and poly(ADP-ribose) polymérase cleavages and consequently, apoptosis of FGFR4-related 5-FU-resistant CRC cells.[54]

Cancer-associated fibroblasts are also able to secrete chemo- and cytokines and soluble growth factors that trigger JAK/STAT and PI3K/AKT signaling pathways to protect cancer cells from chemotherapeutic agents currently used in CRC.[55] Cancer-associated fibroblast-derived culture medium (CAF-CM) containing CAF soluble factors, reduces apoptotic and proliferative levels of CRC cells exposed to 5-FU or not. The CAF-CM reduces CRC cell accumulation in S phase induced by 5-FU, promoting a prolonged G_1 transit with cell accumulation in G_0/G1. Cancer-associated fibroblast soluble factors therefore promote cycle arrest of 5-FU-treated CRC cells before entering mitosis, in order to repair cell DNA damage and prepare them for correct cell division. This cell cycle arrest seems to be mediated by the activation of G_2/M checkpoint kinase 2 (CHK2) and the subsequent decrease in expression of cell division cycle 25B (CDC25B, a phosphatase that enables a cell to resume the cell cycle after its arrest induced by DNA damage). Higher levels of phosphorylated CHK2 consistent with lower CDC25B expression have been observed in 5-FU-treated cells cultured in CAF-CM.[56] Moreover, CAF soluble factors stimulate and activate PI3K/AKT/mTOR and JAK/STAT pathways as well as the subsequent nuclear translocations of p38, AKT, and STAT activated forms, which increase Survivin expression.[56] Therefore, STAT3 constitutes a promising target to overcome CAF-mediated CRC 5-FU resistance, as STAT3 inhibition sensitizes CRC cells to 5-FU even in the presence of CAF-CM. In contrast, AKT inhibition shows antagonists effects; it does not sensitize CRC cells to 5-FU, which probably establishes compensatory mechanisms (MYC and/or tyrosine kinase receptor pathways).[57] Cancer-associated fibroblasts of CRC also release chemokine (C-C motif) ligand 1 (CCL1), a proinflammatory mediator that strongly contributes to lymph node metastasis as well as 5-FU resistance, and highly expresses the finger transcription factor Snail.[58] It is noteworthy that CCL1 interacts specifically and exclusively with chemokine (C-C motif) receptor 8 (CCR8) and the CCL1/CCR8 binding exerts antiapoptotic and proproliferative activities and enhances CRC 5-FU resistance through the induction of MDR1 expression and the activation of the NF-κB and TGF-β prosurvival pathways. Indeed, the inhibition of one or both, as well as CCR8, abolishes CRC 5-FU resistance induced by CAF-released CCL1.[58]

5.3 | Other cytokine (CCL21 and interleukin-1α), growth factor (TGF-β), and chemokine (programmed cell death-ligand 1) pathways upregulated by 5-FU and involved in its resistance

The proinflammatory cytokine CCL21 interacts with CCR7, expressed in several immune cells. After binding to its ligand, CCR7

targets T cells and dendritic cells to lymph organs, contributing to immune tolerance and promoting cell migration.[59] In CRC cells, CCL21 promotes 5-FU resistance through AKT/GSK3β/Snail-mediated P-gp expression and induction of stem cell properties. Indeed, it induces the activation of the PI3K/AKT pathway, which inhibits GSK3β activity and upregulates Snail expression as well as its nucleus translocation.[60]

Interleukin-1α (IL-1α), an inhibitory and proinflammatory cytokine, secreted by monocytes and macrophages, regulates the expression of other proinflammatory cytokines and chemokines such as IL-8.[61] Recent works showed that 5-FU upregulates IL-1α expression at mRNA and protein levels, only in 5-FU-treated CRC cell lysates. Inhibition of IL-1α expression, as well as its binding to its receptor, does not impact IL-8 expression in CRC cells exposed to both 5-FU and IL-1α. In contrast, and surprisingly, treatment with exogenous IL-1α stimulates IL-8 production and secretion.[61]

Transforming growth factor-β, a growth factor with antiinflammatory properties, is released in the TME by several immune cell types. It was reported to be a poor prognostic factor in CRC, being able to modulate the stroma, promote CRC initiation and progression, and predict metastasis. Maturation of TGF-β receptor can be disrupted by MED12, which blocks and accumulates it in the Golgi apparatus.[62] The point is that MED12 expression is often lost in cancer cells, leading to abnormal surface expression of the TGF-β receptor, which consequently protects CRC cells against 5-FU-mediated cytotoxic effects.[62] In addition, TGF-β signaling also inhibits p53 expression and low levels of p53 correlate with low chemotherapeutic responses, notably in CRC.[63] Consequently, TGF-β plays crucial roles in 5-FU resistance through p53 and subsequent inhibition of apoptosis. Combining 5-FU with either TGF-β signaling inhibitors (LY2157299 or LiCl) or TGF-β receptor inhibitor might inhibit 5-FU-mediated prosurvival mechanisms, resensitize chemoresistant CRC cells to 5-FU, and prevent tumor recurrence after treatment.[22]

B7-homolog 1, also called programmed cell death ligand-1 (PD-L1), is an immunoregulatory protein that interacts with its receptor, programmed cell death-1 (PD-1), a costimulatory molecule expressed on the T cell surface. Binding of PD-L1/PD-1 prevents T cell activation/proliferation and induces apoptosis, resulting in cancer immunoresistance.[64] Programmed cell death-ligand 1 is overexpressed in solid cancers including CRC and its expression induced by chemotherapies, like 5-FU, mediates chemoresistance. Indeed, although CRC cell lines express low levels of PD-L1, 5-FU induces its expression at the plasma membrane, at similar levels as with γ-interferon treatment, a cytokine known to induce PD-L1 expression.[65] These results suggest that the PD-L1 expression increase observed following treatment with 5-FU might contribute to 5-FU CRC cell resistance. Combining 5-FU-based chemotherapies with PD-1/PD-L1 inhibitors would thus enhance the efficiency of 5-FU and constitute a promising strategy to overcome immunoresistance induced by this drug. Phase II clinical trials have also recently proved the clinical efficiency of PD-1/PD-L1 immune checkpoint blockade in MSI-high CRC patients, using an anti-PD-1 Ab (pembrolizumab).[66]

6 | EPIGENETIC ALTERATIONS

Epigenetic alteration is defined as the modification of gene expression without altering the nucleotide sequence. Epigenetic alterations are one of the mechanisms conferring 5-FU resistance. Here, 3 types of well-established epigenetic modifications involved in CRC will be discussed: DNA methylation, histone posttranslational modification, and regulation of gene expression by long noncoding RNA.

6.1 | DNA methylation and demethylation

DNA methylation consists of enzymatic addition of a methyl group to the 5′-position of the cytosine ring by DNA methyltransferase (DNMT), producing 5-methylcytosine. Two kinds of aberrant DNA methylation have been described: (i) hypermethylation, with increase of DNA methylation compared to healthy tissue, which occurs on CpG islands and gene promoters resulting in transcription inhibition; and (ii) hypomethylation, which is a decrease of DNA methylation compared to healthy tissue and occurs on the open reading frame (ORF) of the genome, resulting in transcription activation. Globally, tumor suppressors and drug metabolism genes are hypermethylated, promoting tumor formation and acquisition of drug resistance. Prooncogenes are hypomethylated, promoting tumor formation and acquisition of drug resistance. Moreover, DNA and histones can also be submitted to posttranslational modifications, affecting drug responses and efficiency.

6.2 | Hypermethylation-mediated gene silencing

In CRC, it was found that some genes involved in pyrimidine metabolism, drug metabolism mediated by cytochrome P450, epidermal growth factor receptor, and p53 signaling pathways as well as apoptosis (*TGF-β*, death domain-associated protein 6 [*DAXX*], and *p38*) are hypermethylated and consequently downregulated, hence promoting 5-FU resistance.[67] Microsatellite instability is characterized, among others, by a deficiency in the mismatch repair (MMR) *MLH1* gene, due to its promoter hypermethylation. Although characterized by early onset, MSI CRCs generally have a good prognosis. 5-Fluorouracil metabolism provides 5-FUTP, which is misincorporated into DNA, generating a mismatch recognized by the MMR system, leading to cell cycle arrest and apoptosis in cases of a irreparable lesions. This damage is not recognized in *MLH1* gene deficiency patients, thus conferring resistance to 5-FU.[68]

In this way, DNMT inhibitors (also called demethylating agents) such as 5-aza-2'-deoxycytidine (5-azadC or decitabine) have been evaluated as a treatment approach for a variety of cancers in vitro and in vivo, including CRC. Although DNMT inhibitors used alone exerted antitumor activity only in vitro, combined chemotherapy with 5-FU/5-azadC induced a significant decrease in cancer cell viability both in vitro and in vivo. This suggests that 5-azadC mediates restoration of CRC cell 5-FU sensitivity through apoptosis induction, including in cells with MMR deficiency.[68]

Dihydropyrimidine dehydrogenase (DPD) is involved in pyrimidine degradation and catalyzes 5-FU catabolism. High expression of DPD is consequently associated with poor efficiency of 5-FU, promoting its rapid catabolism. In contrast, low expression of DPD increases 5-FU half-life as well as its accumulation and toxicity. Methylation of DPD promoter regulates gene expression by modulating binding of Sp1 transcription factor, previously shown to be important for DPD expression.[69] In CRC cells, hypermethylation was detected in the CpG island of DPD promoter, leading to its downregulation and increase in 5-FU toxicity.[70] This result showed that adding epigenetic analyses to classic genetic ones could increase the sensitivity and specificity of the test.

6.3 | Lack of methylation

6.3.1 | Hypomethylation-mediated gene upregulation

Hypomethylated upregulated genes of recurrent CRC, called proncogenes, were found to be involved in the cell proliferation process as well as the cell cycle, leading to excessive proliferation, protecting cells from chemotherapeutic cytotoxicity, and increasing the risk of recurrence.[71]

6.3.2 | DNA demethylation

As previously described, enhancement of ROS production following 5-FU drug treatment was observed in 5-FU-resistant CRC cells. It is noteworthy that ROS production was reported to be associated with DNA methylation patterns through increasing levels of ten-eleven translocation (TET) enzymes, without any difference in DNMT protein levels. The TET enzymes oxidize 5-methylcytosine, hence promoting DNA demethylation.[72] Furthermore, hypomethylation of *Nrf2* promoter island was observed in 5-FU-treated CRC cells, consistent with the increase in mRNA and protein levels. Nrf2 is an important transcription factor involved in cellular protection and it regulates numerous genes, such as the heme oxygenase 1 (*HO-1*) gene, which is induced upon stress response with role antioxidant-mediated xenobiotics injuries functions. Under 5-FU treatment, Nrf2 is translocated into CRC cell nucleus and interacts with the OH-1 promoter region, inducing cellular protection. Together, these results highlight that upregulation of *Nrf2* and *HO-1* expression by epigenetic DNA demethylation induces acquisition of 5-FU resistance in CRC cells.[73]

6.4 | Histone posttranslational modification

Histone structure modifications play crucial roles in cancer initiation and progression: histone methylation or acetylation directly influences chromatin structure and regulates DNA accessibility as well as gene expression. Histone methylation could be associated with gene activation or gene repression, whereas acetylation is always associated with gene activation.[74]

The serine-threonine kinase receptor associated protein (STRAP) is a scaffold protein that epigenetically regulates the Notch pathway and maintains stem-like properties of CRC cells by acting on the polycomb-repressive complex 2 (PRC2). Polycomb-repressive complex 2 has histone methyltransferase activity and trimethylates histone H3 on lysine 27 (H3K27me3), a mark of both transcriptionally silent chromatin and Notch-related gene repression. But PRC2 methyltransferase activity is inhibited by STRAP, whose protein and mRNA levels are overexpressed in clinical CRC specimens compared with healthy tissues. Moreover, STRAP upregulation is associated with worse survival with adjuvant chemotherapy, highlighting its key role in 5-FU chemoresistance.[75]

Histone deacetylases, enzymes removing acetyl groups from an ε-N-acetyl lysine amino-acid on a histone tail, lead to gene repression. In contrast, histone deacetylase inhibitors (such as valproic acid, trichostatin A, romidepsin, or vorinostat) maintain histone acetylation. Histone acetylation is associated with reduced histone-DNA binding and gene activation, such as *UVRAG* (UV irradiation resistance-associated gene). Thus, UVRAG upregulation enhances DNA repair and autophagy, conferring CRC cell resistance to 5-FU-mediated cell death. However, vorinostat has been shown to resensitize 5-FU-resistant cells by downregulating *TS* gene expression, through impairment of its histone acetylation status.[76]

6.5 | Noncoding RNA – miRNA

5-Fluorouracil interferes with DNA and RNA synthesis and modifies the expression profiles of miR in CRC cells. MicroRNA are small noncoding single-stranded RNAs of approximately 22 nucleotides. They can regulate the expression of many target genes, and 1 gene can also be regulated by many miR. Two molecular mechanisms can explain how miR operate. They can regulate CRC cell 5-FU-drug sensitivity by interacting with the 3′-UTR of their target gene's mRNA. In the event of a perfect base pairing between mRNA and miR, the target is cleaved. In the case of imperfect base pairing, the target is translationally silenced or a decrease in its protein levels can be observed. MicroRNA can also be at the origin of a translational reprogramming, producing steric obstruction on the mRNA target, or dysregulating transcription/translation efficiency. Comparison of miR expression between CRC cells and healthy ones showed some downregulated and upregulated miR following 5-FU exposure. These miR expression variations following chemotherapeutic treatment suggests that they might be involved in the modulation of 5-FU responses in CRC cells. Depending on their targets, some miR function as oncogenes (those upregulated that induce 5-FU resistance) and others as tumor suppressors (those downregulated that promote 5-FU resistance) (Table 1).[77]

Some miR, such as miR-338p, can display opposite functions according to p53 mutational status. It has been shown that 5-FU

caused an increase of miR-338p levels in p53 mutant deficient CRC cells, which silences the expression of its target gene, *mTOR*. Inhibition of mTOR induces autophagy, hence conferring resistance to 5-FU. In contrast, 5-FU decreases miR-338p in p53 WT CRC cells. Consequently, the autophagy is not induced to the benefit of apoptosis, sensitizing p53 WT CRC cells to 5-FU treatment. These results suggest that p53 status of CRC cells constitutes a key determinant in 5-FU responses and efficiency, notably through miR-338-3p, which might or might not be activated, creating a balance between autophagy and apoptosis through mTOR expression regulation. Moreover, these data also indicate that p53 mutant CRC cells are less sensitive to 5-FU than the p53 WT cells.

TABLE 1 MicroRNA (miR) expression following exposure to 5-fluorouracil (5-FU), targeted genes, and the impact of upregulation and downregulation on cellular functions and colorectal cancer (CRC) cell 5-FU sensitivity

	miR expression following 5-FU exposure	Genes targeted	Consequences on cellular functions	5-FU sensitivity or resistance	References
miR-21	Upregulated	*MSH2*[a]	MMR deficiency	Resistance	80, 81
miR-125b		*APC*[a]	Tumor invasion through activation of Wnt/β-catenin signal pathway		82
miR-23a		*APAF1*[a]	Apoptosis inhibited		83
miR-10b		*BIM*[a]			84
miR-425-5-p		*PDCD10*[b]			85
miR-587		*PP2R1B/PP2A*[a]	Cell survival PI3K/AKT pathway increase		86
miR-23a		*ABCF1*[b]	5-FU resistance in MSI CRC cell enhanced		87, 88
miR-192 and -215	Downregulated	*p21/p27*[a]	Cell cycle progression increased		89
miR-330		*TS*[a]	Cell proliferation increased		90, 91
miR-494, -27a, and -27b		*DPD*[a]	5-FU catabolism stimulated		92
miR-20a		*BNIP2*[b]	Autophagy stimulated/ Apoptosis decreased		93
miR-302a		*Erk1/2 and Akt*[b]	Cell proliferation, growth and survival stimulated		94
miR-122		*PMK2*[b]	Cell growth increased, glucose metabolism stimulated		95,96
miR-34a		*SIRT1*[a], *E2F*[b], *LDHa*[b]	Cell growth increased, tumor maintenance		97
miR-519c		*ABCG2*[a]	Drug efflux inhibited		98, 99
miR-143	Upregulated	*Erk5*[a], *NFκB*[a], *c-kit*[a] *and Bcl2*[a]	Apoptosis induction and increased	Sensitivity	100
miR-204		*HMGA2*, *Pi3K/Akt*[b]	Cell growth repressed		101
miR-365, -1915, -129, and -139-5-p		*Bcl2*[a]	Apoptosis increased		102
miR-203 and -218		*TS*[a]	5-FU metabolism decreased		103, 104
miR-22		*BTG1*[a]	Autophagy inhibited, apoptosis increased		105

Abbreviations: ABCF1, ATP binding cassette subfamily F member 1; ABCG2, ATP binding cassette subfamily G member 2; APAF1, apoptotic peptidase activating factor 1; APC, adenomatous polyposis coli; Bcl2, B-cell lymphoma 2; BIM, Bcl-2-like protein 11; BNIP2, BCL2 interacting protein 2; BTG1, BTG antiproliferation factor 1; DPD, dihydropyrimidine dehydrogenase; E2F1, E2F transcription factor 1; HMGA2, high-mobility group AT-hook 2; LDHa, lactate dehydrogenase A; MMR, mismatch repair; MSH2, MutS protein homolog 2; MSI, microsatellite instability; NFκB, nuclear factor-κB; PDCD10, programmed cell death 10; PMK2, pyruvate kinase isozymes M1/M2; PPP2CA, protein phosphatase 2 catalytic subunit α; SIRT1, sirtuin 1; TS, thymidylate synthase.

[a]Tumor suppressor and drug metabolism gene.

[b]Prooncogene.

7 | CONCLUSION

In this review, we made an inventory of the most important relevant research, enabling a better understanding of key 5-FU resistance mechanisms in CRC, even if they remain numerous, complex, and not all understood. Every protein and its related pathway involved in 5-FU resistance of CRC cells, responsible of tumor growth, metastasis, and recurrence, could be targeted. New therapeutics combined with 5-FU could avoid resistance.

In our opinion, the main hopeful therapeutic targets are focused on epigenetic modifications and TME-related cells (cancer cells, CAFs, TAMs, CSCs, and T lymphocytes) and the soluble factors that they release (CCL1, IL-17A, CCL21, TAM-released putrescine, IL-8, TGF-β, and PD-L1).

Epigenetic modifications play important roles in CRC carcinogenesis and are strongly involved in drug resistance acquisition. Unlike genetic modifications, they are reversible and can be targeted by specific drugs. Moreover, epigenetic alterations can be detected and be used as drug-response and/or prognosis biomarkers. The challenge is to better characterize the patient and the disease. Epigenetic status screening of genes involved in CRC aggressiveness and drug resistance could help to individualize treatment in order to improve patient care and reduce the number of nonresponders.

For targeting cancer cells in the TME context, FGFR4 inhibition enhances cytotoxic 5-FU effects, inducing apoptosis of FGFR4-related 5-FU-resistant CRC cells.[54] CCL21 promotes 5-FU resistance through Snail-mediated P-gp (encoded by the *MDR1* gene[78]) upregulation and induction of stem cell pluripotent marker expression (Bmi1, Nanog, and Oct4). Consequently, Snail could constitute a promising target to overcome CCL21-mediated 5-FU resistance as its knockdown abolishes expression of stem cell pluripotent markers and restore CRC 5-FU sensitivity.[60] Interleukin-8 receptor (CXCR2) inhibition leads to tumor growth decrease, with a simultaneous increase of carcinogenesis preventing cytokine expression.[61] Specifically induced in 5-FU-resistant cells, TGF-β expression exerts protective roles against 5-FU-induced cell death in an autocrine manner. Consequently, combining 5-FU with either TGF-β signaling inhibitors (LY2157299 or LiCl) or TGF-β receptor inhibitor might inhibit 5-FU-mediated prosurvival mechanisms, resensitize chemoresistant CRC cells to 5-FU, and prevent tumor recurrence after treatment.[22]

In the CAF context, STAT3 represents a promising target to overcome CAF-mediated 5-FU resistance, as STAT3 inhibition sensitizes CRC cells to 5-FU. Moreover, the inhibition of CCL1 and/or its receptor CCR8 abolishes 5-FU resistance induced by CAF-released CCL1.[58] 5-Fluorouracil stimulates CAFs that upregulate and secrete IL-17A, which contributes to CSC niche maintenance and 5-FU resistance. Disruption of this circuit by targeting IL-17A or NF-κB downstream target genes, might inhibit protumorigenic effects of CAF-released IL-17A and overcome CRC 5-FU resistance.[79]

In TAMs, depletion of TAM-released putrescine through ODC inhibition abolishes CRC cell 5-FU resistance and potentiates 5-FU efficiency.[52]

In T lymphocytes, combining 5-FU-based chemotherapies with PD-1/PD-L1 inhibitors would enhance 5-FU efficiency and constitute a promising strategy to overcome immunoresistance. In fact, phase II clinical trials (NCT01876511) have recently proved the clinical efficiency of PD-1/PD-L1 immune checkpoint blockade in MSI-high CRC patients, using an anti-PD-1 Ab (pembrolizumab).[66]

To conclude, immunotherapy is the most promising approach to counteract 5-FU resistance in CRC. Treating CRC patients with innate or acquired 5-FU resistance is the major challenge to overcome.

CONFLICT OF INTEREST

The authors have no conflict of interest.

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