Chapter 4

Acquired and Intrinsic Resistance to Colorectal Cancer Treatment

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Additional information is available at the end of the chapter

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Abstract

First line therapy for colorectal cancer (CRC) is usually fluoropyrimidine monotherapy and oxaliplatin, or irinotecan-based therapy. Additionally, targeted therapies such as bevacizumab, aflibercept, ramucirumab, regorafenib, cetuximab and panitumumab are indicated in combination with chemotherapy in metastatic CRC. Resistance of CRC to treatment is the principal rationale for treatment failure. Resistance can be intrinsic (primary resistance) or acquired (secondary resistance). Here, we discuss the classical model of resistance, which focuses primarily on mechanisms involving alterations in drug metabolism, increased drug efflux, secondary mutations in drug targets, inactivation of apoptotic pathways, p53 and DNA damage repair. Other resistance mechanisms, including the Warburg effect, cancer stem cells, intra-tumor heterogeneity and pharmacoepigenomic mechanisms will also be discussed. We conclude the chapter with a systems medicine approach to predict response to treatment for the discovery and validation of predictive biomarkers that are urgently needed.

Keywords: colorectal cancer, chemotherapy, targeted therapy, intrinsic resistance, acquired resistance, predictive biomarkers

1. Introduction

The mainstay of colorectal cancer (CRC) treatment is curative surgery, although in some cases patients are administered neo-adjuvant therapy. Surgery is usually followed by adjuvant therapy in patients presenting with Stage III and Stage IV disease. Additionally, adjuvant therapy is sometimes administered to high risk stratified Stage II patients. Adjuvant treatment for stage III CRC patients consists of chemotherapy including 5-fluorouracil (5-FU), oxaliplatin and

capecitabine—usually administered combination therapy [1]. Patients having advanced CRC are frequently treated with targeted therapy in combination with chemotherapy, or as a single agent, and more recently with immunotherapy (**Table 1**). The rationale for using combination therapy is to avoid treatment resistance, promote a synergistic effect and reduce potential toxicity (refer to **Table 2**).

Therapeutic agent	**Class**	**Colorectal cancer indications**	**Date of first launch worldwide [1–6]**
5-Fluorouracil (5-FU)	Antimetabolite, pyrimidine analogue	Used as a single agent or in combination	1962
Epirubicin	Cytotoxic antibiotics	Used as a single agent or in combination	1984
Irinotecan	Topoisomerase I inhibitor	1. Indicated for the treatment of mCRC: • in combination with 5-FU and folinic acid in chemotherapy naïve patients • as a single agent in patients who have failed a 5-FU-based regimen 2. In combination with cetuximab is indicated for the treatment of patients with EGFR-expressing KRAS wild-type mCRC, who had not received prior treatment for metastatic disease or after failure of irinotecan-including cytotoxic therapy 3. In combination with 5-FU, folinic acid and bevacizumab is indicated for first-line treatment of patients with mCRC 4. In combination with capecitabine with or without bevacizumab is indicated for first-line treatment of patients with mCRC	1994
Oxaliplatin	Platinum derivative, alkylating agent	Oxaliplatin in combination with 5-FU and folinic acid is indicated for: 1. adjuvant treatment of stage III colon cancer after complete resection of primary tumor 2. treatment of mCRC	1996
Raltitrexed	Antimetabolite	Palliative treatment of advanced colorectal cancer where 5-FU and folinic acid-based regimens are either not tolerated or inappropriate	1996
Capecitabine	Antimetabolite	1. Used as a single agent or in combination 2. Used for the adjuvant treatment of stage III colon cancer patients 3. Used in mCRC	1998
Cetuximab	Monoclonal antibody, EGFR inhibitor	Indicated for the treatment of patients with EGFR-expressing, RAS wt mCRC: • in combination with irinotecan-based chemotherapy • in first-line in combination with FOLFOX • as a single agent in patients who have failed oxaliplatin- and irinotecan-based therapy and who are intolerant to irinotecan	2003

Therapeutic agent	Class	Colorectal cancer indications	Date of first launch worldwide [1–6]
Bevacizumab	Monoclonal antibody, VEGF inhibitor	In combination with fluoropyrimidine-based chemotherapy is indicated for treatment of mCRC	2004
Panitumumab	Monoclonal antibody, EGFR inhibitor	Indicated for the treatment of wt *RAS* mCRC: 1. first-line in combination with FOLFOX or FOLFIRI 2. second-line in combination with FOLFIRI for patients who have received first-line fluoropyrimidine-based chemotherapy (excluding irinotecan) 3. monotherapy after failure of fluoropyrimidine-, oxaliplatin-, and irinotecan-containing chemotherapy regimens	2006
Regorafenib	Angiogenesis inhibitor, tyrosine kinase inhibitor	mCRC patients who have been previously treated with, or are not considered candidates for available therapies	2013
Aflibercept	Angiogenesis inhibitor, tyrosine kinase inhibitor	In combination with FOLFIRI is indicated in mCRC that is resistant to or has progressed after an oxaliplatin-containing regimen	2013
Trifluridine/ tipiracil hydrochloride	Antimetabolite	Treatment of mCRC patients who have been previously treated with, or are not considered candidates for available therapies	2015
Pembrolizumab	Anti-PD1 immunotherapy	Unresectable or metastatic, MSI-H or dMMR CRC that has progressed following treatment with a fluoropyrimidine, oxaliplatin, and irinotecan	2017

Table 1. Colorectal cancer drugs currently on the market.

Combination regimens	Therapeutic agents
deGramont/modified de Gramont	5-Fluorouracil, folinic acid
FOLFIRINOX	Folinic acid, 5-fluorouracil, irinotecan, oxaliplatin
FOLFIRI	Folinic acid, 5-fluorouracil, irinotecan
FOLFOX	Folinic acid, 5-fluorouracil, oxaliplatin
XELOX	Oxaliplatin, capecitabine
FOLFIRI + cetuximab	Folinic acid, 5-fluorouracil, irinotecan, cetuximab
FOLFOX + cetuximab	Folinic acid, 5-fluorouracil, oxaliplatin, cetuximab
FOLFIRI + panitumumab	Folinic acid, 5-fluorouracil, irinotecan, panitumumab
FOLFOX + panitumumab	Folinic acid, 5-fluorouracil, oxaliplatin, panitumumab
FOLFIRI + aflibercept	Folinic acid, 5-fluorouracil, irinotecan, aflibercept
FOLFIRI + bevacizumab	Folinic acid, 5-fluorouracil, irinotecan, bevacizumab

Table 2. Drug combinations to treat colorectal cancer.

Patients' responses to treatment are limited, and in fact less than one-third of patients respond to 5-fluorouracil as a single agent [2, 3]. However, when used in combination, for instance, with oxaliplatin-based therapy, 50% response rate is obtained [4]. Resistance to 5-fluorouracil can be due to loss of SMAD4 [5], thymidylate synthase (TYMS) amplification [6], defective mismatch repair (MMR) genes [7], high level expression of thymidylate synthase (TS) [8], increased DPD activity [9], microsatellite instability [9], modulation of the Bcl2 family members [10], cell cycle perturbation [11], decreased ATP synthase [12] and adaptation to oxidative stress [13]. General mechanisms attributed to oxaliplatin resistance include cellular transport, detoxification, DNA repair, cell death, epigenetic alteration and NF-κβ signaling pathway [14].

Although, the use of cetuximab and panitumumab in combination with other agents is very effective, they are not sufficiently potent as single agents and are reported to work in only around 10% of cases [15]. Over the last decade, a number of papers on anti-epidermal growth factor receptor (EGFR) resistance mechanisms have been published [16–18]. Resistance mechanisms attributed to EGFR resistance include, but are not limited to, low EGFR gene copy number, low expression of AREG and EREG, EGFR S492R mutation, RAS mutation, BRAF V600E mutation, PIK3CA exon 20 mutation, PTEN loss, STAT3 phosphorylation, activated IGF1R, MET amplification, HER2 amplification, altered VEGF/VEGFR and EMT [16, 19].

Unfortunately, the lack of predictive markers would allow clinicians to select patients who are most likely to benefit from a specific therapy remains a challenge. A recent review by Wu et al. reported that in the context of metastatic cancer, approximately 90% of treatment failure is due to multi-drug resistance [20]. Currently, the only markers that predict potential toxicity to 5-FU treatment are DPD deficiency, DPYD mutation, UGT1A1 and high TS expression [21]. Moreover, the only marker that predicts lack of response to 5-FU is mismatch repair deficiency (dMMR), while pembrolizumab, dMMR predicts increase in response [7]. With respect to anti-*EGFR* treatment, *KRAS*, *NRAS* and *BRAF* mutations are the only biological markers that predict lack of response and hence pose a contraindication to treatment administration [21].

Both intrinsic resistance, which is characterized by cancer cells having only a slight or no response to treatment from the beginning, and acquired resistance that is described as, initially, having a clinical response, ensued by development of resistance will be discussed [22]. Several studies that discuss resistance to specific chemotherapeutic agents have been published and therefore we will be solely referring to salient resistance mechanisms to CRC therapies.

2. The classical model of resistance

2.1. Drug metabolism

Chemotherapeutic agents are extensively metabolized by Phase I, Phase II and Phase III enzymes. Phase I enzymes, which are mostly involved in chemical modification, include the heme protein cytochrome superfamily CYP450, which is sub-divided into 74 gene families and is involved in oxidation reactions. CYP3A is involved in irinotecan metabolism while CYP2A6, CYP2C8 and CYP1A2 are involved in tegafur activation [23].

Increased expression of dihydropyrimidine dehydrogenase (DYPD) or thymidine phosphorylase (TP) is correlated with resistance to 5-FU chemotherapy [24]. On the other hand, in a cohort of 177 CRC patients, there was a correlation between high TP expression and a better survival rate in the doxifluridine arm ($p = 0.025$) [25]. TP has also been reported to increase in both hypoxic and hypoglycemic environments, which will be discussed later [26]. Furthermore, polymorphic changes in DYPD account for life-threatening adverse effects in patients treated with 5-FU or its derivatives [27]. On the other hand, patients having a low expression of DYPD cannot metabolize 5-FU efficiently [28]. Decreased orotate phosphoribosyl transferase (OPRT) expression in gastric cancer is associated with resistance to 5-FU [29].

Phase II enzymes are involved in conjugation and include glutathione (GSH), glutathione-S-transferase (GSTs), uridine diphosphate glucuronosyltransferases (UGT) and NADH quinone oxidases (NQO). One of the oxaliplatin resistance mechanisms entails elevation of glutathione mediated by γ-glutamyl transpeptidase [30]. Additionally, GSTπ1 is associated with oxaliplatin and cisplatin resistance mechanisms [31]. SN-38, the active metabolite of irinotecan, is inactivated by way of glucuronidation by UGT [32]. UGT1A1 is one of the main genes involved in glucuronidation and is reported as being highly polymorphic; subsequently patients having UGT1A1*28 polymorphisms tend to suffer from increased risks of toxicity as reported in a cohort of colorectal cancer patients [33]. Both CYP450 and GSTs have been implicated in the metabolism of chemotherapeutic agents, but their predictive value is still uncertain [32].

Members of the ATP-binding cassette (ABC) superfamily are involved in Phase III drug metabolism [34] and to date 49 ABC transporters have been documented in humans [35]. The role of ABC transporters is to use energy from ATP hydrolysis to move their substrates across biological membranes and against concentration gradients, thereby limiting cellular accumulation of their substrates [36]. ABC members include P-glycoprotein (MDR1/ABCB1), breast cancer resistance protein (BCRP/ABCG2) and transporters of the multidrug resistance-associated protein (MRP/ABCC) family like the multi-drug resistance protein 5 (ABCC5), which bestows resistance to 5 FU via transporting the monophosphate metabolites in colorectal and breast cancers [37]. MDR1 is found to be highly expressed in the epithelial cells of the colon, overexpressed in a number of tumors, and has been associated with treatment failure [38]. At least 12 ABC transporters from 4 ABC sub-families have been shown to have a role in *in vitro* drug resistance (reviewed in Ref. [39]). MRP5 has been reported to confer cross-resistance to a number of anti-cancer agents including 5-FU, oxaliplatin and a number of antifolates [37]. The authors postulated that resistance might be instigated via drug efflux mechanisms which interfere with 5-FU's ability to impede both DNA and RNA synthesis. P-glycoprotein is a drug efflux pump and exerts its mode of action by lowering the intracellular concentration of a number of drugs, which subsequently leads to increased drug resistance [40]. BCRP is also involved in irinotecan efflux and is reported to be overexpressed in colon cancer, subsequently increasing chemoresistance.

2.2. Drug targets

5-FU naive CRC patients exhibiting high expression of TS and disturbed folate pools are intrinsically resistant to 5-FU [9]. A meta-analysis of over 3000 pooled CRC cases by Popat and

colleagues concluded that the variation of TS expression in CRC patients could explain inter-individual variation in clinical outcome, and patients with low TS expression treated with 5-FU had better overall survival [41]. CRC patients who are chemoresponsive to 5-FU have lower TS enzymatic activity compared to those patients who fail to respond [42]. Furthermore, the low availability of 5,10-methylenetetrahydrofolate and its polyglutamates also contributed to intrinsic resistance [43]. An indirect resistance mechanism reported in hepatocellular carcinoma cells is the induction of the expression of the transcription factor Late SV40 Factor (LSF) that regulates TS expression, by way of the astrocyte elevated gene-1 (AEG-1) [44].

Additionally, TS mRNA increases in a number of patients treated with 5-FU, resulting in acquired resistance [45]. In a review by Holohan et al., the authors explained further that 5-FU can post-transcriptionally upregulate the TS expression as a result of the inhibition of a negative feedback loop where the substrate free TS binds to and inhibits the translation of thymidylate synthase mRNA [38].

Watson and colleagues reported that patients with TYMS amplification treated with adjuvant chemotherapy had a median overall survival of 18 months shorter when compared to patients with low or normal TYMS copy number [46]. A total of 113 mCRC patients were enrolled in this study (62 exposed and 51 unexposed to 5-FU prior to resection) and the investigators concluded that TYMS copy number gain was associated with patients treated with 5-FU-based neoadjuvant treatment [46].

Guo and colleagues have demonstrated that a possible mechanism of acquired 5-FU resistance can be due to disruption in cell cycle. Using two 5-FU resistant and two sensitive cell lines, Guo and colleagues showed that the protein expression of CDK2 (total and phosphorylated threonine 160), Cyclin D3, and Cyclin A was significantly decreased in the 5-FU resistant cell lines. On the other hand, $p21^{WAF1}$ expression was modestly increased in both resistant cell lines [11]. The authors postulated that the G1 and S phase delay in the 5-FU resistant cell lines occurs because of Cyclin E—CDK2 complex deficiency. Additionally, the Cyclin A—CDK2 complex is also deficient and may assist in bringing about a delay in the S phase of 5-FU resistance cell lines [11]. Guo and colleagues speculated that the slowing down of the cell cycle might interfere with the active 5-FU metabolites being incorporated into DNA and also allows the cells to repair the DNA damage [11].

Montagut and colleagues confirmed one mechanism of acquired resistance to cetuximab, where they showed that an acquired EGFR ectodomain mutation (S492R) prevented the effective binding of cetuximab to the receptor [47]. On the other hand, overexpression of EGFR in CRC has been poorly correlated with response to anti-EGFR therapy [48]. One of the determinants of poor response is because KRAS mutant patients have a constantly activated KRAS, irrelevant to the phosphorylation status of EGFR. Fluorescence *in situ* hybridisation (FISH) analysis was carried out on a cohort of 58 mCRC patients treated with panitumumab and it was observed that patients that did not exhibit an EGFR copy number gain or chromosome 7 polysomy or amplification were associated with treatment failure ($p = 0.0009$, $p = 0.0007$) [49]. Another possible mechanism of resistance using an *in vitro* model postulated that increased Src family kinases activity leads to lengthened EGFR activity, increased EGFR-modulated HER3 activity, and activation of the PI3K/AKT pathway [50].

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A study by Lievre and colleagues on a cohort of 30 mCRC patients reported a highly significant association between non-response to cetuximab and mutant KRAS (n = 19, p = 0.0003) [51]. This association was further confirmed by other larger studies [52, 53]. A study by Misale and colleagues unprecedentedly described that a significant number of wild-type KRAS CRC patients, who are initially responsive to anti-EGFR therapies, acquire resistance due to *de novo* KRAS mutations resulting from continuing mutagenesis [54]. Another somatic mutation, associated with treatment resistance in CRC is PIK3CA [55], is mutated in 25–32% of CRC patients [56].

2.3. DNA damage repair

Mismatch repair deficiency (dMMR) can occur because of both sporadic and hereditary CRC. In the autosomal dominant hereditary non-polyposis colon cancer (HNPCC), which is also referred to as Lynch Syndrome, dMMR arises primarily due to inactivating germline mutations in either MLH1, MSH2, PMS2, or MSH6 [57]. Furthermore, loss of function of the remaining allele can occur via various mechanisms, namely loss of heterozygosity, mutations, gene conversion, and also promoter methylation [58]. On the other hand, epigenetic hypermethylation of MLH1 accounts for the majority of sporadic dMMR in CRC [59]. A recent study by Ye and colleagues concluded that miR-1290 promotes 5-FU resistance by directly targeting hMSH2 [60].

An *in vitro* study on CRC cell lines showed that MMR-proficient cell lines were more sensitive to the therapeutic doses of 5-FU (5–10 μM) compared to MMR-deficient cell lines [61]. Furthermore, patients who are high microsatellite instable (MSI-H) do not show any survival advantage when administered 5-FU-based chemotherapies [62]. The scientific literature not only alludes to the fact that dMMR tumor cells have a distinct response to standard chemotherapies, but also to many emerging therapies for CRC [58]. In an *in vitro* study, Tajima and colleagues showed that resistance of dMMR cancer cells to 5-FU can arise due to the incorporation of 5-FU metabolites in DNA [63].

Mechanisms attributed to resistance to oxaliplatin include increased DNA repair, impaired DNA adduct formation, over-expression of copper transporters (increased levels of ATP7B correlated with poor outcome in CRC patients) [64], enhanced drug detoxification, and increased tolerance to DNA damage. While NER and recombination repair mechanisms do not distinguish between cisplatin and oxaliplatin adducts, mismatch repair, damage-recognition proteins, and translesion DNA polymerases do distinguish between the two [65].

Increased excision repair cross complementation group 1 (ERCC1) mRNA expression correlates with resistance to oxaliplatin [66]. A polymorphism (Gln mutant allele) in X-ray repair cross complementation group 1 (XRCC1), which is involved in single strand break, adduct formation, and base excision repair, was associated with treatment resistance in a cohort of 61 patients treated with 5-FU and oxaliplatin [67].

2.4. p53

An *in vitro* study on the NCI-60 panel investigated the relationship between a group of p53 mutant and p53 wild-type cell lines and chemosensitivity to 123 drugs used in cancer treatment.

One of the findings was that the median GI50 for p53 mutant cell lines treated with 5-FU was sixfold higher than the GI50 of p53 wild-type cell lines [68].

The TP53 Colorectal Cancer International Collaborative Study published a study consisting of a patient cohort of 3583 samples from 25 different research groups in 17 countries. One of the aims of this study was to investigate whether there was a prognostic impact of TP53 mutations and treatment subgroups. In 1334 Dukes' C patients (792 wild-type TP53 and 542 mutant TP53), the wild-type TP53 patients treated with chemotherapy showed significantly better survival in the proximal and rectal tumor groups ($p = 0.006$ and $p = <0.001$, respectively) and a trend towards statistical significance ($p = 0.022$) was observed for the distal tumor group [69]. In the mutant TP53 group, patients receiving chemotherapy had better survival only in the proximal colon group ($p = <0.001$), with the authors concluding that TP53 mutation had no predictive value within Dukes' C patients treated by surgery alone or surgery and chemotherapy [69]. The authors advised caution in interpreting these observations, since the chemotherapy treatment was not always 5-FU based. When the 5-FU-based regimens were grouped together, the authors reported that chemotherapy can have an impact on survival based on TP53 mutational status and tumor sites [69]. Furthermore, this study showed that wild-type TP53 rectal patients received a significant survival benefit from 5-FU-based chemotherapy, irrespective of whether or not radiotherapy was received [69].

In 2008, Ahmed and colleagues carried out a study on 41 Dukes' C CRC patients that had a curative resection of the primary tumor and were administered 5-FU adjuvant treatment. The p53 mutation status was confirmed by gene sequencing and the study concluded that there was significant advantage for the wild type p53 patients in the time to develop metastasis and overall survival within the two groups receiving 5-FU adjuvant treatment [70].

2.5. Apoptosis

Failure of cells to undergo apoptosis may affect treatment efficacy [71]. Apoptosis can occur via two main signaling pathways: extrinsic (receptor-mediated pathway) and/or intrinsic (mitochondrial-mediated pathway [72]. Although a number of studies have reported the involvement of extrinsic pathway in treatment resistance, namely CD95 (Fas), it has been shown that under certain situations most chemotherapy-treated cells undergo intrinsic apoptosis [71, 73].

By way of an *in vitro* model, Gourdier and colleagues demonstrated that acquired resistance to oxaliplatin can occur via a defect in the intrinsic apoptosis pathway [74]. Furthermore, expression of cleaved caspase-3 and Bax was lost in the 68-fold oxaliplatin-trained resistant cell line, while the expression of Bcl-2, Bak, and Bcl-X_L remained unaltered, suggesting that they are not involved in the acquired resistance mechanism to oxaliplatin [74]. Transcription factor NFκβ is known to be constitutively activated in colorectal cancers and although it has been associated with pro-apoptotic function, it is not always the case [75]. 5-FU has been shown to induce NFκβ expression via IKKβ and consequently chemoresistance in colorectal cell lines [76]. Apart from the fact that oxaliplatin, like 5-FU, can cause NFκβ constitutive activation, this activation imparts chemoresistance via c-FLIP and Mcl-1 [77].

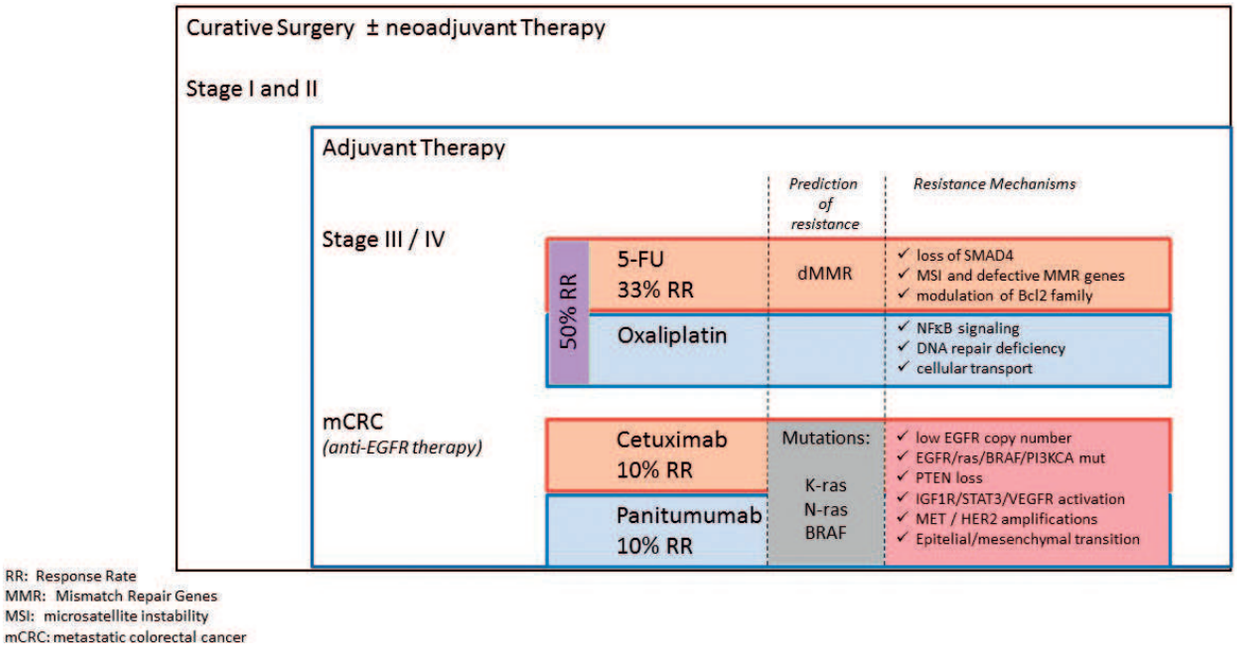

Figure 1. Treatment for colorectal cancer patients according to the stage at diagnosis.

A study identified double stranded RNA dependent protein kinase (PKR), as a key molecule in inducing apoptosis in colon cancer cells—irrelevant of the p53 status [78]. The authors proceeded by demonstrating that PKR knockdown cells responded poorly to treatment with 5-FU. The importance of integrins and apoptosis in chemoresistance is being investigated by a number of groups. A study by Liu and colleagues focussed on the involvement of the β6-integrin-ERK-MAP kinase pathway in conferring chemoresistance to 5-FU in colon cancer lines (**Figure 1**) [79].

3. Novel resistance mechanisms

3.1. Warburg effect

Over the past decade, the significance of the Warburg effect in the field of oncology has gained momentum and a number of original research papers [80, 81] and reviews have been published on this topic [82]. The Warburg effect, also referred to as the glycolytic phenotype, is singularized by an increased rate of aerobic glycolysis together with irreversible injury to mitochondrial oxidative phosphorylation [83] and is favored by the majority of tumors [84]. Subsequently, Tong and colleagues showed that aerobic glycolysis is involved in cancer cell proliferation and tumorigenesis in a model of HCT116 colorectal cell lines [85]. A number of mechanisms that affect increased glycolysis, and therefore contribute to the Warburg effect, include mitochondrial defects, adaptation to hypoxic conditions, oncogenic signals, and altered metabolic enzymes [86]. A number of these mechanisms occur via hypoxia inducible factor-1 (HIF-1) [87].

A putative major player in the Warburg effect and cancer is the uncoupling protein coding-2 (UCP2) [88]. UCP2 is located in the mitochondrial inner membrane and its main function is that of a mitochondrial transporter protein, that creates proton leaks across the inner mitochondrial membrane, ergo uncoupling oxidative phosphorylation from ATP synthesis [89]. Furthermore, UCP2 might act as a negative regulator of ROS production [90].

Horimoto et al. postulated that UCP2 is involved in colon tumor adaptation and is correlated with neoplastic changes [91]. UCP2 gene expression and protein expression was assessed on a small cohort of 10 patients, where a paired normal and tumor sample for each patient was processed. Gene expression results demonstrated an average of 3.88 ± 0.85-fold difference in UCP2 mRNA expression between the tumor (T) and peritumoral (P) paired samples. The same ratio was found for UCP2 protein expression and furthermore a strong linear correlation between T/P ratio of UCP mRNA and protein expression ($r = 0.91$, $p = 0.0015$) was confirmed. Additionally, immunohistochemistry (IHC) for UCP2 was carried out on a cohort of 9 hyperplastic polyps, 17 adenomas, and 107 adenocarcinoma and positive scores were 11.1, 58.8, and 86%, respectively.

A comparable study was undertaken on a larger cohort of colon cancer patients and it yielded the same results and correlations in addition to association of UCP2 expression and metastasis [88]. Altered colon cancer metabolism, as confirmed through measurement of UCP2 expression in these studies, can also contribute to resistance to cancer therapies. An *in vitro* study investigated the rate of cell death caused by 5-FU, with respect to different metabolic rates, as quantified by the bioenergetic signature [92]. This study demonstrated the bioenergetic signature directly correlates with the apoptotic response to treatment with 5-FU [92].

Tumor adaptation to hypoxic and acidic microenvironments strongly selects for tumor cells that are resistant to chemo- and radiotherapy [93]. The slowing down of cell cycling induces a decreased rate of cell division, thereby decreasing chemotherapy activity [93]. Furthermore, hypoxia dysregulates several DNA damage response pathways and prevents effective functioning of proteins involved in homologous recombination (HR), non-homologous end joining (NHEJ), and the mismatch repair (MMR) pathways, thereby driving genetic instability [94]. The cascade of events triggered by chronic hypoxia may also bring about amplification of multidrug resistant gene ABCB1 via induction of chromosomal fragile sites [94].

An *in vitro* study on colon carcinoma cell lines demonstrated that low oxygen concentration resulted in decreased protein expression of Bid, Bad, and Bax [87]. A further series of experiments illustrated that all three CRC cell lines studied expressed a functional HIF-1 pathway and the authors showed that in a hypoxic environment Bid down-regulation occurs via HIF-1, while down-regulation of Bax and Bad occurs independently of HIF-1 function [87]. Additionally, under anoxic conditions, SW480, HCT116, and HT29 were resistant to etoposide treatment and SW480 was also resistant to oxaliplatin. Further investigation by Erler and colleagues demonstrated that down regulation of Bid and/or Bax contributed to etoposide resistance in this model [87]. An important observation from this study was that Bak was least responsive to hypoxia and thereby it might be crucial for drug-induced apoptosis [87].

The PI3K/Akt signaling pathway enhances aerobic glycolysis, and dual PI3K/mTOR inhibitors can influence the cancer cell metabolic programme [95]. Oncogenic mutations involving

this pathway, MAPK, and Src pathways have been shown to increase HIF-1 expression in both hypoxic and normoxic conditions [96]. Inhibiting HIF1 decreases proliferation, influences anaerobic glycolysis, encourages apoptosis, and reduces resistance to chemo- and radiotherapy [93].

Moderate evidence in the literature demonstrates that 18q LOH/SMAD4 loss has potential for it being used as a marker to predict response to 5-FU-based therapies [97]. Papageorgis and colleagues demonstrated that a SMAD4 defect suppresses hypoxia-induced cell death, induces aerobic glycolysis, and promotes 5-FU resistance in the HCT116 cell line model [98]. Furthermore, the authors observed a physical interaction between SMAD4 and HIF1α and postulated that the acquired chemoresistance in 18q-deficient CRC may be explained by Smad4 negatively regulating HIF1α-induced GLUT1 expression and the rate of aerobic glycolysis [98]. Other oncogenes/tumor suppressor genes known to be involved in the stimulation of glycolytic energy include Ras, c-myc, Src, and p53 [99].

Downregulation of pyruvate kinase M2 (PKM2) is also known to promote the Warburg effect metabolic phenotype and tumorigenesis [80]. Additionally, PKM2 is a HIF-1 target gene and concurrently a co-activator of HIF-1 [93]. A study by Tamada and colleagues on a number of cancer cell lines, which also comprised of CRC cell lines HCT116 p53 wild-type and HCT116 p53 null, demonstrated CD44-regulated glycolysis in p53 deficient cells via interaction with PKM2 [100]. Furthermore, the authors speculated that CD44 functions as a scaffold between a tyrosine kinase and PKM2 near the cell membrane, ergo down-regulating the activity of PKM2 [100]. By means of a set of elegant experiments, the authors showed evidence that CD44 silencing in p53 deficient cell lines sensitized the cells to cisplatin, 5-FU, and adriamycin in normoxia, and that CD44 silencing of p53 wild-type cells under hypoxic conditions increased sensitivity to these three chemotherapeutic agents [100].

The relationship of hypoxia and resistance to both radio- and chemotherapy has been explored for the last decade and several mechanisms have been postulated. As evidenced by a number of studies referred above, the Warburg effect is an adaptive mechanism used by solid tumors to overcome stress caused by hypoxia and also contributes to resistance to both chemotherapy and targeted inhibitors.

3.2. Clonal evolution

Another contributor to therapy failure is the innate Darwinian aspect of cancer [101]. In a review of clonal evolution, Greaves and Maley describe the complexity of cancer and the selective pressure for resistant cells to thrive when treated with chemotherapeutic agents. Similarly, adaptive microenvironmental mechanisms such as hypoxia and acidosis lead to both phenotypic and genotypic heterogeneity [102]. This evolution not only affects the genomic instability of the tumor but also contributes towards resistance to therapy, including targeted therapies [102].

A retrospective study analyzing circulating tumor DNA from a cohort of 28 mCRC patients suggested that development of resistance to panitumumab can occur in metastatic lesions, having a sub-clone encompassing just 1 of 42 mutations associated with resistance to panitumumab. Subsequently, the time for recurrence is basically the time taken for that sub-clone to

repopulate the lesion [103]. Furthermore, the authors concluded that resistance mutation in KRAS and other genes were likely to be present prior to starting panitumumab therapy [103].

A number of mechanisms contributing to acquired resistance to 5-FU-based therapies include alteration of the drug's specific target, drug inactivation, influx and efflux of drugs in the cells, drug-induced damage, and evasion of apoptosis [104]. In an attempt to comprehend these complex mechanisms, Tentes et al. investigated 5-FU acquired resistance in the SW620 cell line model [105]. A significant finding reported in this study consisted of the maintenance of a 5-FU resistant phenotype, albeit by culturing the trained cell line in drug-free media for 15 weeks. The authors concluded that the resistant clones may have acquired an altered genetic background and unique gene expression patterns due to long-term exposure to 5-FU, and that this scenario might explain relapses caused by residual disease of chemo-resistant cells. Besides, overlapping mechanisms of resistance to 5-FU could be observed in the trained resistant cell line [105].

3.3. Intra-tumor heterogeneity

A published study evidenced that intra-tumor heterogeneity is a considerable hurdle to both predictive and prognostic biomarker development [106]. One of the principal results in this study highlighted that 63–69% of all somatic mutations are not detectable across every tumor area, hence confirming that one biopsy is not representative of the whole tumor [106]. Intra-tumor heterogeneity is one of the main challenges to patients being successfully treated and can also contribute to patients having relapses [107].

Chromosomal instability (CIN) is associated with both intrinsic- and acquired-drug resistance and also involved in intra-tumor heterogeneity [108]. A number of hypotheses surrounding CIN, Darwinian selection, and intra-tumoural heterogeneity are currently being investigated. Evidence has been obtained to indicate that cells having a high degree of chromosomal instability are more predisposed to exhibit intrinsic resistance [108].

Lee and colleagues conducted a study on a panel of 27 CRC cell lines (18 of which were CIN^+) and demonstrated that CIN^+ cell lines were significantly more intrinsically resistant to the inhibitors used (Kolmogorov-Smirnov test $p < 0.0001$) and, even at similar proliferation rates, CIN^+ cell lines were more resistant to treatment when compared to CIN^- (one sided Wilcoxon-Mann-Whitney test, $p = 0.049$) [55]. Furthermore, according to previous reports, patients treated with 5-FU-based therapy who exhibited CIN^+ did not obtain as much benefit from the treatments, when compared to patients having diploid CRC [109]. This acquired multidrug resistance has been attributed to cell heterogeneity due to multiple chromosomal re-assortments in these aneuploid cells [55]. One of the hypotheses that Lee and colleagues discussed is that there is a distinct CIN^+ survival phenotype that triggers an endurance to ongoing chromosomal rearrangements which is also related to drug resistance [55].

Phenotypic heterogeneity arises from both genetic and non-genetic influences [110]. Non-genetic influences can emerge from phenotypic plasticity and differentiation of cancer stem cells [107]. The cellular phenotype is affected by several factors namely, stochastic fluctuations (noise), genotypes, microenvironment, and the gene regulatory network [110]. In their

review, Marusyk and colleagues remark that even though genetic heterogeneity is not likely to contribute considerably to phenotypic heterogeneity, it still supports tumor evolution during tumorigenesis and treatment resistance [110]. Phenotypic heterogeneity manifests as phenotypic diverse subpopulations of subpopulation of tumor cells, histologic alterations, different patterns of disease progression, prognosis, diagnosis, and also responses to therapy [111]. This necessitates further investigations on therapeutic resistance of CRC with respect to phenotypic heterogeneity.

3.4. Pharmacoepigenomics

Epigenetic modifications are implicated in the progression of chemoresistance [112]. They bring about changes in gene expression that are autonomous of changes in DNA sequence and persevere over numerous cell divisions. In contrast to genetic modifications, epigenetic transformations are reversible [113]. As a result, the field of pharmacoepigenomics is now gaining more popularity. One of the main mechanisms of action of chemotherapeutic agents is by inducing DNA damage which subsequently leads to either DNA repair, apoptosis, or cell-cycle arrest [114]. A number of genes implicated in these biological processes in cancer cells are epigenetically regulated [115]. Drug resistance has been associated with hypermethylation of promoter regions of pro-apoptotic genes, hypomethylation of drug efflux promoters, and also modified promoter methylation patterns of DNA repair genes [116]. Furthermore, global histone modification patterns may also be involved in drug resistance [117].

Sugita and colleagues conducted a study on a cohort of 80 gastric cancer patients and investigated the relationship between methylation of BNIP3 and DAPK with respect to response to 5-FU-based therapy. This study confirmed a relationship between poor response rate and methylation of one or both genes when compared to patients that did not have methylation ($p = 0.003$) [118]. Furthermore, a study on 112 primary colorectal patients substantiated that BNIP3 is methylated in CRC patients, with approximately 58% of the cohort exhibiting methylation [119]. Additionally, 30 patients having BNIP3 methylation were non-responsive to irinotecan therapy [119]. An *in vitro* study demonstrated that cells having a methylated $p16^{Ink4A}$ were more resistant to irinotecan-induced cell cycle arrest [120] . Cheetham and colleagues presented support that hypermethylation of the SPARC promoter is frequently found in both CRC tumor and cell lines when compared to normal colon ($p = 0.03$) [121]. A previous *in vitro* study by the same group showed that mRNA and protein expression of SPARC were low in chemoresistant tumors and thereby the authors concluded that hypermethylation of the SPARC promoter might be a potential mechanism of low SPARC expression, leading to resistance to therapy [121, 122].

Dynamic chromatin modification can also influence resistance to treatment and a publication by Sharma and colleagues illustrated this resistance mechanism. “Drug-tolerant persisters” were detected while studying the acute response of human cell lines with respect to different treatments. These cells remained viable in conditions where other cells failed to thrive, and since they were encountered at a higher frequency than expected, the authors associated this observation to epigenetic regulation [123]. Following several elegant experiments, the authors concluded that this transiently acquired drug resistant phenotype is capable of arising *de novo*

and requires the histone demethylase KDM5A/RBP2/Jarid1A. This particular histone demethylase secures a metastable chromatin state which contributes towards the ability of cells to tolerate drug exposure. Additionally, this chromatin state is dependent on IGF-1R signaling, which has also been associated with drug resistance in a number of other studies [124, 125].

3.5. Additional mechanisms

Resistance to the newest drug, trifluridine was attributed to decreased changes in expression of mRNA and miRNA located on chromosome 9. This could have been due to either genome deletion or LOH of let-7d-5p, a miRNA inversely associated to trifluridine-induced proliferative effects; hence low expression would lead to decreased effectiveness [126] .

Resistance-promoting adaptive responses include the (1) epithelial-mesenchymal transition (EMT) which is associated with invasive capacity, increased motility and related to chemotherapy, and targeted therapy resistance [38]; (2) Oncogenic bypass and pathway redundancy, also referred to as compensatory signaling pathway via crosstalk mechanisms is involved in acquired resistance to cetuximab. With EGFR deregulated, HER2, HER3, cMET, MAPK, and Akt are subsequently switched on and as a result, acquired resistance can ensue since some RTKs share signaling pathways involved in proliferation and survival [127]. (3) Activation of pro-survival signaling: ADAM17, known to be deregulated in CRC, is also known to be activated with chemotherapy and has been implicated with growth factor shedding, growth factor receptor activation, and drug resistance [128].

Another downstream resistance mechanism is autophagy. In CRC, BRAF V600E induces autophagic properties. Recently, Goulielmaki and colleagues reported that PI3K/AKT/MTOR inhibitors induce autophagic tumor properties, whereas RAF/MEK/ERK signaling inhibitors reduce expression of autophagic markers. They showed that pre-treatment of autophagy inhibitor 3-MA followed by its combination with BRAFV600E targeting drug PLX4720 can synergistically sensitize resistant colorectal tumors [129].

Another recent review highlighted the involvement of telomerase in drug resistance in cancer [130]. The main telomerase-related mechanism highlighted in this review included hTERT translocation, hTERT and cell resistance to stress, G-quadruplex inhibitors specific, telomerase inhibition and the mechanism by which telomerase helps cancer cells resistance to DNA damage/apoptosis. Recent literature also implicates the mammalian vault complexes in drug resistance [130].

4. Conclusion

During the last decade, a number of groups have started taking a systems medicine approach to better understand treatment resistance in colorectal cancer. As described by the Coordinating Action Systems Medicine, this approach comprises the iterative and reciprocal feedback between clinical investigations and practice with computational, statistical and mathematical multi-scale analysis and modeling of pathogenic mechanisms, disease progression and remission, disease spread and cure, treatment responses and

adverse events, as well as disease prevention both at the epidemiological and individual patient level [131].

We are already witnessing a number of success stories and by integrating data, especially with respect to understanding mechanisms of resistance, we are now moving away from clinical trials directed to specific tumors towards umbrella trials (multiple molecular targets in a single tumor) and basket trials (single molecular abnormality across multiple cancer types). This evolution can be clearly seen in CRC, where we started with one gene, one drug approach, and moved towards a multi-gene, multi-drug approach and currently we are at a multi-molecular, multi-drug approach [132].

This systems medicine approach is helping to accelerate bench to bedside developments and an example is the EXACT trial, where treatment-refractory cancer patients are administered an individualized treatment concept based on prospective biomarkers assessed in a real-time biopsy [133].

All of the above has had a major impact on the clinic and as we can now witness, we have achieved a better patient stratification, earlier and more sensitive diagnostics and drug repurposing. Nonetheless, it is important that we continue working to overcome the major challenges we are still facing. These include, but are not limited to morphologic and molecular heterogeneity of cancer, treatment resistance, drug addiction, and other challenges such as standardization of methods, infrastructure, and cost and big data. Hence, it will be important to have appropriate biomarkers to inform clinicians on administering the most effective treatment to the individual patient at the right time.

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