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Peter Jordan *Editor*

Targeted Therapy of Colorectal Cancer Subtypes

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Targeted Therapy of Colorectal Cancer Subtypes

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Colorectal Cancer Subtypes – The Current Portrait

1

Peter Jordan

Abstract

Colorectal cancer (CRC) is one prominent example for how chemotherapy has been changing by moving from the use of general cytotoxic agents to more tumour-specific drugs. For example, antibody-based drugs neutralize a growth factor receptor protein on the surface of tumour cells. The development of such new therapeutic opportunities requires a more thorough and systematic subclassification of CRC because tumour cells can exploit several alternative genetic pathways for their survival. This chapter gives an overview on CRC subtypes as an introduction to the following book chapters that will describe aspects of specific subtypes, and how these may lead to the development of novel pathway-specific drugs for a more precise therapeutic intervention.

Keywords

Chromosomal instability · Colorectal cancer subtype · Consensus molecular subtype · Microsatellite instability · Oncogene · Polyp · Serrated pathway

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According to the Globocan 2012 data collected by the International Agency for Research on Cancer, cancer of the colon and rectum (CRC) presented in both sexes over 1.35 million cases. This corresponds to the third most common incidence (behind lung and breast cancer) and the fourth cause of cancer mortality worldwide (Ferlay et al. 2015). CRC incidence continues to rise especially in low and middle income countries and is considered one of the clearest markers for rapid societal and economic changes that are associated with cancer development (Arnold et al. 2017). The corresponding life-style and environmental factors contribute significantly to the vast majority of CRC cases, which are designated as sporadic CRC. Nevertheless, hereditary CRC syndromes exist but cause only a small fraction of cases.

Sporadic CRC has been extensively studied and reviewed (Jass 2007; Fearon 2011; Cancer Genome Atlas Network 2012; Brenner et al. 2014; Matos et al. 2016). The majority of the sporadic CRC tumours originates from premalignant precursor lesions known as polyps, which over time progress to clinically relevant tumours. The Fearon-Vogelstein model has provided an initial paradigmatic model for CRC tumorigenesis based on the loss of the tumour suppressor gene APC and stepwise accumulation of mutations in critical genes including KRAS, DCC and TP53 (Fearon and Vogelstein 1990). A persistent activation of the Wnt pathway that regulates the stem

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cell compartment and cell fate along the crypt-villus axis of the colon mucosa emerged as a key driver of CRC.

However, subsequent pathological or molecular analyses have revealed the existence of several CRC subtypes instead of being a uniform disease entity. From the pathologist's perspective, for example, the detected polyps are precursor structures formed as morphologically distinct types and can be divided into either tubular and villous adenomas, or serrated and hyperplastic polyps. The anatomic site of origin further divides tumours into two groups: those found in the distal or in the proximal colon segment. For example, proximally located tumours are usually larger and present a mucinous histology.

On the other hand, molecular analyses demonstrated that tumours can exhibit either widespread chromosomal abnormalities, designated as the chromosomal instability phenotype (CIN), or, instead, a high rate of DNA sequence mutations (the microsatellite instability phenotype, or MSI) caused by deficient DNA mismatch repair. Another molecular feature that distinguishes different tumour groups is the degree of increase in DNA methylation observed in clusters of the CpG dinucleotide found in many gene promoters, a phenotype known as CpG island methylator phenotype (CIMP).

Finally, genotype analyses of tumours detected the presence of typical and sometimes mutually exclusive somatic mutations in specific cancer-related genes, such as the *APC* and *TP53* tumour suppressor genes, or in oncogenes such as *KRAS, PIK3CA* or *BRAF* and contributed to the assignment of CRC subtypes.

By combining the above criteria of pathology and molecular genotyping, sporadic CRC tumours initially formed two main groups. One group included over 70% of the cases and developed tumours, mostly in the distal colon, that appeared to follow an adenoma-carcinoma sequence involving recurrent somatic mutations in the *APC* and *TP53* tumour suppressor genes or in the oncogene *KRAS* (Jass 2007; Cancer Genome Atlas Network 2012; Brenner et al. 2014; Phipps et al. 2015). These cases also exhibit widespread chromosomal abnormalities (CIN), and derive from adenomatous polyps.

A second major group of sporadic CRC included about 15% of patients with tumours occurring preferentially in the proximal colon, presenting a stable chromosome number, but a high rate of DNA sequence mutations, the MSI phenotype. This phenotype develops following the somatic silencing by DNA methylation of the promoter of the *MLH1* gene that encodes a component required for a functional DNA mismatch repair system. The majority of these tumours derive from precursor polyps with a serrated morphology called sessile serrated adenoma (Snover et al. 2005; Bettington et al. 2013) and present activating mutations in the oncogene *BRAF*, but not in *KRAS*.

Subsequent studies unravelled further heterogeneity within these two groups of tumours. For example, some 8% of sporadic cases have a mutation in *BRAF* but are not MSI. Another 10% have mutation in *KRAS* but occur in the proximal colon and derive from a type of serrated polyp called traditional serrated adenoma (Jass 2007; Phipps et al. 2015). In addition, many adenomatous-derived polyps in the distal colon lead to carcinomas with CIN, but without the presence of mutated *KRAS*.

Although this heterogeneity precluded a simple genotype-phenotype correlation of CRC, it laid the foundation for subtype-specific therapeutic approaches. For example, the stimulation of tumour cell proliferation through the epidermal growth factor receptor (EGFR) and its downstream signalling along the mitogen-activated protein kinase (MAPK) pathway, has led to the development and clinical approval of therapy using the anti-EGFR antibodies cetuximab and panitumumab. In clinical practice only around 10% of cases respond to anti-EGFR therapy (Bardelli and Siena 2010; Misale et al. 2014), while others are *a priori* resistant due to mutually exclusive mutations in either *KRAS (30%), NRAS (2%),* or *BRAF (15%)* that all operate in the EGFR pathway (Zhao et al. 2017). Such mutations revealed to be alternative mutational events and occur early during tumour development, given they can be detected in microdissected premalignant polyps or aberrant crypt foci (Yang

et al. 2004; Beach et al. 2005; Rosenberg et al. 2007; Velho et al. 2008; Carr et al. 2009; Sandmeier et al. 2009; Boparai et al. 2011; Kim et al. 2011). Another example are inhibitors of the *BRAF* kinase activity that have the potential to target some 10–15% of CRC cases (Obaid et al. 2017).

Besides the MAPK pathway, the activation of the phosphatidylinositol 3-kinase (PI3K) pathway has therapeutic potential. Mutations in exon 20 of the *PIK3CA* gene were found to associate significantly with the MSI pathway, while exon 9 mutations E542K and E545K are overrepresented in *KRAS* mutant tumours (Zhao and Vogt 2010; Whitehall et al. 2012; Day et al. 2013).

More recently, genome-wide techniques have allowed determining the gene expression signatures of colorectal tumours. The subsequent bioinformatic clustering of the expression profiles provided yet another approach for the identification of CRC subtypes. Several studies have been published with partly overlapping conclusions (Perez Villamil et al. 2012; Schlicker et al. 2012; De Sousa E Melo et al. 2013; Sadanandam et al. 2013; Marisa et al. 2013; Budinska et al. 2013; Roepman et al. 2014), but have eventually resulted in the definition of at least 4 consensus molecular subgroups (CMS) (Guinney et al. 2015).

These expression signatures overlap in part with some of the previous genotypic or phenotypic CRC subtype characterization. For example, the MSI_BRAF subtype from the serrated pathway corresponds fully to the unique CMS1 gene expression profile and both classification schemes determined that roughly 15% of all sporadic CRCs belong to this group.

By contrast, the major group of 70% of the CRC cases CRC with recurrent mutations in the *APC*, *TP53* and *KRAS* genes and CIN has been subdivided into three distinct CMS profiles. CMS2 joins tumours with *APC* mutations, CIN and frequent gene amplification or deletion, while CMS3 features mutation in *KRAS* and a mixed status of MSI and CIN. Interestingly, this group revealed major changes in metabolic reprogramming of tumour cells. Finally, CMS4 unites tumours with CIN and a high degree of mesenchymal characteristics and activation of the TGFβ pathway. Concerning this subtype, subsequent studies emphasized the contribution of stromal-cell gene expression to the CMS definitions (Calon et al. 2015; Isella et al. 2015, 2017). This could imply that clinically meaningful CRC gene signatures are being obscured by the presence of abundant stromal cell-derived signals. Alternatively, if this CMS4 turns out to be a therapeutically meaningful classification, then progression of this CRC subtypes might be strongly influenced by microenvironmental cues from the tumour stroma. A comparison of the most relevant characteristics of each CMS is given in Fig. 1.1.

	CMS1	CMS2	CMS3	CMS4
Frequency	14%	37%	13%	23%
Tumour location	proximal	distal	proximal or distal	distal
Precursor polyp	sessile serrated	adenomatous	serrated or adenomatous	adenomatous
DNA sequence stability	MSI	MSS	MSS or MSI	MSS
DNA methylation	CIMP-H	no CIMP	CIMP-L	no CIMP
Chromsome number	stable	CIN	stable or CIN	CIN
Mutated genes	BRAF	APC, TP53	KRAS	
Pathway signature	immune activation	WNT and MYC	metabolic deregulation	TGF-β, mesenchymal

Fig. 1.1 Comparison of the pathological, molecular and genomic features that distinguish the four consensus molecular subtypes (CMS) of colorectal tumours defined by gene expression-based clustering. *CIMP* CpG island methylator phenotype, *-H high*, *-L* low, *MSI* microsatellite instability, *MSS* microsatellite stability, *CIN* chromosomal instability

It should be noted that despite of the progress of using genome-wide and unbiased gene expression signatures for the CMS classification, a group of 13% of all sporadic CRC cases could not be accommodated into the 4 CMS groups due to a mixture of features observed in the other 4 groups. This may indicate several properties: either further criteria are required to define this group, or these tumours are heterogeneously composed of different clones, or transition can occur between CMS signatures during tumour progression.

Altogether, this heterogeneity among sporadic CRC cases implies that a standardized therapeutic approach does not exist for patients; however, it also provides an opportunity for the identification of subtype-specific therapeutic targets or strategies.

In this book, a collection of review articles presents major CRC subtypes and how they can be distinguished by molecular analyses. They also highlight how this knowledge may guide the development of therapeutic strategies with higher precision and efficiency, thus reducing harmful side effects and increasing therapeutic efficacy.

In particular, the second chapter by Matos and Jordan describes the subgroup characterized by proximal colon location, mutation in the oncogene *BRAF* and microsatellite instability.

Then, Aguilera and Serna-Blasco elaborate on the *KRAS*-mutant CMS3 subtype in the distal colon and its changes in cell metabolism.

Chapters 4 and 5 are devoted to alterations in the phosphatidylinositol (PI)-dependent signalling pathway that affects more than one CRC subgroup. Fernandes et al. describe the role of the oncogenic lipid kinase PI3K and targeted therapeutic strategies, whereas Kotelevets et al. focus on the antagonizing phosphatase and tumour suppressor PTEN.

Chapter 6 reviews CRC subtype CMS2, which is characterized by recurrent mutations in the canonical Wnt signalling components, and presents the perspectives for using targeted therapy.

The more mesenchymal tumour cell properties that distinguish the highly invasive CMS4 subtype are addressed in Chap. 7 by Georges et al., together with the role of tumour budding and of the microenvironment.

Chapters 8 and 9 present important aspects of the targeted treatment approach through anti-EGFR therapy. Martins et al. describe first the clinical challenges encountered in the treatment of patients with anti-EGFR therapy. Finally, Pereira and Rodrigues elaborate on the development of miRNA-based strategies to improve the response to EGFR-directed therapy in patients.

Altogether, the book presents the main aspects of our current knowledge on heterogeneity in colorectal cancer, a prerequisite for the development of novel targeted therapy approaches.

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2 Targeting Colon Cancers with Mutated *BRAF* and Microsatellite Instability

Paulo Matos and Peter Jordan

Abstract

The subgroup of colon cancer (CRC) characterized by mutation in the *BRAF* gene and high mutation rate in the genomic DNA sequence, known as the microsatellite instability (MSI) phenotype, accounts for roughly 10% of the patients and derives from polyps with a serrated morphology. In this review, both features are discussed with regard to therapeutic opportunities. The most prevalent cancer-associated *BRAF* mutation is BRAF V600E that causes constitutive activation of the pro-proliferative MAPK pathway. Unfortunately, the available BRAF-specific inhibitors had little clinical benefit for metastatic CRC patients due to adaptive MAPK reactivation. Recent contributions for the development of new combination therapy approaches to pathway inhibition will be highlighted. In addition, we review the promising role of the recently developed immune checkpoint therapy for the treatment of this CRC subtype. The MSI phenotype of this subgroup results from an inactivated DNA mismatch repair system and leads to frameshift mutations with translation of new amino acid stretches and the generation of neo-antigens. This most likely explains the observed high degree of infiltration by tumour-associated lymphocytes. As cytotoxic lymphocytes are already part of the tumour environment, their activation by immune checkpoint therapy approaches is highly promising.

Keywords

Alternative splicing · BRAF · Microsatellite instability · RAC1b · Serrated polyp pathway

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2.1 Introduction

As described in the introductory Chap. 1, sporadic colorectal cancer (CRC) is not a homogenous disease entity but presents with distinct subtypes that differ in molecular and pathological criteria. One defined subgroup comprises about 10–15% of the patients and displays a high mutation rate in the genomic DNA sequence,

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known as the microsatellite instability (MSI) phenotype. Tumours of this type occur mostly in the proximal colon, carry frequently a mutation in the *BRAF* gene, and derive from polyps with a serrated morphology. They present a stable chromosome number, but an inactivated DNA mismatch repair system, which causes the high number of mutations in the DNA sequence, as detailed below (Jass 2007; Fearon 2011; Cancer Genome Atlas Network 2012; Brenner et al. 2014; Matos et al. 2016). Besides this subgroup of sporadic CRC, DNA mismatch repair genes can also suffer germline mutations and cause Lynch syndrome or hereditary nonpolyposis colorectal cancer (HNPCC) that is responsible for about 3% of all CRC cases.

From the morphological point of view, sessile serrated adenoma (SSA) is the main precursor lesion of this tumour subtype (Noffsinger 2009; Snover 2011; Bettington et al. 2013). By colonoscopy, SSAs are detected in the proximal colon as broad-based flat polyps that grow on the mucosa surface with a mucinous cap, and are estimated to give rise to about 12% of all sporadic CRC tumours.

Following the availability of genome-wide gene expression analyses, CRC subtypes have also been determined based on transcriptomic signatures. Eventually, this resulted in the definition of consensus molecular subgroups (CMS) (Guinney et al. 2015) and one of the unique gene expression profiles characterizing roughly 15% of all sporadic CRCs, called CMS1, corresponds unequivocally to the BRAF/MSI subtype.

2.2 Biology and Role of Mutated BRAF Protein Kinase

The *BRAF* gene encodes a serine/threonine-protein kinase that is one of the downstream effector proteins of several growth factor receptors, including the epidermal growth factor receptor (EGFR). In normal colon epithelial cells, stimulation of EGFR leads to recruitment of the small GTPase KRAS to the plasma membrane and subsequent interaction with BRAF. This initiates the mitogen-activated protein kinase (MAPK) pathway by activating MEK through its phosphorylation (Dhillon et al. 2007; Lavoie and Therrien 2015). Several studies have revealed the formation of a dimeric complex between BRAF and CRAF proteins (Wan et al. 2004; Poulikakos et al. 2010) involved in activation of the MAPK pathway, which then induces cell growth and proliferation. In addition, this pathway also affects other key cellular processes, such as cell migration (through RHO small GTPases), apoptosis (through the regulation of BCL-2), and survival (through the HIPPO pathway) (Matallanas et al. 2011).

In colon cancer, this pathway is highly activated, either by oncogenic mutation or overexpression of EGFR (occurs in up to 50% of human cancers (Dhillon et al. 2007)), or by mutually exclusive oncogenic mutations in the *KRAS* or *BRAF* genes. The 1799 T > A mutation in *BRAF* encodes substitution of a valine with a negatively charged glutamate residue at codon 600 within the BRAF kinase domain. This mimics a regulatory phosphorylation event that increases kinase activity approximately tenfold (Davies et al. 2002). The BRAF V600E mutation accounts for up to 80% of all cancer-associated BRAF mutations, characterizes between 8 and 14% of early and advanced stages CRC cases (Roth et al. 2010; Gavin et al. 2012; Venderbosch et al. 2014; André et al. 2015; Taieb et al. 2016), and causes constitutive activation of the MAPK pathway (Davies et al. 2002). Clinically, BRAF V600E-mutated CRCs are associated with right-sided, high-grade primary tumours, more frequent in older women, and arising from precursor sessile serrated adenomas (Jass 2007). Despite having a favourable prognosis in early-stage disease, BRAF V600E-mutated CRCs are associated with poor survival rates at a metastatic stage (Roth et al. 2010; Venderbosch et al. 2014; Guinney et al. 2015; Bläker et al. 2018). They also display a distinct pattern of metastatic spread, with higher frequencies of peritoneal involvement, distant lymph node metastases, and a lower frequency of lung metastases (Atreya et al. 2016).

Up to 80% of CRCs positive for the BRAF V600E mutation were also found to overexpress RAC1b, a highly active splicing variant of the small GTPase RAC1 (Matos et al. 2008). RAC1b overexpression seems to occur independently of

tumour stage and microsatellite status (Matos et al. 2008), and was shown to arise from persistent inflammatory cues from the tumour microenvironment (Matos et al. 2013). RAC1b differs from RAC1 both by requiring 10-times fewer growth factor stimulation to become activated and by exhibiting a selective downstream signalling that favours cellular proliferation and survival (Matos et al. 2003; Matos and Jordan 2005). RAC1b overexpression was found to synergise with BRAF V600E in promoting cellular transformation (Matos and Jordan 2008; Matos et al. 2008), and to allow colonocytes to overcome BRAF V600E-induced senescence (Henriques et al. 2015).

2.3 Causes and Consequences of Microsatellite Instability

Short repetitive sequence stretches consisting of mono-, di-, tri- or tetra-nucleotides are designated as microsatellites and occur frequently throughout the genome, mostly in non-coding regions. These stretches constitute frequent hotspots for DNA polymerase slippage during DNA replication, leading to the gain or loss of repeated nucleotides, i.e. short insertions or deletions (Yamamoto and Imai 2015). Usually, these errors generate unpaired nucleotides that are recognized and excised by the DNA mismatch repair system (MMR) followed by resynthesis of the affected stretch. If MMR is not functioning properly, then microsatellite regions suffer length variation and an instable number of repeat units, designated as microsatellite instability (MSI). Thus, MSI represents phenotypic evidence that MMR is malfunctioning. Typically, two mononucleotide repeat markers (BAT25 and BAT26), and three dinucleotide repeats (D2S123, D5S346, and D17S250) are analysed to determine the MSI status of a tumour (Umar et al. 2004). Samples are classified as MSI-high when two or more markers are unstable, and as MSI-low when only one marker shows instability.

Although the majority of repeats occur in untranslated regions and introns, the coding region of some genes also contain repeats (Cortes-Ciriano et al. 2017). In this case, MSI will cause sequential acquisition of frameshift mutations that usually create a premature stop codon in the corresponding mRNA, leading to its subsequent degradation by the nonsense-mediated mRNA decay (NMD) pathway and lack of translated protein product. Well-known examples of tumour-suppressor genes mutated in MSI tumours are encoding the receptor TGFBR2 (A10 repeat), the pro-apoptotic protein BAX (G8 repeat), or mismatch repair proteins MSH6 (C8 repeat) and MSH3 (A8 repeat) (Grady et al. 1999; Duval and Hamelin 2002). Another possible outcome of MSI-induced frameshift mutations in the coding region, in particular when occurring in the NMD-resistant terminal exon, can be the translation of new amino acid stretches that affect protein function but also confer a neo-antigen character (Williams et al. 2010), as described below.

But what causes MSI in sporadic tumours and could this be therapeutically targeted? While in the hereditary non-polyposis syndrome (HNPCC or Lynch syndrome), MSI initiates tumorigenesis and is caused by germline mutations in MMR genes (mainly *MSH2* and *MLH1*, but in some cases also in *MSH6*, *MSH3* or *PMS2*), this is rarely the case in sporadic tumours. The sporadic MSI tumour phenotype corresponds to 12% of all CRC cases, occurs later during tumorigenesis and is mainly caused by biallelic hypermethylation of the *MLH1* promoter, resulting in silencing of the gene's expression (Veigl et al. 1998; Cunningham et al. 1998). Hypermethylation is a major mechanism of gene silencing. It occurs through the methylation of cytosines in the CpG islands present in the majority of human gene promoters, including the *MLH1* gene. Indeed, there is a significant correlation between MSI and the presence of both the CpG island methylator (CIMP) phenotype and *BRAF* mutation in this subgroup of CRCs (Weisenberger et al. 2006; Shen et al. 2007). More specifically, this subgroup is characterized as CIMP-high (CIMP-H), characterized by methylation in the majority of five selected CIMP marker gene promoters, although definition and prognostic role are still controversial (Curtin et al. 2011; Jia et al. 2016). CIMP represents a genome-wide change in epi-

genetic regulation that precedes the MSI phenotype and a genome-wide study identified that over 100 genes were silenced due to CpG island-methylation in this group of colorectal tumours (Hinoue et al. 2012). Recent evidence suggests that CIMP, in turn, may be preceded by the presence of the BRAF-V600E mutant protein because this stimulates ERK activity and subsequent phosphorylation of MAFG, a transcriptional repressor protein. MAFG then binds the *MLH1* promoter and recruits a protein complex containing DNA methyltransferase DNMT3B involved in transcriptional silencing (Fang et al. 2014). Thus, if oncogene-induced epigenetic silencing can trigger CIMP in CRCs, the therapeutic down-regulation of oncogene signalling in tumour cells should, in theory, also be able to reverse the CIMP phenotype. Yet, to our knowledge, there is no reported evidence of CIMP reversion by therapeutic targeting of oncogenic MAPK signalling. Moreover, although the use of DNA methyltransferase inhibitors, such as 5-aza-2′-deoxycytidine (5-aza-dC), have had some success in reverting the hypermethylation of CRC-associated genes in vitro (Kai et al. 2017), the use of therapeutic use of cytosine-analogues did not shown convincing clinical benefit when used alone (Vaiopoulos et al. 2014).

Considering the epigenetic nature of this CRC phenotype, this is perhaps one of the many cases where preventive medicine and adjuvant measures concerning life-style factors such as nutrition, physical activity or tobacco smoking, could have an important impact. For example, DNA methylation reactions depend on folate intake and the level of the methyl-group donor S-adenosylmethionine (SAM) (Sapienza and Issa 2016; Bultman 2017). Although the effects of folic acid dietary supplementation in colorectal cancer still remain controversial (Crider et al. 2012; Johnson and Belshaw 2014), in cultured human colonocyte cells, folate depletion was shown to result not only in the expected DNA hypomethylation but also in the targeted hypermethylation of certain gene locus (Zhang et al. 2017). Moreover, these aberrant DNA methylation and gene expression patterns correlated with tumour stage, disease progression, recurrence rate and overall survival of sporadic CRCs.

Another potential epigenetic mechanism causing MSI was reported based on mutations in the H3K36 trimethyltransferase SETD2, a histone modification that characterizes the DNA synthesis phase of the cell cycle and is required for recruitment of the MMR protein MSH6 to sites of DNA replication (Li et al. 2013). MSH6 forms one of the two major MMR protein complexes with MSH2 and cells lacking the H3K36 trimethyltransferase SETD2 displayed the MSI-typical mono- and dinucleotide repeat instability. However, a subsequent analysis of tumour samples could not confirm the association of SETD2 mutations with any clinically relevant MSI phenotype (Kanu et al. 2015).

It should be noted that an important clinical implication of the presence of MSI in CRC is that the widely used 5-fluorouracil (5FU) adjuvant chemotherapy has no benefit for sporadic MSI CRC as well as Lynch syndrome patients (Ribic et al. 2003; Carethers 2017). The reason is that the MSH2-MSH6 mismatch repair complex is required for binding 5FU after its incorporation into DNA and for triggering cell death (Tajima et al. 2004).

More recently, another type of MSI specific for the less frequent tri- or tetra-nucleotide repeats has been linked to inflammation-induced mislocalization of the MSH3 protein from the nucleus to the cytosol (Haugen et al. 2008; Tseng-Rogenski et al. 2015). This phenotype was detected in up to 60% of CRC and is thus not specific for the BRAF/MSI subtype; however, it can influence patient outcome because an A8-repeat in exon 7 of the *MSH3* gene is one of the target regions for frameshift mutations in MSI (Carethers et al. 2015; Koi et al. 2018).

2.4 Pharmacological Approaches to Target the BRAF CRC Subtype

Patients with *BRAF*-mutated metastatic CRCs show shorter progression-free and overall survival (Roth et al. 2010; Venderbosch et al. 2014; Guinney et al. 2015) and have limited therapeutic options [nearly 70% of patients with advanced BRAF V600E-mutated CRC do not survive first-

line chemotherapy (Seligmann et al. 2017)]. This raised high hopes for the use of BRAF inhibitors to treat this subtype of CRC. BRAF inhibitor monotherapy has proven efficient in the treatment of metastatic melanoma patients, the majority of which carry BRAF V600E mutations and reach response rates above 50% (Flaherty et al. 2010; Long et al. 2014)). However, these inhibitors alone were proven ineffective in 20 of 21 (95%) of *BRAF*-mutated metastatic CRCs and this was not associated with either microsatellite instability status, CpG island methylation status, PTEN loss, or EGFR expression and copy number alterations (Kopetz et al. 2015). Thus, understanding the poor response rate to BRAF inhibitors constitutes a major therapeutic challenge for the treatment of this *BRAF*-mutated CRC subgroup.

Primary or acquired resistance to BRAF inhibitors is well known and has been a subject of intense investigation in the last decade (Shi et al. 2014; Cosgarea et al. 2017). In melanoma, several mechanisms of resistance to BRAF inhibitors were already characterized, including activating mutations in MEK1 (Emery et al. 2009) or *NRAS* (Nazarian et al. 2010), the appearance of BRAF splice variants that favour dimerization with CRAF (Poulikakos et al. 2011), *BRAF* gene amplification (Villanueva et al. 2013), MAP 3K8 overexpression (Johannessen et al. 2010), upregulation of platelet-derived growth factor receptor (PDGFR) (Nazarian et al. 2010; Sabbatino et al. 2014) or EGFR (Girotti et al. 2013), and MAPK pathway-related mutations (Van Allen et al. 2014). Thus, the vast majority of resistance mechanisms in melanoma appear somehow related to the re-stimulation of MAPK pathway signalling. This is further supported by recent clinical studies showing that the combination therapy with BRAF and MEK inhibitors significantly increases the response rate and overall survival of melanoma patients (Mäkinen 2007).

In the case of CRC, *BRAF* gene amplification was also found to mediate resistance to BRAF and MEK inhibition (Corcoran et al. 2010). In addition, upregulation of EGFR activity, either by its overexpression (Corcoran et al. 2012) or via feedback activation through CDC25C inhibition (Prahallad et al. 2012), have been implicated in resistance to BRAF inhibitors and lead to reactivation of MAPK signalling. In contrast to melanoma, however, combined therapy using a selective BRAF inhibitor (dabrafenib) and a selective MEK inhibitor (trametinib), produced only minimal improvement over the individual monotherapies in patients with BRAF V600 mutant metastatic CRC (Corcoran et al. 2015). Better results were apparently obtained in a trial combining a BRAF inhibitor (vemurafenib) with an anti-EGFR antibody (panitumumab). In this trial, tumour regressions were seen in 10 of the 12 evaluated patients, with partial responses in 2 patients (100% and 64% regression lasting 40 and 24 weeks, respectively), and stable disease lasting over 6 months in 2 other patients (Yaeger et al. 2015). A very recent report of a clinical trial, combining dabrafenib (D) to inhibit BRAF with anti-EGFR panitumumab (P), alone or together with MEK inhibitor trametinib (T), obtained no response for the T + P combination but, in turn, achieved response rates of 10% for D + P, and of 21% for the triple drug combination (D + T + P) (Corcoran et al. 2018). Importantly, although the authors found that the efficacy of the D + T + P combination correlated with increased MAPK suppression, they also observed significant correlation between reduced response, the emergence of *KRAS* and *NRAS* mutations, and disease progression (Corcoran et al. 2018). Thus, targeting the adaptive feedback pathways in BRAF V600E-mutated CRC can improve efficacy, but MAPK reactivation remains an important mechanism for acquired resistance.

Altogether, these findings reveal that BRAF V600 mutant metastatic CRCs may need the targeting of additional pathways, and pre-clinical data collected in the last few years have shown promising contributions for the development of new combination therapy approaches. For example, a patient with metastatic BRAF-mutated colorectal cancer, who initially responded to combined EGFR and BRAF inhibition, developed resistance due to clonal selection of tumour cells bearing hepatocyte growth factor receptor gene (*MET*) amplification (Pietrantonio et al.

2016). Switching therapy to dual MET and BRAF blockade, produced a rapid and marked regression of the disease, suggesting that the identification of molecular targets emerging during the first treatment may allow to optimize the therapeutic strategy and maximize patient benefit (Pietrantonio et al. 2016). Consistently, a preclinical study using mouse xenografts of HT-29 and RKO CRC cell lines, which bear the BRAF V600E mutant allele, showed that the combination of a MET inhibitor (PHA-665752) and BRAF inhibitor (vemurafenib) produced a significantly higher inhibition of tumour growth than PHA-665752 or vemurafenib alone (Zhi et al. 2018). The effect correlated with increased cell cycle arrest and decrease in MET, AKT and ERK activation.

Another recurrent event in CRC are activating mutations in the PIK3CA gene encoding phosphatidylinositol-3-kinase (PI3K). BRAF V600E CRC cell lines show higher levels of PI3K/AKT pathway activation, and cell lines with mutations in PTEN or PIK3CA were highly insensitive to growth inhibition by vemurafenib (Mao et al. 2013). Moreover, treatments combining vemurafenib with PI3K inhibitors caused synergistic growth inhibition in both primary and secondary resistance BRAF-mutant CRC cells, indicating that activation of the PI3K/AKT pathway is a mechanism of both innate and acquired resistance to BRAF inhibitors in this CRC subgroup (Mao et al. 2013). Consistently, in a preclinical model studying sessile serrated adenomas/polyps from a genetically engineered mouse model for BRAF V600E CRC, the authors observed an upregulation of PI3K/mTOR signalling upon BRAF inhibition (Coffee et al. 2013). Importantly, combination treatment with PI3K/mTOR and BRAF inhibitors circumvented resistance leading to induction of apoptosis and tumour regression.

Another study reported evidence that BRAF V600E induces the expression of key autophagy markers, like LC3 and BECN1, in CRC cells (Goulielmaki et al. 2016). It showed that pretreatment with autophagy inhibitor 3-MA or bafilomycin A1, when followed by the BRAF V600E inhibitor vemurafenib, had a synergistic effect on apoptosis induction in these BRAF V600E CRC cells. Thus, the study provided evidence that the autophagic properties could be exploited to sensitize resistant colorectal tumours to BRAF inhibitors. Unfortunately, the authors also observed that PI3K/AKT/mTOR inhibitors stimulated autophagy in these tumour cells, which could antagonise the anti-autophagic effects of inhibiting BRAF/MEK signalling in combined therapies.

The Wnt/β-catenin pathway, which is frequently dysregulated in non-MSI CRC, was also recently implicated in the mechanisms of BRAF V600E CRC resistance (Chen et al. 2018). Treatment with BRAF inhibitors was found to upregulate this pathway in preclinical models of BRAF V600E-mutant CRC, including cell line- and patient-derived xenografts. Stimulation of the Wnt signalling occurred through activation of focal adhesion kinase (FAK) upon inhibitor treatment. Notably, FAK activation and did not require EGFR or ERK1/2 activation, indicating that the observed hyperactivation of Wnt signalling was a MAPK pathway reactivation-independent event. Importantly, combined inhibition of BRAF/Wnt pathway or BRAF/FAK exerted strong synergistic antitumor effects, both in cell lines and mouse xenograft models (Chen et al. 2018). Interestingly, the resistance-associated upregulation of Wnt signalling in these models occurred also in the presence of new generation RAF inhibitors, such as LY3009120 (a new pan-RAF inhibitor) (Vakana et al. 2017) and PLX7904 (a more effective BRAF inhibitor preventing paradoxical reactivation of the MAPK pathway in preclinical studies (Zhang et al. 2015; Tutuka et al. 2017).

In the near future, a refinement of targets for combination therapy may result from the emerging genome-wide gene expression analyses. For example, the above referred BRAF-driven CMS1 group (Guinney et al. 2015) was recently suggested to be composed of two branches with predominant deregulation of either the cell cycle or of cell growth pathways (Barras et al. 2017).

Finally, regarding the tumour microenvironment, persistent inflammatory cues were shown to promote the overexpression of tumour-related

RAC1B GTPase (Matos et al. 2013). RAC1B overexpression facilitates malignant progression (Matos et al. 2008) by promoting evasion from BRAF V600E-induced senescence (Henriques et al. 2015). Importantly, it was shown that one non-steroid anti-inflammatory drug, ibuprofen, specifically downregulated inflammation-related RAC1B overexpression and led to reduced tumour growth in mouse xenografts of BRAF V600E CRC cell lines (Matos et al. 2013). This could represent another therapeutic opportunity for combination therapy in patients with the BRAF CRC subtype (Matos and Jordan 2015).

2.5 The Promise of Immunotherapies

The CRC subgroup of BRAF/MSI-H tumours develops primarily in the proximal colon and patients have a better prognosis and survival rate than those with other CRC subtypes (Samowitz et al. 2001; Galon 2006; Kumar 2009; Ogino et al. 2009; Brenner et al. 2014). This subtype and is associated with a high degree of infiltration by tumour-associated lymphocytes (Dolcetti et al. 1999; Smyrk et al. 2001; Shia et al. 2003), which consist mostly of activated CD8+ cytotoxic T lymphocytes (Phillips et al. 2004). Indeed, patient-derived cytotoxic lymphocytes recognize the above-referred neo-antigens (Schwitalle et al. 2008) that are translated as a consequence of MSI and presented as peptides on the cell surface by major histocompatibility complex class I molecules. This could also be demonstrated experimentally in mouse models, using genome editing with the CRISPR–Cas9 system to inactivate *Mlh1* (Germano et al. 2017). In these mice, the lack of DNA mismatch repair resulted in frameshift mutations at repetitive sequence stretches throughout the genome, which led to the production of neo-antigens and induced an immune surveillance response observed in human MSI tumours.

In order to evade an immune response, tumour cells express on their surface the inhibitory programmed cell death 1 ligand (PD-L1) that binds to the PD-1 co-receptor on T lymphocytes, an immune checkpoint, and supresses cytotoxic activity. Thus, although infiltrating T cells are abundantly detected in the BRAF/MSI subtype of tumours, their activity is downmodulated by tumour cells, suggesting the malignant progression involved increased expression of PD-L1 as an escape mechanism (Rosenbaum et al. 2016). These tumour cells express PD-L1 in response to the inflammatory microenvironment as an adaptive immune resistance. Indeed, blocking the interaction between PD1 and PD-L1 by the therapeutic use of antibodies can reactivate cytotoxic T lymphocytes to attack cancer cells. Moreover, this response has been specifically observed for the BRAF/MSI subtype, since patients with microsatellite-stable tumours did not respond to the therapy (Llosa et al. 2015; Le et al. 2015; Toh et al. 2016). Altogether, these data suggest that the better prognosis of MSI CRC is partly due to immune surveillance. In addition, the above mentioned mutational inactivation of the TGFBR2 gene due to MMR deficiency renders these tumour cells less prone to the process of epithelial-mesenchymal transition, a key event involved in metastasis (Pino et al. 2010).

In 2017, anti–PD-1 therapy has been approved under the trade name pembrolizumab for the treatment of patients with metastatic late stage MSI CRC, a promising target group since cytotoxic lymphocytes are already part of the tumour environment but require activation. Other immune checkpoint blockers are in clinical trials such as nivolumab (anti-PD-1) (Sarshekeh et al. 2018), atezolizumab (Tapia Rico and Price 2018), durvalumab (Levy et al. 2016), and avelumab (Wang et al. 2017) (all anti-PD-L1), and ipilimumab (anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)) (Passardi et al. 2017; Overman et al. 2018). CTLA4 is expressed by cytotoxic T cells and immunosuppressive T regulatory (T-reg) cells, transmits inhibitory signals that downmodulate the cytotoxic T cell response, and can thus regulate the physiological balance between immune reaction and immune tolerance. The blockade of CTLA4 with therapeutic antibodies led to increased proliferation of T cells, increased production of interleukin-2 and depletion of inhibitory T-reg cells. Other potential tar-

get molecules for the development of therapeutic antibodies have also been identified including lymphocyte activation gene 3 (LAG-3) and T cell immunoglobulin mucin 3 (TIM-3), and members of the costimulatory tumour necrosis factor receptor family (OX40, GITR, 4-1BB, CD40, CD70) (Hahn et al. 2017).

It should be noted that other tumour types acquired resistance to immune checkpoint therapy, mostly through clonal selection of tumour cells with mutations in genes required for processing or cell surface presentation of antigens, as well as metabolic changes affecting T-cell activity (Syn et al. 2017).

2.6 Conclusions

Figure 2.1 illustrates the discussed therapeutic targets in the BRAF/MSI subtype of CRC. From the data presented above, it is reasonable to speculate that successful therapies against metastatic

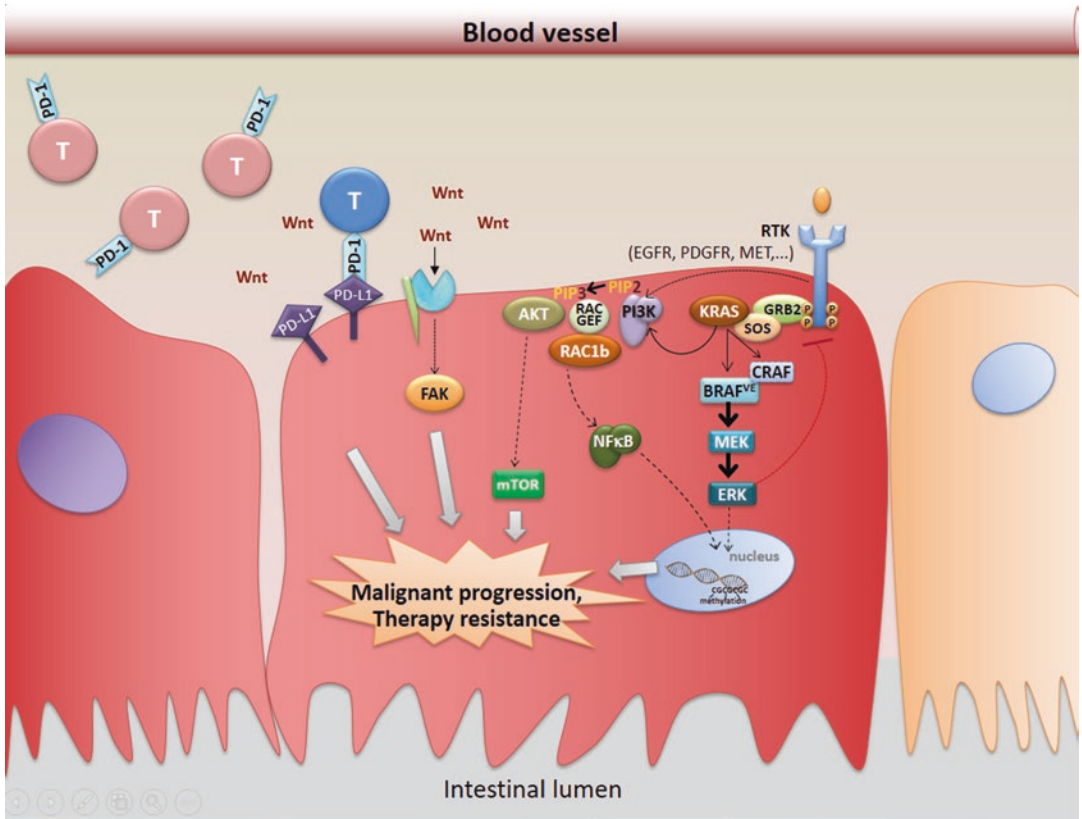

Fig. 2.1 Illustration of pathways relevant for therapeutic targeting of the BRAF/MSI subtype of CRC**.** The MAPK pathway drives proliferation and is of central importance for this tumour type. Direct BRAF inhibition was ineffective in CRC and needs to be combined with other targets from this pathway in order to prevent its reactivation through complex feed-back regulation loops. Examples include the downstream kinase MEK and upstream receptor tyrosine kinases (RTK), such as EGFR, PDGFR or MET. Additional relevant pathways that cooperate with the MAPK pathway include as therapeutic targets the PI3K and Wnt pathways, as well as the small GTPase RAC1b. The latter appears to be specifically downregulated by COX-independent NSAID activities, which could be explored to improve outcome of patients with this CRC subtype. Besides these cancer cell-centred targets, the BRAF/MSI subtype is characterized by high infiltration of cytotoxic T lymphocytes that can be activated by immunotherapy strategies such as humanized antibodies that bind to and block the receptors PD-1 or CTLA4. Solid connecting lines represent direct protein interactions whereas dashed lines indicate indirect action or unknown mechanisms

CRCs with the BRAF V600E mutation will involve the targeting of multiple pathways simultaneously (horizontal inhibition), by combining selective inhibitors or engineering multi-target agents. However, unforeseen adverse cross-interactions, as discussed above, as well as dose-limiting toxicities can bring a significant challenge to the establishment of horizontal inhibition therapeutic strategies (Tolcher et al. 2018; Bahrami et al. 2018). Therefore, prior and/or concurrent use of immune checkpoint approaches at earlier stages of CRC detection may provide a valuable strategy to decrease tumour burden and therapy-driven clonal selection, as they do not target the specific genetic changes within particular cancer cells but rather the tumour microenvironment.

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Targeting KRAS Mutant CMS3 Subtype by Metabolic Inhibitors

3

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Abstract

Cancer cells rewire their metabolism in order to boost growth, survival, proliferation, and chemoresistance. The common event of this aberrant metabolism is the increased glucose uptake and fermentation of glucose to lactate. This phenomenon is observed even in the presence of O_2 and completely functioning mitochondria. This is known as the "Warburg Effect" and it is a hallmark in cancer. Up to 40% of all CRC's are known to have a mutated (abnormal) KRAS gene, found at differing frequencies in all consensus molecular subtypes (CMS). CMS3 colon cancer molecular subtype contains the so-called 'metabolic tumours' which represents 13% of total CR cases. These tumours display remarkable metabolic deregulation, often showing KRAS mutations (68%). Unfortunately, patients harbouring mutated KRAS are unlikely to benefit from anti-EGFR therapies. Moreover, it remains unclear that patients with KRAS wild-type CRC will definitely respond to such therapies. Although some clinically designed-strategies to modulate KRAS aberrant activation have been designed, all attempts to target KRAS have failed in the clinical assays and KRAS has been assumed to be invulnerable to chemotherapeutic attack. Quest for metabolic inhibitors with anti-tumour activity may constitute a novel and hopeful approach in order to handle KRAS dependent chemoresistance in colon cancer.

Keywords

KRAS · Cancer · Chemoresistance · Metabolism · CMS3

3.1 Introduction

According to GLOBOCAN (2012), the estimated number of cancer cases worldwide in 2008 was 12.7 million, with 7.6 million deaths. By 2030, there will an estimated 22.2 million newly diagnosed cancer cases and 12 million deaths (Ferlay et al. 2015).

Colorectal cancer (CRC) is a frequently lethal disease showing diverse outcomes and chemotherapy responses. Currently, CRC is the third leading site of cancer in men and women and is the second leading cause of cancer-related deaths.

This number will increase largely by growth and aging of populations and will be largest in low- and medium-resource countries.

The global distribution of neoplasia and types of cancer with higher penetrance continues to change, especially in economically developing

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countries. Lower income countries accounted for about half (51%) of all cancers worldwide in 1975 but this proportion increased to 55% in 2007 and it is projected to reach 61% by 2050 (Bray and Møller 2006).

Nowadays, oncology tries to personalize anticancer therapy on the basis of tumour genotypes in order to provide enhanced prognostic and treatment planning.

In 2015, the molecular classification carried out by Guinney et al. established four different CRC subtypes with specific molecular features: CMS1 (MSI Immune, 14%), hypermutated, microsatellite unstable, remarkable immune activation; CMS2 (Canonical, 37%), epithelial, chromosomally unstable, marked WNT and MYC signalling activation; CMS3 (Metabolic, 13%), epithelial, discernible metabolic dysregulation; and CMS4 (Mesenchymal, 23%), prominent transforming growth factor β activation, stromal invasion, and angiogenesis.

Despite the frequency of *KRAS* mutations in cancer patients, data outcome is confusing regarding the impact these mutations have on treatment response and patient outcomes.

Although the development of molecular-targeted therapy has supposed a clear benefit on the survival of patients with metastatic CRC, the majority of patients with stage IV CRC undergoing complete resection die from metastasis (Karapetis et al. 2008; Hurwitz et al. 2004).

CRC tumorigenesis is characterized by the accumulation of sequential genetic and epigenetic alterations, and V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) mutations are an early step in tumorigenesis (Vogelstein et al. 1988; Aguilera et al. 2006).

RAS genes are among the most frequently mutated genes in human cancer. Scientific evidence indicates that mutations activating RAS family members, KRAS, HRAS, and NRAS, are found in 20–30% of all human tumours (Cox et al. 2014; Prior et al. 2012). Although *KRAS* mutations are present in every molecular subtype defined by Guinney et al. in 2015, they are more prevalent in CMS3 CRC (68%). Interestingly, CMS3 appeared to be the most similar to normal colon tissue at the gene expression level.

Recently, it has been suggested that the precursor lesion to *KRAS* mutant CRC (the majority of CMS3 cancers) are tubovillous adenomas with serrated traits.

Cetuximab and panitumumab are monoclonal antibodies directed to the exodomain of the epidermal growth factor receptor (EGFR), which in turn blocks downstream signalling, including the RAS/RAF/MEK/ERK pathway. Cetuximab and panitumumab have been proven to be effective in patients with metastatic colorectal cancer (mCRC) when administered alone or in combination with standard chemotherapy (Jonker et al. 2007; van Cutsem et al. 2009).

Cetuximab, is commonly used in the clinical practice to treat metastatic colon, and head and neck cancer. The U.S. Food and Drug Administration (FDA) approved Panitumumab (INN), formerly ABX-EGF, in 2006 for "the treatment of EGFR-expressing metastatic colorectal cancer with disease progression". However, benefits of such therapies in the survival outcome of patients harbouring KRAS mutant CRC are dramatically decreased.

Here we aim to describe different molecular approaches focused on overcoming the molecular barriers posed by *KRAS* mutation, often shown in metabolic CMS3 tumours, targeting key players involved in the metabolic reprogramming in colorectal tumours.

3.2 Reprogramming Cell Metabolism in Cancer: The "Warburg Effect"

After almost a century ago since the Warburg effect was described in 1924, this atypical metabolism is considered a hallmark in every type of cancer, exhibiting higher glycolysis and lactate metabolism and defective mitochondrial ATP production.

Although cancer is a heterogeneous malady, often considered as different diseases converging in the abnormal cell growth, cancer cells share the very same metabolic trait: abnormal rates of glucose conversion to lactate even in the presence of O_2 (Warburg 1956).

In general, cancer cells metabolize glucose, lactate, hydroxybutyrate, pyruvate, lactate, fatty acids and glutamine at much higher rates than normal ones. In spite of the higher glycolytic rates often shown by cancer cells, ATP rendering is substantially lower than normal cells. Unlike, molecular biosynthesis requirements of tumour cells are markedly higher (Fig. 3.1a, b).

However, the metabolic homeostasis of tumours is complex because they contain multiple niches, which are linked by the transfer of these catabolites. This metabolic plasticity enables cancer cells to produce ATP, while keeping the reduction–oxidation (redox) balance and committing resources to biosynthesis processes that are essential for cell survival, growth, and proliferation (Martinez-Outschoorn et al. 2017).

This metabolic feature, known as the "Warburg effect", represents in fact the basis for the 18F-fluorodeoxyglucose positron emission tomography (FDG-PET), a non- invasive imaging technique that, providing an accurate assessment of tumour glucose utilization, is widely exploited in the clinic for initial diagnosis, measuring tumour size, staging, and monitoring tumour responses to therapies. The Warburg effect allows promoting the deviation of glycolytic metabolites into multiple correlated pathways that provide substrates for the biosynthesis of macromolecules (lipids, nucleic acids, and

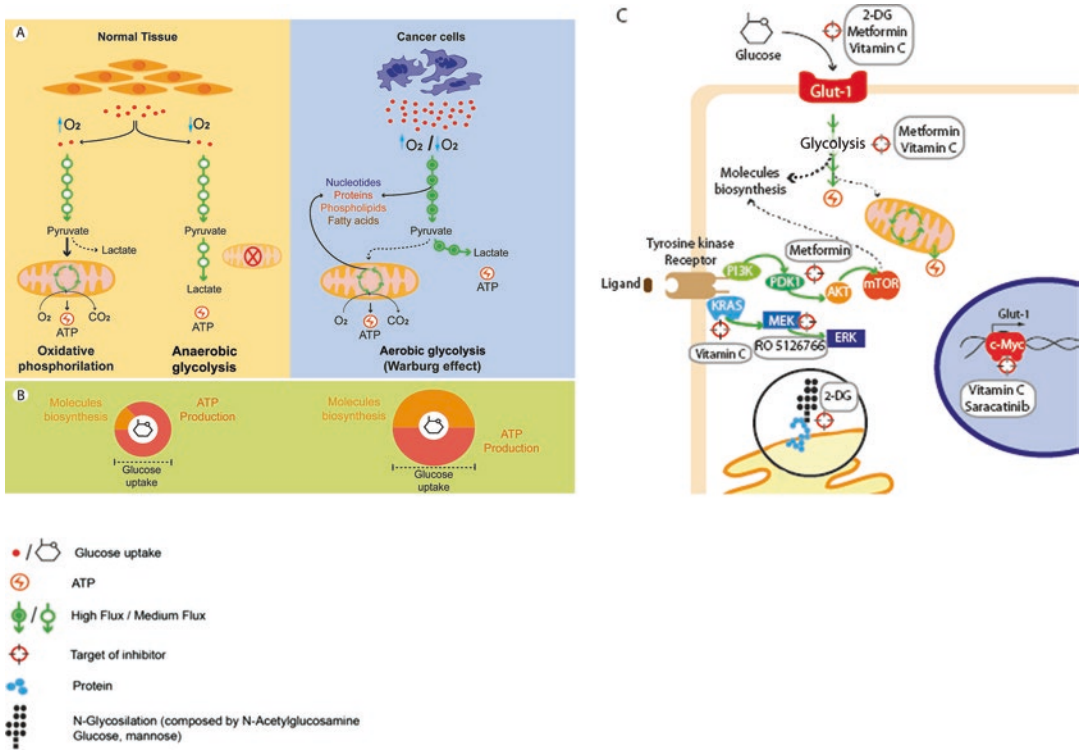

Fig. 3.1 Targeting metabolism in colon cancer**.** (**a**) In *normal tissues*, cell may obtain energy through Oxidative Phosphorylation (OxPhos) that generates 36 ATP. In poor oxygen conditions, normal tissues may obtain energy via anaerobic glycolysis, which gives 2 ATP. For the same glucose, normal cells will get 18 times more energy using oxygen in the mitochondrion compared to anaerobic glycolysis. Normal tissues only use this less efficient pathway in the absence of oxygen. In *cancer cells* the situation changes dramatically. Even in the presence of oxygen, cancer cells use a less efficient method of energy generation (glycolysis, not phosphorylation) delivering only 4 ATP per molecule of glucose. (**b**) Unlike cancer cells, in normal cells the main goal for glucose uptake is producing enough ATP to keep cell metabolic homeostasis. (**c**) Described molecules with antitumor activity targeting metabolism in colon cancer

proteins) required for rapid tumour growth and proliferation.

Furthermore, an augmented flux of cytosolic NADPH provided from the cytosolic oxidative pentose phosphate pathway (PPP) is observed that will balance the intracellular redox potential and neutralize the excessive levels of reactive oxygen species (ROS) resulting from the enhanced metabolic activity of cancer cells.

Warburg hypothesized that cancer was caused by defects in mitochondrial oxidative phosphorylation and then forcing the cell to switch into glycolysis, thus cells would become undifferentiated and cancerous. However, studies carried out by Prof. Craig B. Thompson's laboratory at the Memorial Sloan-Kettering Cancer Center, preferentially indicates that the Warburg effect is not just a passive response to damaged mitochondria but results from oncogene-directed metabolic reprogramming required to support glycolytic metabolism and anabolic growth (Ward and Thompson 2012). The question about altered metabolism as primary cause or just consequence of genomic alteration in cancer still remains open.

Nevertheless, alterations in oncogenic signal transduction pathways and loss of tumour suppressor genes, then affecting the regulation of enzymes and transporters, are tightly correlated to aerobic glycolysis in cancer. Over-expression of the facilitative glucose transporter 1 (GLUT-1) promotes an increased glucose uptake in a variety of cancer cell types, and has been associated with the loss of the tumour suppressor PTEN (Morani et al. 2014) or constitutive activation of oncogenes such as KRAS, AKT, SRC, and Myc (Dang 2012).

HIF-1 and Myc positively regulate the expression of pyruvate kinase M2 (PKM2), the final rate-limiting enzyme of glycolysis, which catalyses the conversion of phosphoenolpyruvate to pyruvate.

Aberrant activity of some signalling pathways such as the PI3K/AKT/mTORC1 pathway has been reported to enhance the expression of glycolytic enzyme Hexokinase II (HKII) that mediates the phosphorylation of glucose upon its entrance into the cell (Wang et al. 2014).

HIF-1 and c-Myc are also considered master regulators of the metabolic reprogramming in neoplasia. Both proteins have been shown to cooperatively induce the expression of both, HKII and pyruvate dehydrogenase kinase 1 (PDHK1). PDHK1 in turn inactivates the pyruvate dehydrogenase (PDH) and so the pyruvate dehydrogenase enzyme complex (PDC) involved in the conversion of pyruvate to acetyl-coenzyme A, therefore inhibiting pyruvate entrance in the tricarboxylic acid (TCA) cycle and then limiting mitochondrial OXPHOS.

Moreover, both HIF-1 and c-Myc enhance the transcription of lactate dehydrogenase A (LDHA), which catalyses the transformation of pyruvate into lactate, further then boosting the glycolytic cancer phenotype in Myc over-expressing cancers (Dang et al. 2008).

Over-expression of PKM2 has been found in multiple human cancers, including colon cancer, and it is considered a master regulator of the metabolic rewiring (Aguilera et al. 2016).

PKM2 may form a complex tetramer, with high catalytic activity, or can exist as a dimer, less active form. In cancer cells, different post-translational modifications, such as fibroblast growth factor receptor type 1 (FGFR1)-mediated phosphorylation at Tyr105, enhance the tetramer-to-dimer switch, therefore inhibiting pyruvate kinase activity and promoting the diversion of glycolytic intermediates towards collateral pathways, such as PPP and serine biosynthesis (Dayton et al. 2016).

3.3 Mutant *KRAS* in Colon Cancer

Genetic alterations in any one of the 3 isoforms of the RAS family (HRAS, NRAS, or KRAS genes) are very frequent events on neoplasic transformation. The Sanger Centre keeps and periodically updates an exhaustive database involving the nature and frequency of *RAS* mutations in different human tumours (catalogue of somatic mutations in cancer: http://sanger.ac.uk/cosmic).

In fact, activating *KRAS* mutations are found in human epithelial neoplasia with an overall fre-

quency of 30%. Studies of the extensive panels of human tumour tissue samples analysed during the last three decades have shown that there is a prevalent correlation of specific mutated RAS isoforms with particular types of tumours (Shimizu et al. 2007).

Some reports describing KRAS overexpression in a colon carcinoma did not find a positive correlation between KRAS overexpression and prognosis, pointing out that RAS overexpression should not be used as a predictive factor (Akkiprik et al. 2008).

KRAS mutations are detected in 40–45% of all colorectal carcinoma (CRC) suggesting that KRAS proteins must play a pivotal role in tumour development (Vaughn et al. 2011). Most *KRAS* mutations are located in codons 12 and 13 (80% and 20%, respectively), being G12D the most often amino acid modification. On the other hand, much lower activating mutation percentages have been found in NRAS (1–3%) (Irahara et al. 2010). *KRAS* mutation is an early event in CRC. Although still arguable, it has been proposed that in some CRCs, *KRAS* mutations may occur as early events in formation of aberrant crypt foci that could later trigger the development of hyperplasic polyps and eventually to CRC (Yuen et al. 2002; Nash et al. 2010). However, contrary to pancreatic carcinomas where *KRAS* mutations are prevalent, many other genetic and epigenetic alterations besides *KRAS* mutations may occur in CRC that could be responsible for tumour initiation and progression in this case like APC, beta-catenin mutations and promoter silencing of genes involved in controlling the WNT signalling pathway.

As it has been reported on pancreatic ductal adenocarcinoma (PDAC), there is a tight correlation between *KRAS* mutations and poor prognosis of aggressive and invasive colorectal carcinomas (Conlin et al. 2005; Zavodna et al. 2009). Interestingly, some clinical studies have reported that the rate of *KRAS* mutation is higher in CRC patients with lung metastasis and that the presence of the mutation in CRC patients with liver metastasis correlates with poor prognosis (Cejas et al. 2009; Nash et al. 2010).

3.4 Metabolic Switch in CRC: KRAS Connection

As it has been widely described, several clinical trials have shown that *KRAS* mutations in cancer may predict a lack of responses to the anti-epidermal growth factor receptor (EGFR)–based therapy. So, anti-EGFR therapies using cetuximab and/or panitumumab are now limited to patients with *KRAS* wild-type CRC (Jonker et al. 2007; Karapetis et al. 2008; Ye et al. 2013).

Interestingly, mutant KRAS is closely involved in the upregulation of the cell Warburg metabolism. Mutated KRAS maintains tumour growth by boosting glucose uptake and transporting glucose intermediates into the pentose phosphate pathway (PPP) and hexosamine biosynthesis pathway (HBP). Nowadays, the interest to understand the reprogramming of metabolism in neoplasic transformation is increasing. Although the molecular mechanism behind the upregulation of glucose metabolism is not yet understood, the pivotal role played by KRAS signalling in the homeostasis of aerobic glycolysis has been reported in several types of cancer. For example, in a PDAC murine model, it has been demonstrated that mutated KRAS keeps tumour growth by stimulating glucose uptake and leading glucose intermediates into the hexosamine biosynthesis pathway (HBP) and pentose phosphate pathway (PPP) (Ying et al. 2012). Remarkably, knockdown of rate-limiting enzymes in HBP or PPP halted tumour growth, indicating their potential as therapeutic targets.

In human colorectal cancer, the increase of glucose transporter 1 (GLUT1) expression and glucose uptake is critically dependent on *KRAS* mutational state (Yun et al. 2009). In fact, PET (fluorodeoxyglucose (FDG) positron emission tomography scan) is used to evaluate tumour size and location by analysing glucose metabolism by measuring the uptake of FDG, a glucose analogue and it has been described that CRC cells with mutated *KRAS* show an increased FDG accumulation via of GLUT1 upregulation (Kawada et al. 2012; Iwamoto et al. 2014).

On the other hand, the tight relationship between GLUT-1 and KRAS is confirmed by

studies reporting that glucose deprivation contributes to the development of mutated KRAS pathways in tumour cells (Yun et al. 2009).

Actually, GLUT1 has been addressed as a potential target in oncology drug discovery since in 2014 a crystal structure of human GLUT-1 was obtained. This achievement will surely be helpful in the discovery of new GLUT1 inhibitors as anti-cancer agents (Flight 2011). First, GLUT-1 antibodies were shown to inhibit breast cancer and lung cancer (NSCLC) cell lines growth (Rastogi et al. 2007). Interestingly, in 2010 a series of synthetic polyphenolic esters were also described to inhibit glucose transport through the cell membrane, and to exert a certain anti-proliferative activity in the H1299 lung cancer cell line. Moreover, these molecules were used in combination with anti-cancer drugs cisplatin or paclitaxel demonstrating synergistic effects in the inhibition of breast and lung cancer cell growth (Granchi et al. 2016).

Likewise, the small-molecule N-[4-chloro-3-(trifluoromethyl)phenyl]-3-oxobutanamide (fasentin) was identified as a chemical sensitizer to the death receptor stimuli FAS and tumour necrosis factor (TNF) apoptosis-inducing ligand. Fasentin interacts with a unique GLUT-1 site in the intracellular channel of this protein, thus inhibiting glucose transport (Wood et al. 2008)

GLUT-1 also plays an essential role for the homeostasis of pancreatic, ovarian, and glioblastoma cancer stem cells (CSCs). WZB117, a specific GLUT1 inhibitor, was shown to inhibit the self-renewal and tumour-initiating capacity of the CSCs without compromising their proliferative potential in vitro. *In vivo*, systemic WZB117 administration was able to inhibit tumour initiation after implantation of CSCs without causing significant adverse events in host animals (Shibuya et al. 2015). Recently, WZB117 was shown to kill lung and breast cancer cells by inhibiting GLUT1-mediated glucose transport, leaving non-tumorigenic cells unaffected (Shibuya et al. 2015)

In addition to their glucose dependency, tumour growth and survival also relies on glutamine uptake. Glutamine is a fundamental carbon source for the tricarboxylic acid (TCA) cycle and a nitrogen source for nucleotides and nonessential amino acids. Glutamine is also involved in other cellular processes in cancer cells, such as (mTOR) signalling and including anti-oxidative stress. Therefore, glutamine dependent pathways and signalling involved in cancer cell survival, progression and metastasis is a hot topic in cancer research (Deberardinis and Cheng 2010; Wise and Thompson 2010).

As it has been reviewed for glucose transport, glutamine metabolism exhibits pleiotropic effects on cancer cell signalling and therapeutic suppression of glutamine metabolism is considered to be an attractive anti-cancer strategy.

For instance, Benzylserine and L-γ-glutamyl-p-nitroanilide (GPNA), the inhibitor of the glutamine transporter SLC1A5, have been shown to be effective agents in the treatment of non-small cell lung cancer cell lines and murine xenografts (Hassanein et al. 2015). However, these drugs have been shown to induce unselective toxicity in normal, healthy cells that require glutamine for other pathways.

Some other small inhibitors, such as, CB-839 and bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl) ethyl sulfide (BPTE) have been reported to specifically target glutaminase (GLS) isoforms not often expressed in normal cells (Xiang et al. 2015)

In view of the abovementioned factors supporting the tight interplay between mutated KRAS and the Warburg metabolism in cancer and the partial success of molecules directly targeting KRAS, quest for new scopes and molecules capable to overcome chemotherapy resistance in tumours displaying gene mutations downstream EGFR should be considered as a top priority in oncological research worldwide.

3.5 Uncoupling the "Warburg Effect" in CRC: A Chink in the Armour of KRAS Dependent Chemoresistance

As it has been widely explained, CRC is the second leading cause of cancer-related deaths and trials using apoptosis-inducing ligand monotherapy to overcome resistance to apoptosis in colon cancer have not shown clinical benefits.

There is a need for a novel focus to overcome clinical resistance to chemotherapies, mainly due to RAS/RAF mutations.

It has been also explained that the enhanced metabolic requirements of colon cancer cells necessarily involves increased glucose uptake and glycolytic flux relative to normal tissues. This feature is used to visualize colon cancer cells using positron emission tomography (PET) where signals emitted from 2-deoxy-2-fluoro-D-glucose (FDG), which is taken up preferentially by colon cancer cells, are monitored.

Currently, some promising molecules are being investigated to bypass KRAS dependent chemoresistance, using a metabolic approach (Fig. 3.1c).

In 2016, Carr et al., published an interesting article where they overcome colon cancer cells resistance to TRAIL (tumour necrosis factor-related apoptosis-inducing ligand) using 2-deoxy-D-glucose (2DG), which is molecularly similar to FDG and is preferentially uptaken by cancer cells. In tumour cells, 2DG metabolism may affect death receptor (DR) expression (TRAIL is a DR ligand) and dissociate the Bak-Mcl-1 complex in cells with high glycolytic activity (Yamaguchi and Perkins 2012). In addition, 2DG is a receptor-competitive inhibitor of glucose, increasing oxidative stress, inhibiting N-linked glycosylation, and hence inducing autophagy. It inhibits cell growth and facilitates selective apoptosis in cancer cells.

The Ras/Raf/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated (ERK) cascade is involved the signal transduction from the cell surface receptors to the nucleus and regulates cell cycle, cell proliferation, cell differentiation and survival. Genetic and epigenetic alterations in many of the key players in this pathway have been found to be associated with cancer.

The dual **RAF/MEK kinase Inhibitor RO5126766,** synthesized in Chugai Pharmaceuticals Co., Ltd. was shown to decrease FDG uptake in KRAS and BRAF mutant colon cancer murine xenografts (Tegnebratt et al. 2013). This metabolic inhibition correlates with a decreased cell membrane expression of the glucose receptor GLUT-1 and it was tightly associated with a notably reduced expression of the marker of proliferation Ki67.

In the 70–80th of the twentieth century, several scientific reports demonstrated that the anti-diabetic biguanide drugs phenformin (PF) and buformin (BF) can exert some anti-tumour activity in animal models and increase from 5 to 10-years survival of cancer patients.

Metformin (1,1-dimethylbiguanide hydrochloride) is often used to reduce hepatic gluconeogenesis and increase skeletal muscle glucose uptake in patients with type 2 diabetes. Metformin has been proposed as adjuvant therapy in cancer treatment because of its ability to limit cancer incidence by negatively modulating the PI3K/AKT/mTOR pathway.

In 2015, Jia et al. reported that Metformin might prevent induced colorectal cancer in diabetic rats by reversing the Warburg effect. They described that Warburg inhibition was mediated through inhibition of the master regulator PKM2. In fact, PKM2 metformin-induced inhibition has been also reported in other neoplasia. For instance, Metformin Induces apoptosis, down-regulating PKM2 in breast cancer cells grown in poor nutrient conditions (Silvestri et al. 2015).

c-Src, is found to be over-expressed and activated in a wide variety of human tumours. The relationship between Src activation and cancer progression appears to be significant, regulating cancer glucose metabolism in premalignant estrogen receptor (ER)-negative mammary epithelial cells.

In this model, **Saracatinib**, a highly selective, dual Src/Abl kinase inhibitor, was shown to blocks c-Myc translation and glucose metabolism a result of an inhibition in ERK1/2-MNK1-eIF4E-mediated cap-dependent translation of c-Myc and transcription of the glucose transporter GLUT1, therefore interfering with the Warbug effect.

Saracatinib has been used in a phase II trial treating patients with previously treated metastatic colon cancer or rectal cancer. Results from this study suggested that may stop the growth of tumour cells by blocking blood flow to the tumour and by blocking some of the enzymes needed for cell growth (ClinicalTrials.gov Identifier: NCT00397878).

3.5.1 Vitamin C: Reassessing the Role of Vitamin C in Metabolic Enhanced Colon Cancer

In 1976, Linus Pauling and Ewan Cameron performed a pioneering clinical study of the survival times of 100 terminal cancer patients who were given supplemental ascorbate (usually 10 g/day) plus adjuvant chemotherapy and 1000 matched controls, similar patients who had received the same treatment except for the ascorbate. Survival times greater than 1 year after the date of untreatability were observed for 22% of the ascorbate-treated patients and for 0.4% of the controls (Cameron and Pauling 1976).

Many authors have reported that vitamin C shows certain anti-tumour activity, but in spite of the several efforts carried out in order to unravel the molecular mechanism underlying this killing effect and its intriguing selective activity, this is still far from clear.

Scientific data published by Chen Q et al., supporting the previous clinical study carried out by Pauling and Cameron, have shown that vitamin C exerts killing effects on cancer cells from very different origin, displaying a wide variety of gene mutations (many of them do not display KRAS mutation) and alterations in different signalling pathways (Chen et al. 2008).

In 2015 in an article published in Science, Yun J et al., presented data stating that oxidized vitamin C was able to kill CRC cells depending on the KRAS mutational status (Yun et al. 2015). They found that cultured human CRC cells harbouring KRAS or BRAF mutations were selectively killed when exposed to high levels of vitamin C. This effect was due to increased uptake of the oxidized form of vitamin C, dehydroascorbate (DHA), via the GLUT1 glucose transporter. Increased DHA uptake caused oxidative stress when intracellular DHA is reduced to vitamin C, depleting glutathione. Thus, ROS accumulate and inactivate glyceraldehyde 3-phosphate dehydrogenase (GAPDH), an enzyme of ~37 kDa that catalyses the sixth step of glycolysis and thus serves to break down glucose for energy and carbon molecules. Inhibition of GAPDH in highly glycolytic KRAS or BRAF mutant cells leads to an energetic catastrophe and cell death not seen in normal cells.

Later, in 2016 it was published another scientific work partially confirming previous observations of Yun et al., but mainly focused on the putative interaction of vitamin C in the Warburg metabolism that is considered, as stated previously, a hallmark in cancer and glycolytic enzymes reported as central points of regulation in cancer. In this work we described a novel antitumour mechanism of vitamin C in KRAS mutant colorectal cancer involving the Warburg metabolic disruption through downregulation of key metabolic checkpoints in KRAS mutant cancer cells and tumours without killing human immortalized colonocytes (Aguilera et al. 2016). Vitamin C is capable to induce RAS detachment from the cell membrane via ROS inhibition. Thus, RAS detachment leads inhibition ERK 1/2 and PKM2 phosphorylation. As a consequence of this activity, we could observe strong downregulation of the glucose transporter (GLUT-1) and pyruvate kinase M2 (PKM2)-PTB dependent protein expression causing a major blockage of the Warburg effect and therefore energetic stress.

Tumour-specific pyruvate kinase M2 (PKM2) is a master regulator for the Warburg effect and In addition to its well-established role in aerobic glycolysis, PKM2 directly regulates gene transcription [90].

Interestingly, in 2014 Tian et al., published that highly glycolytic cells triggered by activation of the hypoxia-inducible factor (HIF) pathway greatly enhanced vitamin C-induced toxicity in multiple cancer cell lines, including von Hippel-Lindau (VHL)-defective renal cancer cells. According to their observations, HIF increases the intracellular uptake of DHA through its transcriptional target glucose transporter 1 (GLUT1), synergizing with the uptake of its reduced form through sodium-dependent vitamin C transporters (Tian et al. 2014).

The majority of these results point to the Warburg metabolism and related overexpressed enzymes in cancer as a promising scientific field that requires further in-depth studies in order to find new therapeutic targets. Encouraging results

observed in vitamin C research, such as its ability to overcome anti-EGFR resistance and displayed selectivity, emphasizes the need for further research on this topic and may open the door to a novel generation of molecules that may constitute a new hope in handling RAS dependent chemoresistant cancer.

3.6 Conclusions

KRAS dependent chemoresistance is a major threat to the clinical handling of CRC and other neoplasia. Among all the CRC subtypes, the CMS3 also called "metabolic subtype", shows frequent KRAS mutation and concomitant metabolic alterations. Many authors have shown compelling data highlighting the role of KRAS signalling in the regulation of aerobic glycolysis in several types of cancer.

KRAS harbouring cancers have altered metabolism involving enhanced nutrients uptake glycolysis, glutaminolysis, and elevated synthesis of nucleotides and fatty acids.

Unfortunately, clinical trials with molecules targeting KRAS did not render clear benefits to the overall survival (OS) and progression-free survival of metastatic colorectal cancer patients and, to date, no effective treatments that target mutant variants of KRAS have been introduced into clinical practice. It is time to propose different approaches to break down the KRAS barrier to chemotherapy. Counteracting KRAS-ruled metabolic pathways may be a promising focus in order to sensitize KRAS mutant tumours to chemotherapy and many studies are already centred in this approach.

But the problem seems to be far more complex.

Glucose requirements and carbon sources in tumours are much more heterogeneous than initially thought. Currently, new studies have been developed showing a dual capacity of tumour cells for glycolytic and oxidative phosphorylation (OXPHOS) metabolism.

Metabolic OXPHOS-dependent cancer cells are capable to use alternative substrates, such as glutamine and/or fatty acids. Therefore, the variety of carbon substrates able to fuel neoplastic cells points out to a high metabolic heterogeneity, even within tumours sharing the stage and clinical diagnosis. Indeed, it has been reported that 80% of the ATP generation in MCF7 breast cancer cells relies on mitochondrial respiration (Guppy et al. 2002).

Furthermore, some studies have reported that glycolysis inhibition often restores OXPHOS in cancer cells (Bonnet et al. 2007; Michelakis et al. 2010) demonstrating that in spite of the augmented glycolytic rates often shown by cancer cells, mitochondrial oxidative metabolism remains intact. The overall data strongly point out to a high cancer metabolic plasticity, implying that molecules targeting metabolic factors in cancer may face similar mechanisms of resistance as previously described for conventional chemotherapy.

Combination of metabolic inhibitors and classical chemotherapeutic agents may constitute a great advance to address KRAS-driven chemoresistance, often attributed to the concomitant altered metabolic expression patterns.

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4 Targeting the PI3K Signalling as a Therapeutic Strategy in Colorectal Cancer

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Abstract

Colorectal cancer (CRC) remains one of the leading causes of cancer mortality worldwide. Regarded as a heterogeneous disease, a number of biomarkers have been proposed to help in the stratification of CRC patients and to enable the selection of the best therapy for each patient towards personalized therapy. However, although the molecular mechanisms underlying the development of CRC have been elucidated, the therapeutic strategies available for these patients are still quite limited. Thus, over the last few years, a multitude of novel targets and therapeutic strategies have emerged focusing on deregulated molecules and pathways that are implicated in cell growth and survival. Particularly relevant in CRC are the activating mutations in the oncogene *PIK3CA* that frequently occur in concomitancy with *KRAS* and *BRAF* mutations and that lead to deregulation of the major signalling pathways PI3K and MAPK, downstream of EGFR. This review focus on the importance of the PI3K signalling in CRC development, on the current knowledge of PI3K inhibition as a therapeutic approach in CRC and on the implications PI3K signalling molecules may have as potential biomarkers and as new targets for directed therapies in CRC patients.

Keywords

Colorectal cancer · PI3K signalling pathway · PI3K p110α · Targeted therapies · KRAS

4.1 Introduction

Colorectal cancer (CRC) is one of the most common cancer types, and despite intensive research, remains one of the leading causes of cancer mortality worldwide (Torre et al. 2015). It results from the accumulation of genetic and epigenetic alterations in oncogenes and tumour suppressor

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genes, leading to the transformation of the normal epithelia towards invasive carcinoma (Markowitz and Bertagnolli 2009). Although a large number of molecules have been shown to be altered in these patients, to date, the use of targeted therapies has been limited to anti-epidermal growth factor receptor (EGFR) and anti-vascular endothelial factor (VEGF) agents. Moreover, patients harbouring *KRAS* and *NRAS* mutations are currently excluded from anti-EGFR therapies, as these alterations were shown to cause resistance (Allegra et al. 2016). Therefore, it is urgent to unravel novel therapeutic approaches for CRC patients in an attempt to improve patient outcomes and overcome therapy resistance. While several inhibitors are already being tested in preclinical and clinical trials, the interplay of the signalling pathways has proved to be rather complex and no other agents have yet been approved for these patients. In this review, we focus on the key role of the phosphatidylinositol 3-kinase (PI3K) signalling pathway in CRC development, the latest developments in the field of PI3K targeted specific agents as well as on the implications of PI3K inhibition as an alternative therapeutic approach for CRC patients.

4.2 Molecular Aspects of CRC Progression

Colorectal carcinogenesis is characterized by the gradual accumulation of alterations, genetic and epigenetic, in specific oncogenes and tumour suppressor genes in a multistep manner (Markowitz and Bertagnolli 2009).

The classical model of colorectal cancer progression is the adenoma-carcinoma sequence, however this pathway oversimplifies the heterogeneity of CRC and is not able to explain the development of all types of CRC (Fearon and Vogelstein 1990; Walther et al. 2009). Due to the distinct molecular, clinical and pathological characteristics observed in CRC tumours, other mechanisms for CRC development have emerged, namely the serrated pathway (O'Brien et al. 2006). In each of these models, a unique progression pathway is associated with a distinct mutational spectra. While alterations in the genes *APC*, *KRAS* and *p53* are classic molecular alterations in the Vogelstein pathway, mutations in *BRAF* are typical of the serrated polyp pathway (Jass 2006; Velho et al. 2010; Vilar and Gruber 2010).

Moreover, these models are often associated with different types of genetic instability. Indeed, according to the type of genetic instability, CRC can be subdivided in different molecular subsets, microsatellite instability (MSI) and microsatellite stability (MSS), the latest characterized by having chromosomal instability (CIN) and observed in the majority of the cases (approximately 85%) (Vilar and Gruber 2010; Cunningham et al. 2010). In contrast, MSI is detected in about 15% of CRC patients and is characterized by a defective mismatch repair system through epigenetic silencing or germline mutations, leading to the accumulation of mutations across the genome mainly in repetitive sequences (microsatellites) (Cunningham et al. 2010). As previously highlighted, MSS and MSI are preferentially observed in the adenoma-carcinoma sequence and the serrated pathway, respectively (Velho et al. 2010; Vilar and Gruber 2010). Notably, while for most patients (about 70%) CRC occurs sporadically (MSI and MSS), in other cases CRC develops in a hereditary context being the most common form the hereditary non-polyposis CRC (HNPCRC) also termed Lynch syndrome (Tops et al. 2009).

Of particular importance in CRC, is the fact that MSI status can be used as a prognostic marker and predictor of therapeutic resistance in CRC patients. More specifically, MSI CRC tumours have been shown to be associated with a better prognosis than MSS tumours (Malesci et al. 2007; Gryfe et al. 2000). In addition, these subsets are known to respond differently to the available therapies and studies indicate that, in contrast to MSS, MSI tumours do not benefit from 5-fluouracil (5-FU) based adjuvant chemotherapies (Ribic et al. 2003).

Overall, to successfully design and develop novel targeted therapies, more studies are needed to clarify the value of specific biomarkers for predictive and prognostic purposes, including MSI

status, as well as to better understand the mechanisms underlying the development of CRC in the different molecular subsets.

4.3 The MAPK and PI3K Signalling Pathways and Their Deregulation in CRC

The mitogen activated protein kinase (MAPK) and PI3K are ubiquitous signalling pathways, downstream of EGFR, implicated in a variety of key biological processes including cell proliferation and survival, cell cycle regulation, differentiation, metabolism and apoptosis, among others (Sebolt-Leopold and Herrera 2004; Liu et al. 2009). Figure 4.1 illustrates, in a simplified manner, the classical MAPK and PI3K signalling pathways and their intervenient molecules.

Overall, these pathways are of major relevance in CRC as activating mutations in genes of these cascades are frequently detected in CRC patients, leading to the constitutive activation of the signalling pathway independently of a stimuli. Indeed, as determined by others and our group, a high frequency of mutations has been observed in *KRAS, BRAF* and *PIK3CA* (the gene coding for PI3K p110α, the catalytic subunit of PI3K) (De Roock et al. 2011; Lievre et al. 2010; Oliveira et al. 2004, 2007; Velho et al. 2005, 2008).

Briefly, as part of the RAS-RAF-MAPK cascade, KRAS is a member of the RAS superfamily of GTPases, along with N-RAS and H-RAS, all belonging to the larger class of regulatory GTP hydrolases (Pylayeva-Gupta et al. 2011). By switching between on and off states, GTP- and GDP-bound respectively, KRAS is important in controlling a complex network of signalling pathways by transducing signals from cell surface receptors namely EGFR to specific intracellular effectors (Sebolt-Leopold and Herrera 2004; Samatar and Poulikakos 2014). Upon stimulation, guanine nucleotide exchange factors (GEFs) promote the activation of RAS by stimulating GDP for GTP exchange; conversely, GTPase-activating proteins (GAPs) accelerate RAS-mediated GTP hydrolysis. In their active state, RAS proteins interact and activate their effectors and stimulate downstream signalling

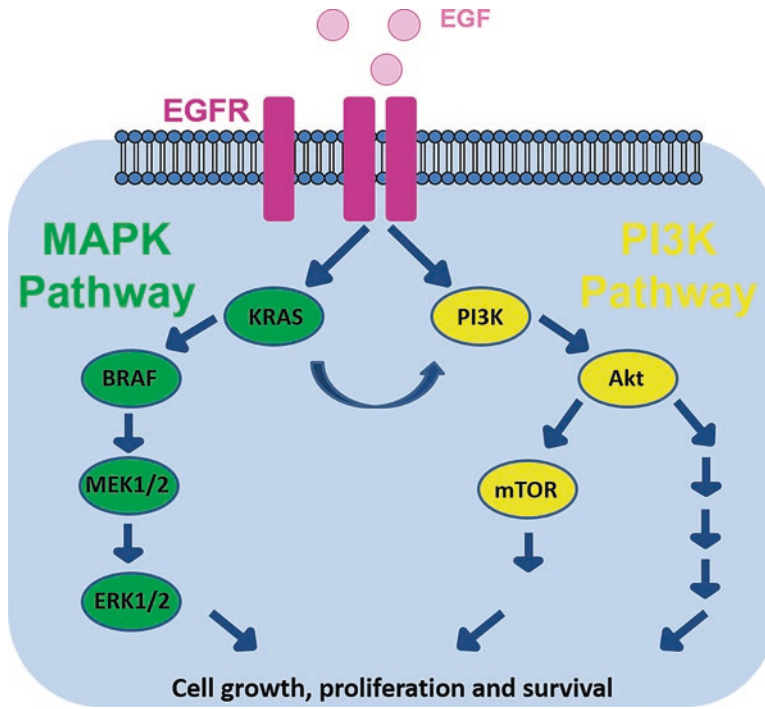

Fig. 4.1 Simplified representation of the MAPK and PI3K signalling pathways. *EGF* epidermal growth factor, *EGFR* epidermal growth factor receptor, *PI3K* phosphatidylinositol 3-kinase, *MAPK* mitogen activated protein kinase, *MEK1/2* MAPK kinase 1/2, *ERK1/2* extracellular signal-regulated kinase 1/2, *mTOR* mammalian target of rapamycin

pathways (Bos et al. 2007). More specifically, the classical RAS signal transduction pathway comprises sequential phosphorylations of the serine/threonine kinase RAF, MAPK kinase 1/2 (MEK1/2) and extracellular signal-regulated kinase 1/2 (ERK1/2), ultimately modulating other molecules and regulating the distinct biological functions (Sebolt-Leopold and Herrera 2004; Samatar and Poulikakos 2014). Importantly, RAS is also known to activate other molecules and signalling cascades namely the PI3K signalling pathway, with major implications in targeted therapies (Liu et al. 2009; Fernandes et al. 2013; Murillo et al. 2014; Gupta et al. 2007).

The B-RAF serine/threonine kinase, which belongs to the RAF kinase family of protein kinases together with A-RAF and C-RAF, is one of the best characterized RAS effectors. RAF phosphorylates MEK1/2, which in turn phosphorylates and activates ERK1/2 that will modulate downstream effectors (Sebolt-Leopold and Herrera 2004; Samatar and Poulikakos 2014).

On a separate and parallel signalling cascade, PI3Ks are a rather ubiquitous family of lipid kinases activated by receptor tyrosine kinases (RTK) or other molecules as G-proteins (Liu et al. 2009). PI3Ks are able to phosphorylate the 3′-hydroxyl group of phosphatidylinositol and phosphoinositides and these lipid products act as second messengers to trigger a multitude of signalling cascades with impact in key mechanisms as survival, differentiation and metabolism (Liu et al. 2009; Vanhaesebroeck et al. 2012). In terms of classification, PI3Ks are grouped into three classes (IA/IB, II and III), with distinct structures and substrate specificities but class IA have received much attention as they have been implicated in many human cancers. Class IA PI3Ks, able to phosphorylate phosphatidylinositol (4,5)-biphosphate (PIP2), converting it to phosphatidylinositol (3,4,5)-triphosphate (PIP3), are composed of a heterodimer of a p85 regulatory subunit (p85α, p85β, p55γ or splice variants) and a p110 catalytic subunit (p110α, p110β or p110δ) (Liu et al. 2009; Vanhaesebroeck et al. 2012). Notably, the different p110 and p85 isoforms seem to preferentially mediate specific signalling cascades, though with some redundancy as reviewed in (Hennessy et al. 2005). Moreover, while p110α and p110β are ubiquitously expressed, p110δ expression is mostly restricted to the immune system (Engelman et al. 2006; Liu et al. 2009). In CRC, the p110α subunit of PI3K, encoded by the *PIK3CA* gene, is of particular relevance as it is often mutated in these patients (De Roock et al. 2011). The p110 catalytic isoforms share high homology and have common specific domains namely the p85 binding domain (able to interact with the p85 subunit), a RAS binding domain (to mediate activation by RAS family members) and a kinase catalytic domain (to generate PIP3 and activate downstream targets) (Liu et al. 2009; Thorpe et al. 2015). Similarly, the common p85 subunit domains include a p110-binding domain also termed inter-Src homology 2 (iSH2), and SH2, SH3 and BCR homology (BHD) domains (Liu et al. 2009; Thorpe et al. 2015). Mechanistically, activation (dimerization and autophosphorylation) of the RTK, upon stimulation by growth factors, leads to the recruitment of class IA PI3K to the membrane where the regulatory p85 subunit will bind RTK phosphorylated motifs but also relieve the p85 inhibition of p110; the activated p110 is then able to generate PIP3, a second messenger that provides docking sites for specific proteins, i.e., PIP3 binds to specific domains, as the pleckstrin-homology (PH) domain, of downstream targets including Akt (also termed protein kinase B, PKB) and phosphoinositide-dependent kinase (PDK1); PDK1 is then able to phosphorylate Akt at Thr308 important to activate Akt (Liu et al. 2009; Thorpe et al. 2015). Remarkably, the activation of the serine/threonine-specific protein kinase Akt, one important downstream effector of PI3K, leads to phosphorylation and subsequent activation or inhibition of additional downstream molecules that will ultimately regulate other proteins modulating the many functions of the PI3K signalling cascade (Manning and Cantley 2007). Indeed, a panoply of Akt substrates have been identified including glycogen synthase kinase 3β (GSK-3β), forkhead box O (FoxO) transcription factors, mouse double minute 2 homologue (MDM2), Bcl-2 associated death promoter (Bad), tuberous sclerosis complex

2 (TSC2), proline-rich Akt substrate of 40 kDa (PRAS40) among others, that are involved in cell growth, metabolism, insulin signaling and survival (Manning and Cantley 2007; Manning and Toker 2017). In addition, an important downstream effector of Akt is the mechanistic target of rapamycin (mTOR), a serine/threonine protein kinase. Interestingly, the catalytic subunit mTOR can be found in two distinct complexes, named mTOR complex 1 (mTORC1, composed by mTOR, raptor, mLST8, PRAS40 and DEPTOR) and mTOR complex 2 (mTORC2, composed by mTOR, rictor, mLST8, DEPTOR, Sin1 and Protor) and while mTORC1 is activated by Akt and known to modulate protein synthesis by phosphorylating the key mTOR effectors elF4E Binding Protein (4EBP1) and p70S6 Kinase 1 (S6 K1), mTORC2 is able to phosphorylate Akt at Ser473 and activate it (Sarbassov et al. 2005, 2006; Liu et al. 2009; Manning and Toker 2017; Saxton and Sabatini 2017). Importantly, the phosphorylation of Akt at both Thr308 and Ser473 is required for full Akt activation (Manning and Toker 2017). In contrast to the above mentioned activation mechanisms, negative regulation of the PI3K pathway is also mediated by the tumour suppressor gene phosphatase and tensin homolog (*PTEN*) that removes the 3′ phosphate from PIP3 hampering the PI3K signalling (Cully et al. 2006). Noteworthy, in addition to the described regulatory mechanisms, the PI3K-Akt-mTOR signaling is also tightly controlled by a number of feedback loops and cross-talk with other signaling pathways as reviewed in (Manning and Toker 2017; Rozengurt et al. 2014). Indeed, an important negative feedback mediated by mTORC1 is known to inhibit the PI3K pathway through distinct mechanisms. In particular, mTORC1 and its downstream effector S6K are able to inhibit the PI3K/Akt signalling through phosphorylation, inhibition and degradation of the insulin receptor substrate I (IRS-I) (Harrington et al. 2004; Manning and Toker 2017). In addition, RTKs are also targets of negative feedback regulation by activated PI3K-Akt-mTOR (Zhang et al. 2007; Chandarlapaty et al. 2011; Manning and Toker 2017).

As previously mentioned, mutations in genes of the MAPK and PI3K pathways are frequent in CRC. Specifically, about 30–40% of CRC patients harbour a mutation in *KRAS* (mostly affecting codons 12 and 13 of exon 2), whereas mutations in the *KRAS* effector, *BRAF*, are detected in about 15% of CRC patients (typically V600E on exon 15) (De Roock et al. 2011; Velho et al. 2010). Notably, *KRAS* and *BRAF* oncogenes have been suggested to play different roles in the development and progression of CRC, as *KRAS* and *BRAF* mutations are rarely detected in the same tumour and are instead observed as alternative molecular modifications (Rajagopalan et al. 2002; Velho et al. 2010). Moreover, *KRAS* and *BRAF* mutation frequencies and patterns are distinct in MSI, MSS, sporadic and hereditary subsets of CRC, with mutations in *BRAF* mostly found in MSI sporadic CRC and *KRAS* in MSS and MSI sporadic and hereditary CRC (Oliveira et al. 2004, 2007; Velho et al. 2010; Domingo et al. 2004; Lubomierski et al. 2005). In addition to *KRAS* and *BRAF*, *PIK3CA* mutations are observed in approximately 15% of CRC patients and, in contrast, often arise concomitantly with *KRAS* or *BRAF* mutations (De Roock et al. 2011; Velho et al. 2005, 2008). These *PIK3CA* mutations are of the missense type and are mostly in hotspots involving exon 9 that corresponds to the helical domain of PI3K p100α, and exon 20 that corresponds to the kinase domain of PI3K p100α; two common examples are E545K and H1047R (De Roock et al. 2011; Liu et al. 2009). In contrast to these, alterations in other molecules of the PI3K pathway are rare, except for PTEN for which controversial information has been raised in terms of mutations and loss of expression (De Roock et al. 2011; Nassif et al. 2004).

In any case, aberrant activation of these molecules will have a major impact in cell behaviour with effects on proliferation, survival, invasion and therefore in the initiation and progression of CRC (Thorpe et al. 2015). Of particular importance, and essential to better understand the underlying signalling mechanisms, is the increasing evidence in support of RAS-RAF-MAPK and PI3K-Akt cross-talk resulting in a complex signalling network (Castellano and Downward

2011; Thorpe et al. 2015). However, the extent of such cross-talk and the implications for CRC therapy are still not clear.

4.4 Current Targeted Therapies for CRC and Their Limitations

To date, apart from the conventional therapeutic strategies, CRC is limited to two distinct types of targeted therapy. These include anti-angiogenic and anti-EGFR agents (Ciardiello and Tortora 2008; Welch et al. 2010; Weng et al. 2015). In particular, cetuximab and panitumumab, two anti-EGFR antibodies that bind the extracellular domain of EGFR, have received much attention and are currently approved for the treatment of patients with mCRC (Ciardiello and Tortora 2008). Regrettably, only a subgroup of mCRC patients can benefit from such therapies and only a small percentage will be sensitive (Ciardiello and Tortora 2008; Lievre et al. 2017). More specifically, *KRAS* mutations have been recognized as predictive biomarkers of resistance to anti-EGFR agents, though some controversy exist as to the type of *KRAS* mutation (De Roock et al. 2010; Allegra et al. 2016). Nonetheless, CRC patients harbouring somatic *KRAS* mutations are currently not eligible to cetuximab and panitumumab targeted therapies (Allegra et al. 2009, 2016). Such limitation has a major impact, as about 30–40% of CRC patients do harbour a *KRAS* mutation (De Roock et al. 2011; Velho et al. 2010). Importantly, not all mCRC patients with wild type *KRAS* respond to anti-EGFR agents, suggesting that other mechanisms of resistance are involved (Heinemann et al. 2016; Lievre et al. 2017; Price et al. 2016). Indeed, *NRAS* mutations were also shown to be associated with resistance to anti-EGFR agents, and recommendations are now to exclude these patients from EGFR targeted therapies (Allegra et al. 2016). However, these account for only about 2% of CRC patients, indicating that other molecules are involved (Irahara et al. 2010). Mutations in other genes downstream of EGFR, including *BRAF* and *PIK3CA*, have been associated with resistance to EGFR targeted therapies but inconsistent results have been obtained and additional evidence is required to clarify such controversy (De Roock et al. 2011; Mohamed et al. 2018; Lievre et al. 2010; Therkildsen et al. 2014). As above mentioned, mCRC patients can also be offered anti-angiogenic therapy using the anti-VEGF agent bevacizumab, namely in combination with chemotherapy regimens, which has proven some clinical efficacy (Welch et al. 2010).

Altogether, at present, not only the available targeted agents for mCRC patients are limited but also exclude a considerable proportion of patients. Therefore, there is an urgent need to develop novel therapeutic strategies for CRC patients, particularly for those with *KRAS* mutations.

4.5 Targeting the PI3K Signalling Pathway

The PI3K signalling pathway is a key signalling cascade implicated in many human cancers including CRC (Janku et al. 2018). It is well established that deregulation of the PI3K signalling can occur through activating mutations in the *PIK3CA* gene, but other important activation mechanisms are known to exist, namely through oncogenic KRAS, with strong implications for CRC patients (Thorpe et al. 2015; Castellano and Downward 2011). Thus, efforts have been made to advance in the development of novel targeted therapies directed to the many molecules of the PI3K signalling cascade. Attractive therapeutic targets include PI3K p110 isoforms, Akt and mTOR, and inhibitors can be isoform specific- or pan-PI3K inhibitors, dual PI3K/mTOR inhibitors, Akt inhibitors and mTORC1 and mTORC2 inhibitors.

A vast number of drugs have already been tested in preclinical assays, however, only a few have reached clinical studies for several cancer types, including CRC (Fig. 4.2). Furthermore, the available data on the clinical effects of PI3K inhibitors is still limited (Rodon et al. 2013; Janku et al. 2018). Notably, despite reports suggesting specific alterations to be predictive of responsiveness, as the presence of *PIK3CA*

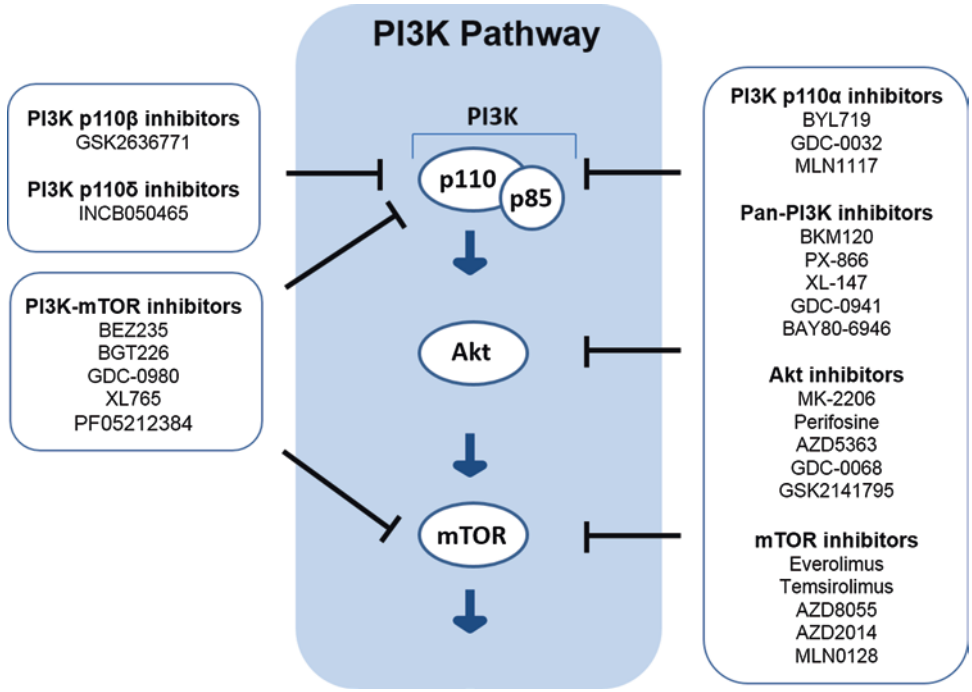

Fig. 4.2 Inhibitors of the PI3K signalling pathway used in clinical trials for several cancer types, including CRC

mutations for PI3K p110α specific inhibitors, reliable predictive biomarkers of therapeutic response or resistance are still awaited. To date, no PI3K signaling pathway inhibitor has yet been approved for CRC patients and only temsirolimus and everolimus (mTORC1 inhibitors) and copanlisib and idelalisib (PI3K inhibitors) have been approved for specific types of cancer as subsequently described in more detail (Janku et al. 2018). A brief overview of the current knowledge is described below.

4.5.1 PI3K Isoforms as Therapeutic Targets

As above mentioned, agents that target the PI3K are classified into pan-class I PI3K inhibitors targeting all class I PI3K isoforms, or into isoform specific-PI3K inhibitors, targeting specifically one p110 isoform (Thorpe et al. 2015; Janku et al. 2018).

Initially, many studies were performed in several cancer type models using the PI3K pan-inhibitors wortmannin and LY294002, but these were only tested in preclinical studies and did not reach clinical trials, in part due to selectivity and toxicity issues (Liu et al. 2009). In particular, the irreversible PI3K inhibitor wortmannin was shown to have antitumour activity in several human tumour cell lines, including a colon carcinoma cell line (Schultz et al. 1995). Moreover, LY294002, a reversible small molecule PI3K inhibitor, demonstrated a remarkable growth-inhibitory and apoptosis-inducing effect in colon cancer cell lines and, experiments using mouse xenografts revealed that LY294002 administration *in vivo* also resulted in suppression of tumour growth and induction of apoptosis (Semba et al. 2002).

In recent years, novel inhibitors were generated with improved characteristics, namely in terms of specificity, potency and stability while simultaneously minimizing toxicity. In most cases, these inhibitors are ATP competitive agents and many of them are now being evaluated in clinical trials in patients with solid tumours, including CRC, either as monotherapy or in

combination with other therapies (Thorpe et al. 2015). The list of inhibitors is vast and includes BKM120, PX-866, XL147, GDC0941, GSK1059615, BYL719, GDC0032, INK1117 (Janku et al. 2018). Some relevant studies focusing on CRC are briefly described.

BKM120 (buparlisib), a pan-PI3K inhibitor, when tested in a panel of 353 cell lines exhibited preferential inhibition of tumour cells harbouring *PIK3CA* mutations, in contrast to either *KRAS* or *PTEN* mutant models (Maira et al. 2012). In addition, BKM120 was shown to reduce cell proliferation in wild type and mutant *PI3KCA* CRC cells and treatment with cetuximab and BKM120 significantly reduced the growth of xenograft tumours originating from *PIK3CA* wild type and *KRAS* mutant cells compared with cetuximab alone (Hong et al. 2016). Noteworthy, the mechanisms underlying resistance to PI3K inhibition are known to involve other molecules. For instance, high nuclear β-catenin concentrations were shown to confer resistance to BKM120 in sphere cultures derived from patients with colon cancer (Tenbaum et al. 2012). Clinical studies have shown that BKM120 was well tolerated and had preliminary antitumour activity (Bendell et al. 2012; Rodon et al. 2014). However, a phase I trial of BKM120 plus mFOLFOX6 (5-FU/LV + oxaliplatin), in patients with refractory solid tumours, including CRC, resulted in increased toxicity compared to either therapy alone (McRee et al. 2015). At present, other clinical trials using BKM120 are under investigation, one in combination with panitumumab in *KRAS* wild type mCRC patients (NCT01591421) and another in combination with irinotecan in previously treated advanced CRC patients (NCT01304602). PX-866, an irreversible pan-PI3K inhibitor, has been shown to cause prolonged inhibition of PI3K signalling in human tumour xenografts, namely in colon tumour xenografts (Ihle et al. 2004). In clinical trials, it was well tolerated and was associated with prolonged stable disease in patients with advanced solid tumours, namely CRC (Hong et al. 2012). A multicenter phase I study of PX-866 and cetuximab in patients with mCRC or recurrent/metastatic squamous cell carcinoma of the head and neck has shown that PX-866 and cetuximab treatment was tolerated with signs of antitumour activity (Bowles et al. 2014). Subsequently, a randomized phase II study evaluated cetuximab with or without PX-866 in patients with *KRAS* wild type mCRC; however the addition of PX-866 to cetuximab did not improve progression free survival, objective response rate, or overall survival in patients with mCRC but instead the combination arm had greater toxicity (Bowles et al. 2016). In addition to these, other pan-PI3K inhibitors are being investigated in clinical trials for several tumour types and include XL-147 (pilaralisib), GDC-0941 (pictilisib), CH5132799, GSK1059615, SF1126 and ZSTK474 (Wheler et al. 2017; Sarker et al. 2015; Thorpe et al. 2015; Patnaik et al. 2016; Blagden et al. 2014; Janku et al. 2018). To date, copanlisib (BAY80-6946) is the only pan-PI3K inhibitor, with predominant activity against PI3K p110α and PI3K p110 δ isoforms, approved for relapsed lymphoma (Markham 2017).

In the last few years, isoform specific PI3K inhibitors have also been developed and of particular interest for CRC patients are the PI3K p110α inhibitors, as mutations in the gene that code for the PI3K p110α are frequently observed. BYL719 (alpelisib), a selective inhibitor of the PI3K p110α, was shown to have antitumour activity in preclinical studies and *PIK3CA* mutation was suggested to be a positive predictor of BYL719 sensitivity (Fritsch et al. 2014). Our group has also demonstrated the potential benefit of targeting PI3K p110α in CRC cells. Notably, not only cells with *PIK3CA* mutations were sensitive to PI3K p110α inhibition, but also cells with *KRAS* mutations (Fernandes et al. 2016). In particular, we have shown that the specific inhibition of PI3K p110α, by small interfering RNA (siRNA) or BYL719, had an impact in the viability of SW480 and HCT116 CRC cells harbouring mutations in *KRAS* and *KRAS/PIK3CA*, respectively (Fernandes et al. 2016). In addition, PI3K inhibition induced apoptosis in HCT116 cells and cell cycle arrest in SW480 cells suggesting that different mechanisms may be involved (Fernandes et al. 2016). Thus, specific inhibition of the p110α subunit of PI3K could provide an

alternative therapeutic approach for CRC patients, particularly those harbouring *KRAS* mutations, who are currently excluded from EGFR-targeted therapies. In addition to preclinical studies, data from clinical trials is now emerging. The results from the first in-human phase Ia study revealed that BYL719 was tolerable and encouraging preliminary activity was observed in patients with *PIK3CA*-altered solid tumours (Juric et al. 2018). Moreover, due to the limited efficacy of BRAF inhibitors as single agents in *BRAF* mutant CRC and since EGFR and PI3K activation have been associated with resistance, a clinical phase Ib study evaluated the selective RAF kinase inhibitor encorafenib plus cetuximab or encorafenib plus cetuximab and BYL719; the results demonstrate that the treatments were tolerable and provided promising clinical activity in *BRAF* mutant mCRC patients (van Geel et al. 2017). Taselisib (GDC-0032) and MLN1117 (TAK-117, INK1117) are other PI3K p110α inhibitors currently in clinical trials for several cancer types (Janku et al. 2018).

In addition to PI3K p110α inhibitors, other p110 isoforms have been targeted including PI3K p110β and PI3K p110δ, but these have been mostly used for other cancer types. Indeed, idelalisib (CAL-101), a selective PI3K p110δ inhibitor, has already been approved for the treatment of patients with haematological malignancies (Gopal et al. 2014; Yang et al. 2015). In addition, the PI3K p110β inhibitor GSK2636771 and the PI3K p110δ inhibitor INCB050465 are also in clinical trials for various cancer types including CRC.

4.5.2 PI3K/mTOR Axis as a Therapeutic Target

The p110 catalytic domain of PI3K is structurally similar to that of the mTOR and therefore a class of inhibitors has been developed that target both molecules (Takeda et al. 2016). Using this strategy, PI3K-Akt-mTOR activation should be more efficiently inhibited as feedback mechanisms could be prevented. A number of dual inhibitors have been evaluated both in preclinical and clinical settings including BEZ235, XL765, BGT226 and PKI587.

BEZ235 (dactolisib), a potent ATP-competitive dual PI3K-mTOR inhibitor, was shown to have antitumour activity *in vitro* and *in vivo* using human tumour cell lines and tumour xenografts (Maira et al. 2008). In addition, BEZ235 was shown to induce tumour regression in genetically engineered mouse models of *PIK3CA* wild type CRC (Roper et al. 2011). Also in preclinical studies, BEZ235 was able to inhibit the PI3K/mTOR axis and to have antiproliferative and antitumoural activity in cancer cells with both wild type and mutated *PIK3CA* (Serra et al. 2008). Regarding predictive biomarkers, *PIK3CA* mutations have been associated with antitumour activity in preclinical and clinical studies, as shown with the association of the *PIK3CA* mutation H1047R with response to PI3K/AKT/mTOR signalling pathway inhibitors in early-phase clinical trials (Janku et al. 2013). Importantly, other preclinical studies have shown that coexistent mutations in *PIK3CA* and *KRAS* in CRC cells conferred resistance to BEZ235 (Kim et al. 2013). In addition, alterations in distinct molecules have also been associated with resistance to PI3K signaling inhibitors as is the case of TRIB2 that was shown to confer *in vivo* resistance to BEZ235 treatment through activation of Akt (Hill et al. 2017). Nonetheless, additional data is awaited from clinical trials. BEZ235 has also been combined with the mTOR inhibitor everolimus. Indeed, a phase Ib study of BEZ235 combined with everolimus was evaluated in patients with advanced solid malignancies but the combination of BEZ235 and everolimus demonstrated limited efficacy and tolerance (Wise-Draper et al. 2017).

BGT226, another dual PI3K-mTOR inhibitor, has also been evaluated in a clinical trial in patients with advanced solid tumours, including patients with colon cancer. However, BGT226 was shown to have limited preliminary antitumour activity and inconsistent target inhibition (Markman et al. 2012). The first-in-human study of PF05212384 (PKI-587) in patients with advanced cancer demonstrated a manageable

safety profile and antitumour activity supporting further clinical development for patients with advanced solid malignancies (Shapiro et al. 2015).

Additional dual PI3K-mTOR inhibitors have been tested in clinical trials for many cancer types, but for some inhibitors, little success or toxicity issues were observed and no further studies were pursued (Janku et al. 2018; LoRusso 2016). More data on CRC is still awaited.

4.5.3 Akt and mTOR as Therapeutic Targets

As a key molecule in the PI3K signalling cascade, Akt has been appointed as a potential therapeutic target. Indeed, MK-2206, a potent allosteric inhibitor of all Akt isoforms, has already been evaluated in preclinical and clinical settings (Brown and Banerji 2017). For instance, in mice with established xenograft tumours, MK-2206 exhibited a significant deceleration of tumour progression and primary patient-derived tumour sphere growth was significantly inhibited by MK-2206 (Malkomes et al. 2016). In the clinic, the first-in-man clinical trial of MK-2206 demonstrated good tolerability with evidence of Akt signalling blockade in patients with advanced solid tumours that included CRC patients (Yap et al. 2011). Further clinical trials using MK-2206, alone or in combination, are ongoing in patients with advanced CRC namely a phase II study in patients with metastatic *KRAS* wild type and *PIK3CA* mutant (NCT01186705). Perifosine is another Akt inhibitor that, as MK-2206, targets the PH domain of Akt, thereby preventing its translocation to the plasma membrane and blocking its phosphorylation and activation (Gills and Dennis 2009; Brown and Banerji 2017). In a phase II trial in patients with mCRC, perifosine plus capecitabine showed promising clinical activity when compared with capecitabine alone (Bendell et al. 2011). Other inhibitors, including ATP competitive inhibitors of Akt, are being tested in patients with different cancer types and these include AZD5363, GDC-0068 and GSK2141795 (Janku et al. 2018; LoRusso 2016). Importantly, special attention should be taken when using these inhibitors alone as data indicates that PI3K may signal through both Akt-dependent and Akt-independent mechanisms. Indeed, an Akt-independent signalling downstream of *PIK3CA* mutations has been described in human cancer cells (Vasudevan et al. 2009).

Inhibitors targeting mTOR are also being evaluated in clinical trials for many cancer types. These inhibitors, which can be rapamycin analogs inhibiting mTORC1, or ATP-competitive inhibiting both mTORC1 and mTORC2, have been investigated in preclinical and clinical studies (Guertin and Sabatini 2009; Papadatos-Pastos et al. 2015). Indeed, temsirolimus, which is an mTORC1 inhibitor, has already been approved for advanced renal cancer and everolimus (RAD001), also an mTORC1 inhibitor, was approved for certain cancer types including advanced renal cancer and particular types of advanced breast cancer (Hudes et al. 2007; Baselga et al. 2012; Motzer et al. 2008; Janku et al. 2018). Temsirolimus and everolimus have also been evaluated in several clinical trials in mCRC patients, either alone or in combination with other therapeutic agents. For instance, in a phase II study in patients with refractory mCRC, the combination of tivozanib (a VEGFR inhibitor) and everolimus was shown to be well tolerated, with stable disease achieved in 50% of patients (Wolpin et al. 2013). In contrast, in a phase II study in patients with mCRC heavily pretreated, everolimus was well tolerated but did not confer meaningful efficacy (Ng et al. 2013). A phase I trial of everolimus in combination with 5-FU/LV, mFOLFOX6 and mFOLFOX6 plus panitumumab in patients with refractory solid tumours including CRC has shown that the further addition of panitumumab resulted in an unacceptable level of toxicity that cannot be recommended for further study (McRee et al. 2014). A phase II trial of temsirolimus, alone or in combination with irinotecan, in *KRAS* mutant mCRC revealed that treatment was well tolerated but had limited efficacy in chemotherapy resistant *KRAS*

mutant (Spindler et al. 2013). Nonetheless, plasma KRAS quantification was suggested as a strong predictor of outcome (Spindler et al. 2013). In addition to these, other mTOR inhibitors entered clinical trials including AZD8055, AZD2014 and MLN0128 (LoRusso 2016; Papadatos-Pastos et al. 2015). More studies and data are required evaluating mTOR inhibitors, namely in combination with other regimens.

4.5.4 PI3K Inhibition in Combination with MAPK Targeted Therapies

The available PI3K targeted therapies have shown limited success in CRC patients. Indeed, despite the development of many specific inhibitors, some of which already in clinical trials, none of these have yet been approved for the treatment of patients with CRC. Therefore, in an attempt to improve the response rates of these patients, combined targeted therapies have been proposed and investigated.

Notably, it is well established that the MAPK and PI3K signalling pathways are interconnected and inhibition of one signalling cascade could induce feedback loops and compensatory mechanisms, ultimately leading to resistance (Britten 2013). Moreover, as mutations in *KRAS* and *BRAF* are frequently observed in CRC patients, inhibition of both MAPK and PI3K pathways could be a more effective strategy. Thus, several studies have been performed to evaluate the combination of inhibitors targeting molecules of these pathways.

In preclinical models, inhibition of both the MAPK and PI3K signalling pathways have been reported to be synergistic in various cancer types (Temraz et al. 2015). For instance, although treatment with BEZ235 led to marked tumour regression in a mouse model of lung cancer with the *PIK3CA* H1047R mutation, in *KRAS* G12D mutant mice only BEZ235 combined with the MEK inhibitor ARRY-142886 induced tumour regression but not BEZ235 alone (Engelman et al. 2008). In CRC, the combination of a PI3K/mTOR (PF-04691502) and a MEK (PD-0325901) inhibitor demonstrated enhanced anti-proliferative effects against CRC cell lines and demonstrated enhanced reduction in tumour growth in patient-derived CRC tumour xenograft models, regardless of *KRAS* or *PI3K* mutational status (Pitts et al. 2014). In a panel of CRC cell lines, dual targeting of PI3K (GDC-0941) and MEK (AZD6244) induced synergistic growth inhibition but the combination of specific PI3K inhibitors, rather than dual mTOR/PI3K inhibitors, with MEK inhibitors resulted in greater synergy (Haagensen et al. 2012).The inhibition of MEK and PI3K/mTOR was shown to suppress tumour growth in patient-derived xenografts of *RAS*-mutant colorectal carcinomas, though it did not cause tumour regression (Migliardi et al. 2012). However, preclinical data also indicates that such therapeutic strategies have limitations namely related to toxicity issues and periodic rather than continuous inhibition has been suggested as an alternative strategy (Will et al. 2014). Indeed, rapid induction of apoptosis by PI3K inhibitors was reported to be dependent of the transient inhibition of RAS–ERK signalling (Will et al. 2014).

In a retrospective analysis, dual targeting of the PI3K and MAPK pathways was evaluated in patients with advanced cancers including CRC and treated with phase I study drugs; the results suggested that dual inhibition may potentially exhibit favourable efficacy compared with inhibition of either pathway, although with greater toxicity (Shimizu et al. 2012). In a biomarker-driven trial, no clinical benefit was observed in CRC patients treated with the Akt inhibitor MK-2206 and the MEK1/2 inhibitor selumetinib; instead, overlapping toxicities limited the ability to dose escalate to achieve exposures likely needed for clinical activity (Do et al. 2015). At present, many clinical trials are ongoing with MAPK and PI3K inhibitors for many cancer types including CRC but data is still awaited (Temraz et al. 2015; Tolcher et al. 2018).

4.6 The Importance of Bioinformatic Tools for Biomarker and Therapy Predictions

The number of studies evaluating specific inhibitors, both in preclinical and clinical settings, is enormous. Whether evaluating single and combination of drugs in cancer cell lines, performing *in vivo* experiments in animal models, or clinical trials in patients with advanced cancers, the amount of generated information is huge and rather under analysed.

Over the last few years, bioinformatic tools have gained much interest and proved powerful in unravelling some key aspects in cancer research, namely in the discovery of cancer biomarkers and evaluation of therapy responsiveness. Currently, it is well established that despite similarities in the mutational patterns among several cancer types, specific molecular alterations have context specific functional consequences with impact in therapy outcome. For instance, using a computational strategy for integrating (phospho) proteomic and mRNA sequencing data across tumour data sets, it was possible to link the dysregulation of upstream signalling pathways with altered transcriptional programs. More specifically, it was possible to associate *PIK3CA* activating mutations with altered activities of distinct sets of transcription factors and therefore this model could help to better predict which patients will benefit from targeted and combination therapies (Osmanbeyoglu et al. 2017). In a different study, a model is proposed to integrate oncogene and tumour suppressor activity in CRC cells and used to identify cancer drivers and compute patient-specific gene activity scores (Pavel et al. 2016). In this model, the integrative score improved prediction of drug sensitivity and the gene activity scores were also used to cluster CRC cell lines (Pavel et al. 2016). In addition to these, other studies focusing on bioimaging and bioinformatics have been developed. For instance, a novel method was proposed to characterize E-cadherin signature in gastric cancer cells in order to identify E-cadherin deregulation and functional impairment (Sanches et al. 2015). More specifically, this strategy included a bioimaging pipeline to quantify the expression level and characterize the distribution of the protein from *in situ* immunofluorescence images (Sanches et al. 2015). As for gastric cancer, bioimaging tools could be used in CRC models with potential implications in biomarker identification and therapy outcome predictions.

4.7 Conclusions and Future Perspectives

The ultimate goal in cancer therapy is to provide patients with treatments that will improve their overall survival and eventually manage cancer as a chronic disease. To achieve successful outcomes, personalized therapy will most probably be needed, i.e., select the best targeted therapy to each patient tumour characteristics. Although many drugs are being developed, there is still the urgent need to develop novel therapeutic strategies.

In recent years, inhibitors to the various molecules of the PI3K-Akt-mTOR cascade have emerged. In most cases, these inhibitors have shown a wide range of adverse effects and limited success. A deeper knowledge of the complex interplay between distinct signalling pathways, as well as a better understanding of feedback-loops disruption and the occurrence of compensatory mechanisms upon PI3K inhibition, will guide and improve the design of novel therapeutic strategies. The available clinical results have shown that dual MAPK and PI3K inhibition is possible, although toxicity has been an issue. Additional combination therapy regimens are being tested and should be considered.

A challenge in the treatment of CRC patients will be the identification of specific biomarkers predictive of therapy responsiveness, for which bioinformatics tools will be essential. Indeed, a proper patient stratification will most probably be a key issue for a successful outcome. Although many studies have been performed to identify such biomarkers, further studies are required. Overall, and despite ongoing clinical trials with some of these drugs, CRC patients are still await-

ing alternative therapies to be approved. More data on novel drugs, combination regimens and clinical trials is expected to shed light on CRC best targeted therapies.

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Targeting PTEN in Colorectal Cancers

5

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Abstract

Phosphatase and tensin homolog (PTEN) is a tumour suppressor that represents one of the most common targets for genetic defect in human cancer. PTEN controls an array of physiopathological processes related to cell proliferation, differentiation, DNA/chromosome integrity, apoptosis and invasiveness. PTEN dephosphorylates not only proteins, but also phosphoinositides generated by phosphatidylinositol 3-kinase, thus counteracting the Akt signalling pathway. Interestingly, PTEN can also exert some biological functions independently of its catalytic activity.

A feature of colorectal cancers is the relatively low incidence of PTEN mutation or deletion, whereas PTEN downregulation occurs in approximately one third of tumours. PTEN inactivation may be even higher when changes in posttranslational modifications and/or mislocalization of the tumour suppressor are accounted for. Strategies based on pharmacologically-induced restoration of wild-type PTEN function in colon cancer cells could therefore be considered, to impact cell growth, trigger apoptosis, and sensitize tumour cells to therapeutic agents.

This review details current knowledge of the mechanisms regulating PTEN expression, activity and function. It also focuses on the use of small molecules targeting positive or negative PTEN regulators and summarizes alternative strategies that could be used to alter PTEN conformation/activity. Finally, we propose an outline of a personalized approach to restore PTEN function in colon cancer cells.

Keywords

AKT signaling · DNA repair · Molecular scaffolds · Phosphatase · Tumor suppressor

5.1 Background

PTEN (phosphatase and tensin homolog deleted on chromosome ten)/MMAC (mutated in multiple advanced cancers) was identified in 1997 by two groups as a candidate tumour suppressor gene located at 10q23 (Li et al. 1997; Steck et al. 1997). In parallel, in a study screening for new

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dual-specificity phosphatases the same gene was identified and named TEP-1 (TGF-β-regulated and epithelial cell-enriched phosphatase) (Li and Sun 1997). Homozygous inactivation of PTEN occurs in a large fraction of glioblastomas, melanoma cell lines, advanced prostate cancers and endometrial carcinomas (Teng et al. 1997). Depending on tissue type, PTEN inactivation can occur either as an early (*e.g.* endometrium), or late event (*e.g.* prostate cancers, glioblastomas). PTEN is one of the most common targets for genetic defect in human cancer. Haploinsufficiency or inactivation of a single PTEN allele is sufficient for cancer development. Germ-line mutations in PTEN cause three autosomal dominant inherited cancer syndromes (Cowden disease, Lhermitte-Duclos disease, and Bannayan-Zonana syndrome) characterized by hamartomas, and an increased prevalence of breast and thyroid malignancies. $Pten^{+/-}$ mice mimic the effects of some germ-line mutations of the human tumour suppressor gene (Di Cristofano et al. 1998; Suzuki et al. 1998; Podsypanina et al. 1999). $Pten^{-/-}$ mice exhibit early embryonic lethality, whereas heterozygotes show increased tumour incidence, consistent with its identification as a tumour suppressor gene.

PTEN encompasses 403 amino acids and is characterized by five functional domains: a short N-terminal PtdIns(4,5)P2 (PIP2)-binding domain, a phosphatase domain, a membrane-targeting C2 domain, and a C-terminal tail containing PEST sequences and a PDZ binding motif in its C-terminus (see Fig. 5.1). The PDZ binding motif permits binding to PDZ domain-containing proteins that often direct the assembly of multiprotein complexes at membrane-cytoskeletal interfaces.

PTEN is a multifunctional protein endowed with phosphatase activity. It has been reported that PTEN dephosphorylates the protein substrates FAK, SHC, IRS1, Dvl2 and PTK6 (Gu et al. 1998; Shi et al. 2014; Shnitsar et al. 2015; Wozniak et al. 2017). The tyrosine residue 138 of PTEN appears to be critical for PTEN protein phosphatase activity (Davidson et al. 2010). Importantly, PTEN dephosphorylates not only proteins, but also the phosphoinositides generated by phosphatidylinositol 3-kinase activity. PtdIns(3,4,5)P3 is known to exert its function by recruiting proteins that contain pleckstrin homology (PH) domains to the membrane, such as Btk, PKB/Akt, PLC-γ, Gab1, P-Rex1, PDK1, and Grp1 (Lemmon 2007). PtdIns(3,4,5)P3 effectors promote activation of Rac GTPases and F-actin polymerization at the leading edge of migrating cells. Through its lipid phosphatase activity, PTEN counteracts the PI3K/Akt signalling cascade to decrease cell proliferation (Furnari et al. 1998), promote apoptosis (Stambolic et al. 2001; Szado et al. 2008) and revert invasiveness (Kotelevets et al. 2001; see also "Targeting the PI3K signaling as a therapeutic strategy in colorectal cancer" by Fernandes et al., Chap. 4, in this issue). PTEN can autodephosphorylate threonine 383 and threonine 366 in its C-terminal tail (Fig. 5.1) (Raftopoulou et al. 2004; Tibarewal et al. 2012).

The many somatic PTEN mutations identified in human cancers impact PTEN stability, subcellular localisation, and/or the lipid phosphatase/both lipid and protein phosphatase activities (Georgescu et al. 2000; Yang et al. 2017; Furnari et al. 1998).

PTEN also exerts some biological activities independently of its catalytic activity. For example, PTEN directly interacts with the tumour suppressor TP53, enhancing its stability and transcriptional activity (Freeman et al. 2003; Tang and Eng 2006). The C-terminal domain of PTEN physically interacts with the forkhead-associated domain of the Microspherule Protein 1 (MSP58) and inhibits its oncogenic activity (Okumura et al. 2005). The isolated C2 domain of PTEN is also able to mimic effects of full-length PTEN in the control of both cell migration and glandular morphogenesis in 3D colorectal cancer cell systems (Raftopoulou et al. 2004; Leslie et al. 2007; Lima-Fernandes et al. 2011; Javadi et al. 2017). Nuclear PTEN also interacts with the anaphase-promoting complex (APC/C), promotes APC/C association with CDH1 (CDC20 homolog 1), and thereby enhances the tumour-suppressive activity of the APC-CDH1 complex, in a phosphatase-independent manner (Song et al. 2011). Interestingly, the knockin of

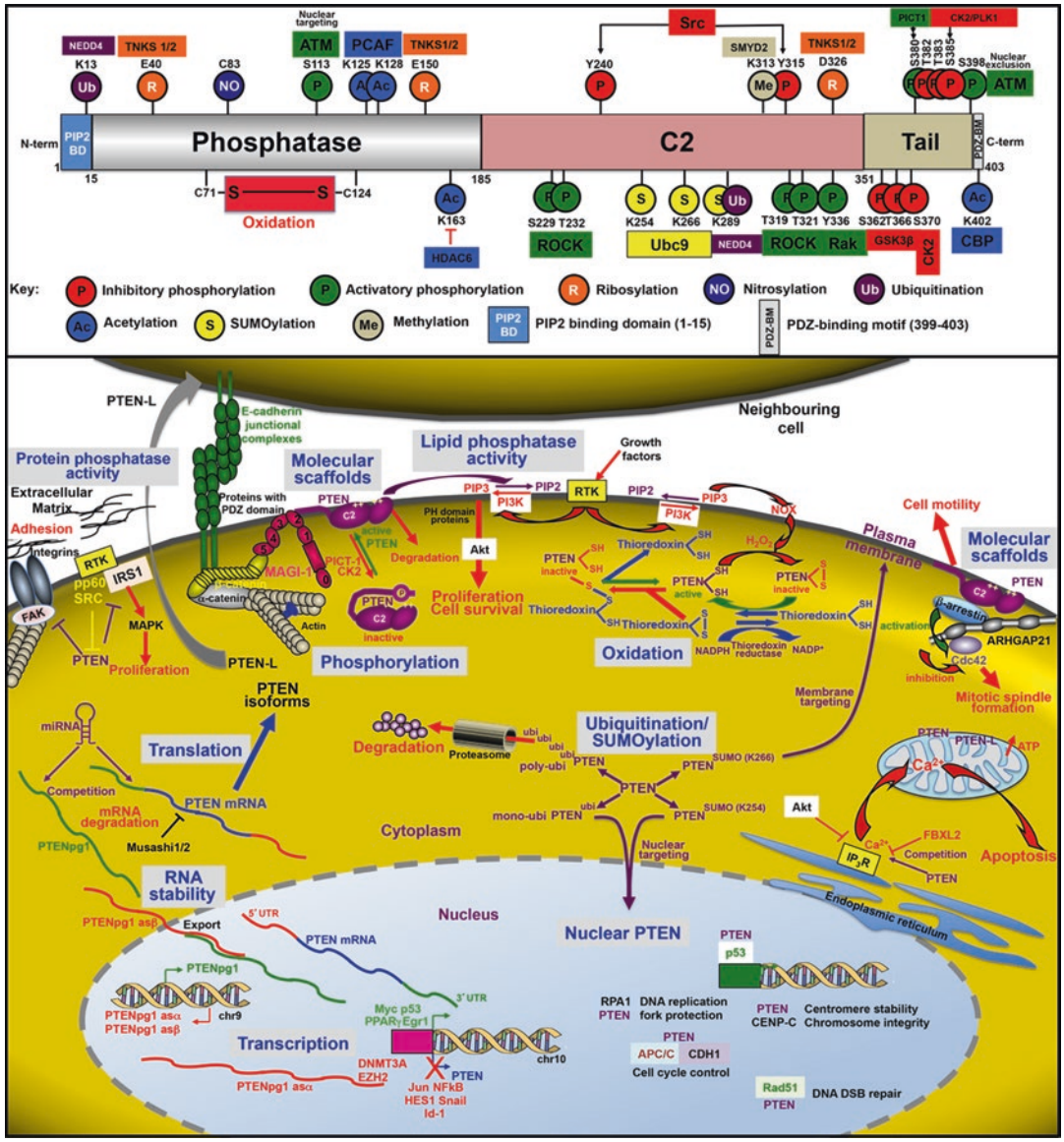

Fig. 5.1 Schematic overview of PTEN structure, biological functions and regulation by epigenetic, transcriptional, post-transcriptional and post-translational mechanisms Upper Panel: Structure of canonical PTEN protein domains and post-translational modifications. Lower Panel: Effector systems controlling PTEN accumulation, activity and subcellular localisation. For details see the text

mutant phosphatase-defective alleles of PTEN in mouse models reveals that heterozygous mice bearing one mutant allele ($Pten^{C124S/+}$ and $Pten^{G129E/+}$) have a higher tumour burden than $Pten^{+/-}$ counterparts with one full null allele. This suggests that heterooligomerization of wild-type with mutant PTEN inhibits PTEN tumour suppressor activity (Papa et al. 2014).

Due to the significant progress in high-throughput technologies, vast amounts of multi-dimensional data relevant to the biology of colorectal cancers have been generated (http://www.colonatlas.org) (Chisanga et al. 2016). Colorectal cancers (CRC) arise through the stepwise accumulation of genetic alterations leading from normal epithelia to aberrant crypt foci,

adenoma, carcinoma and metastatic disease (Fearon and Vogelstein 1990; Kotelevets et al. 2016), and follow three molecular pathways to genome instability characterized by (*i*) chromosomal instability (CIN), (*ii*) high microsatellite instability (MSI-H), or (*iii*) CpG island methylator phenotype (CIMP). A more detailed classification of primary colorectal cancers based on intrinsic gene expression profiles, resulting in the four biologically distinct consensus molecular subtypes (CMS1–4) was recently proposed to facilitate the translation of molecular subtypes into the clinic (Guinney et al. 2015).

According to The Cancer Genome Atlas Network, APC, TP53, KRAS, PIK3CA, FBXW7, SMAD4, TCF7L2 and NRAS are the most frequently mutated genes in CRC (cancer genome atlas network 2012). Molecular analysis of PTEN status in sporadic colorectal cancers revealed that PTEN mutation is a relatively rare event. COSMIC v84 (URL http://cancer.sanger.ac.uk/cosmic/, released February 13 2018), reports that 335 out of 6361 human colonic tumour samples exhibited PTEN mutations (5.27%, 2.02% according to Lin et al. 2015), whereas PTEN mutations were found in 37.8% of endometrial cancers. PTEN mutation in CRC was associated with the subgroup displaying microsatellite instability (mutation rate estimated to 19% in this subgroup), suggesting that PTEN might be a target of defective mismatch repair function in colorectal carcinogenesis. In line with this, the PTEN coding region contains several repeat sequences, including two poly(A) tracts in exons 7 and 8 (Goel et al. 2004). These mutations were found regardless of the antero-posterior localization of the tumour, with a slightly higher incidence in the cecum and proximal colon (Loree et al. 2018).

The loss of *PTEN* copy number was identified in 1.56% of tumour samples. Nevertheless, PTEN downregulation occurs in 33% of colon cancer samples. PTEN inactivation in colonic tumours might therefore be underestimated and could occur via other non-genomic mechanisms such as aberrant regulation of posttranslational modifications and/or mislocalization of the tumour suppressor. Nuclear-cytoplasmic partitioning of PTEN is a promising biological marker: the absence of nuclear PTEN is associated with more aggressive disease in patients with colorectal cancer or other types of cancer (Zhou et al. 2002; Tachibana et al. 2002; Whiteman et al. 2002; Perren et al. 2000; Fridberg et al. 2007).

Experimental studies demonstrate that restoration of PTEN expression sensitizes tumour cells to conventional as well as targeted therapies and immunotherapies. Since PTEN mutation is uncommon in CRC, targeting the multiple levels of PTEN regulation constitutes an attractive strategy to explore with the goal of re-establishing/potentiating its tumour suppressor activities and to manage tumour cell responses to chemotherapies.

5.2 Targeting PTEN in Colorectal Cancers

PTEN exerts pleiotropic activity and fulfils a complex array of physio-pathological processes related to cell proliferation (cell cycle arrest in G1 or in G2-M), differentiation, DNA and chromosomal integrity (Shen et al. 2007), apoptosis (increased susceptibility) and invasiveness (inhibition). PTEN expression is therefore subjected to fine-tuning at transcriptional, post-transcriptional and post-translational levels (Fig. 5.1). The importance of maintaining appropriate PTEN expression is highlighted by the fact that even a subtle reduction in PTEN levels is sufficient to promote cancer susceptibility (Carracedo et al. 2011).

5.2.1 Transcriptional Level

5.2.1.1 Epigenetic PTEN Regulation

Hypermethylation of CpG islands in promoters is associated with gene silencing and PTEN silencing might therefore result from promoter methylation independently of copy number loss. The analysis of *PTEN* promoter methylation might be biased by contamination of the methylated *PTEN* pseudogene. In colonic cell lines, *PTEN* promoter methylation is a rare event (Hesson et al. 2012). In contrast, however, *PTEN* promoter

methylation was observed in approximately 30% of colorectal tumour samples (Lin et al. 2015; Yazdani et al. 2016). Interestingly, sulforaphane, an organosulfur compound present in cruciferous vegetables such as broccoli, Brussels sprouts and cabbages induces DNA demethylation and restores PTEN expression in cultures of mammary cell lines (Lubecka-Pietruszewska et al. 2015). Other epigenetic inhibition of PTEN expression involves the histone methyltansferase activity of the Polycomb Repressive Complex 2 that is reversed by the selective antagonist 3-deazaneplanocin A (Benoit et al. 2013).

This provides a rationale for epigenetic therapies based on the use of DNA demethylating agents, such as 5-azacytidine and 5-aza-2′-deoxycytidine, or of selective inhibitors of histone methyltransferase. Such compounds are under clinical trial for cancer treatment (16 trials referenced at URL: https://clinicaltrials.gov/, accessed on 1st March 2018 concern azacytidine derivatives in colorectal cancers, 13 trials evaluate histone methyltransferase inhibitors in different types of cancers).

The *PTEN* pseudogene *PTENpg1* is located on chromosome 9, and encodes a long noncoding RNA (lncRNA) that regulates PTEN both positively and negatively at transcriptional and post-transcriptional levels (Johnsson et al. 2013). *PTENpg1* is transcribed in the sense orientation and in antisense under three isoforms: unspliced, spliced antisense alpha and antisense beta. It is proposed that the antisense alpha recruits the EZH2 histone methyltransferase and the DNA methyltransferase 3A (DNMT3a) to the *PTEN* promoter leading to *PTEN* silencing. The nuclear export of the *PTENpg1* sense RNA that lacks a poly-A tail is facilitated by *PTENpg1* antisense beta. Due to its strong homology with *PTEN*, cytoplasmic sense *PTENpg1* transcript acts as a "sponge" to "mop up" the microRNAs targeting *PTEN* (Fig. 5.1 and see below) (Poliseno et al. 2010).

5.2.1.2 Transcription Factors

A series of transcription factors bind directly to the PTEN promoter and either activate or repress PTEN transcription (Fig. 5.1).

Inducing factors include the early growth response transcriptional factor 1 (EGR1) (Virolle et al. 2001), the peroxisome proliferator activated receptor gamma (PPARγ) (Patel et al. 2001), activating transcription factor 2 (ATF2) (Shen et al. 2006), the nuclear factor of activated T cell (NFAT) (Wang et al. 2011) and the tumour suppressor, TP53 (Stambolic et al. 2001).

In contrast, PTEN is transcriptionally repressed by c-Jun (Hettinger et al. 2007), nuclear factor-kappa B (NFκB) (Xia et al. 2007; Ghosh-Choudhury et al. 2010)), and the zinc finger-like proteins SNAIL and SLUG involved in epithelial-mesenchymal transition (Escrivà et al. 2008; Uygur et al. 2015).

These transcription factors and their upstream effectors therefore constitute targets to enhance PTEN transcription. Examples include the PPARγ agonist rosiglitazone, induction of EGR-1 following irradiation, activation of NFAT by butyrate a short-chain fatty acid produced by fermentation of dietary fibers by colonic microbiota, statins or selective inhibitors to target NFkB.

5.2.2 Posttranscriptional Modulation

MicroRNAs (miRNAs) are a class of small noncoding RNAs containing 18–24 nucleotides. These short RNAs can negatively regulate gene expression by complementary binding to the 3-untranslated region (3′-UTR) of target transcripts, leading to translation inhibition and/or mRNA degradation (Fig. 5.1). One single miRNA may target the expression of many different genes. Conversely, one transcript may be targeted by distinct miRNAs. MiRNAs are usually transcribed as miRNA precursors, which are processed by the DGCR8–Drosha complex, to produce a 60- to 70-nucleotide pre-miRNA. This pre-miRNA is exported to the cytoplasm and further cleaved by the Dicer complex into the mature form of miRNA. The mature miRNA is then loaded onto the Argonaute protein, forming a miRNA–protein complex known as the RNA-induced silencing (RISC). A subgroup of miRNAs termed oncomiRs, exert oncogenic action

through the binding and downregulation of tumour suppressor transcripts.

So far, 19 miRNA known to directly target PTEN have been identified in colorectal cancers, including miR-17 and miR-92a (cluster miR-17-92) (Tanaka et al. 2016; Zhang et al. 2013), miR-20b (Zhu et al. 2014) and miR-106a (cluster miR-106a-363) (Qin et al. 2018), miR-21 (Zhu et al. 2014), miR-26b (Fan et al. 2018), miR-29a (Wang et al. 2016), miR-32 (Wu et al. 2013), miR-103 (Geng et al. 2014), miR-106b (Zheng et al. 2015), miR-130b (Colangelo et al. 2013), miR-135b (Xiang et al. 2015), miR-181a (Wei et al. 2014), miR-200a (Li et al. 2016), miR-200c (Chen et al. 2014), miR-221 (Xue et al. 2013), miR-494 (Sun et al. 2014), miR-543 (Sun et al. 2017) and miR-582 (Song et al. 2017). In contrast, miR-22 suppresses the growth, migration and invasion of colorectal cancer cells through targeting Sp1 transcripts, resulting in PTEN up-regulation (Xia et al. 2017).

MiRNAs expression could therefore be targeted at different levels, *i.e.* transcription, processing, or via depletion or inactivation using antisense sequence or small molecule inhibitors (Nguyen and Chang 2018). As a proof of concept, the high throughput analysis of a library of pharmacologically active compounds allowed the identification of small molecule inhibitors of the (onco)mir-21 (Gumireddy et al. 2008). Anti-miRNA-221 sensitizes human colorectal carcinoma cells to radiation by upregulating PTEN (Xue et al. 2013). The MEK inhibitor PD0325901 suppresses expression of the miR-17-92 cluster and up-regulates PTEN in human colonic HT-29 cells (Tanaka et al. 2016).

The RNA-binding proteins Musashi-1/2 overexpressed in CRC bind *PTEN* transcripts leading to loss of the PTEN protein and to activation of the Akt pathway (Wang et al. 2015; Li et al. 2015; Fig. 5.1). The emerging role of Musashi proteins in carcinogenesis motivated several groups to screen libraries of small molecules in order to identify compounds that might disrupt the binding of Musashi proteins to RNA. Gossypol, a natural phenol derived from the cotton plant, was identified following screening. Interestingly, this inhibitor of RNA-binding proteins suppresses tumour growth in a mouse xenograft model and might constitute the basis for the development of more selective compounds (Kudinov et al. 2017).

5.2.3 PTEN Translation/PTEN Isoforms

In addition to the initial PTEN sequence encompassing 403 amino acid residues, longer isoforms have been recently identified (Hopkins et al. 2013; Liang et al. 2014; Tzani et al. 2016). These isoforms originate via translation from alternative start codons, distinct from the canonical AUG, and characterized by an extra in frame N-terminal sequence of 72 (PTEN-O), 131 (PTEN-N), 146 (PTEN-M/PTEN-β) and 173 amino acid residues (PTEN-L/PTEN-α) (Pulido et al. 2014; Tzani et al. 2016). Translation of the PTEN-L isoform was reported to be under the control of the eukaryotic translation initiation factor 2A (eIF2a) (Liang et al. 2014). All these isoforms retain phosphatase activity and down-regulate the PI3K/Akt pathways. Nevertheless, the N-terminal extension affects their subcellular localization. PTEN-L interacts with canonical PTEN to increase PTEN-induced kinase 1 (PINK1) levels and collaborates in mitochondrial bioenergetics through regulation of cytochrome c oxidase activity and ATP production (Liang et al. 2014). PTEN-M is localized to the nucleolus, where it binds and dephosphorylates, nucleolin, the nucleolar phosphoprotein resulting in inhibition of rDNA transcription, ribosomal biogenesis and cell proliferation (Liang et al. 2017).

PTEN-L harbours an N-terminal signal peptide secretion signal, is secreted from cells and can enter into other neighbouring cells (Fig. 5.1). As an exogenous agent, PTEN-L antagonizes PI3K signalling and induces tumour cell death *in vitro* and in mouse tumours xenograft after intraperitoneal injection (Hopkins et al. 2013). By providing a means to restore a functional tumour suppressor protein to tumour cells, PTEN-L may have therapeutic implications. In this context, a variant of this isoform was engineered by replacement of the native leader sequence of PTEN-L with a leader sequence from human light-chain

immunoglobulin G (IgG) to enhance cell-mediated protein delivery to neighbouring cancer cells (Lavictoire et al. 2018). Another prospect might be to exploit surrounding neighbouring non-transformed cells to produce PTEN-L. The eukaryotic translation initiation factor 2 (eI2F) plays an important role in the translation of this isoform (Liang et al. 2014).

5.2.4 Posttranslational Regulation

In the case where PTEN is expressed, several approaches could be devoted to increase its activity, including posttranslational modulation, stabilization of active conformations and modulating its subcellular localization.

5.2.4.1 Phosphorylation

PTEN is subjected to phosphorylation, mainly on serine/threonine residues located in the C-terminus (Thr366, Ser370, Ser380, Thr382, Thr383 and Ser385) (Odriozola et al. 2007). Casein kinase 2 (CK2) phosphorylates PTEN sequentially on Ser-385, Ser-380, Thr-383, Thr-382, and Ser-370, and reduces phosphatase activity and proteolysis by 70% (Torres and Pulido 2001; Cordier et al. 2012; Fragoso and Barata 2015) (Fig. 5.1). Despite T cell acute lymphoblastic leukemia (T-ALL) cells displaying normal levels of wild type *PTEN* mRNA and exhibiting PTEN overexpression, hyperphosphorylation of PTEN on the phosphorylation sites in its C-terminus by CK2, results in decreased PTEN lipid phosphatase activity and hyperactivation of the PI3K/Akt pathway (Silva et al. 2008). Incubation of T-ALL cell lines with the selective CK2 inhibitor CX-4945 reverses Akt activation and triggers apoptosis (Buontempo et al. 2014). Interestingly, CK2 is also overexpressed in CRC and colonic cell lines treated with CK2 inhibitor display decreased proliferation and invasiveness (Zou et al. 2011).

It has been proposed that phosphorylation of the C-terminal Ser380, Thr382, Thr383, Ser385 cluster induces a "closed" less active cytoplasmic form that has decreased plasma membrane targeting and increased conformational compaction (Vazquez et al. 2000; Vazquez et al. 2001; Das et al. 2003; Bolduc et al. 2013; Fig. 5.1). Intramolecular interaction of the phosphorylated C-terminal tail with basic residues within the N-terminal PIP2-binding motif, the catalytic and C2 domains maintains PTEN in its "closed" form (Rahdar et al. 2009). Mutation of the C-terminal residues disrupts the intramolecular interaction promoting an "open" form of PTEN with increased plasma membrane association to control PIP3 levels (Rahdar et al. 2009; Lima-fernandes et al. 2014). The "open" PTEN conformation also favours PTEN translocation to the nucleus (Nguyen et al. 2015) where it functions in DNA repair and genome stability independently of its lipid phosphatase activity (see below, PTEN ubiquitination). PTEN is also inhibited by the GSK-3β (phosphorylation of Ser362 and 366) and the MASTs (Microtubules associated Kinase 205, MAST3, phosphorylation of C-terminal tail) Ser/Thr kinases (Al-Khouri et al. 2005; Cordier et al. 2012; Fragoso and Barata 2015; Valiente et al. 2005). In contrast, phosphorylation of Ser-229/Thr-223 and Thr-319/Thr-321 amino acid residues by ROCK (RhoA-associated kinase) in the PTEN C2 domain enhances PTEN phosphatase activity (Li et al. 2005; Lima-Fernandes et al. 2011). Activation of ATM serine/threonine kinase (ataxia telangiectasia mutated) by DNA damage induces PTEN phosphorylation at Ser 113 leading to PTEN nuclear translocation and induction of autophagy (Chen et al. 2015). The interaction of glioma tumour suppressor candidate region 2-gene product, GLTSCR2/ 'protein interacting with carboxyl terminus 1′ (PICT-1) with PTEN favors phosphorylation of Ser-380 (Okahara et al. 2004).

PTEN is also a substrate for tyrosine kinases. Src phosphorylates PTEN at Tyr-240 and Tyr-315 leading to a decrease in phosphatase activity and stability of the tumour suppressor (Lu et al. 2003). Phosphorylation of tyrosine 336 by the tyrosine kinases Rak and FAK results in inhibition of PTEN polyubiquitination by NEDD4–1 and degradation by the proteasome (Yim et al. 2009; Tzenaki et al. 2015).

The polo-like kinase 1 (PLK1) is a regulator of many cell cycle-related events, including mitotic entry and the G2/M checkpoint, coordination of the centrosome and cell cycle, regulation of spindle assembly and chromosome segregation. PLK1 phosphorylates PTEN *in vitro* on Ser-380, Thr-382, and Thr-383, but not Ser-385. *In vivo*, only the Ser-380 amino-acid residue is significantly phosphorylated and this is associated with PTEN accumulation on chromatin (Choi et al. 2014). PTEN and PLK1 can reciprocally regulate each other. PTEN inhibits PLK1 by inducing its dephosphorylation, or by promoting the association of the E3 ligase APC/C with its activator CDH1, which induces the degradation of mitotic cyclins (Cyclins A and B), as well as mitotic kinases including PLK1 (Song et al. 2011; Zhang et al. 2016).

So far, few studies have reported the mechanisms related to PTEN dephosphorylation. The N-myc downstream-regulated gene 2 (NDRG2) is a molecular partner of PTEN that recruits protein phosphatase 2A (PP2A) resulting in dephosphorylation of PTEN at the Ser380, Thr382 and Thr383 (Nakahata et al. 2014). Interestingly, NDRG2 is frequently down-regulated in CRC. The Tyrosine phosphatase SHP-1 dephosphorylates PTEN in Src transfected cells and restores PTEN stability (Lu et al. 2003). Some orally bioavailable small molecule activators of PP2A (SMAPs) efficiently inhibited the growth of KRAS-mutant lung cancers in mouse xenografts and transgenic models (Sangodkar et al. 2017).

5.2.4.2 Oxidation

PTEN is subjected to reversible inactivation by reactive oxygen species (ROS) produced by the membrane associated Duox1/2, NAPDH oxidase (Noxs) and mitochondrial oxidative stress. Hydrogen peroxide (H_2O_2) inactivates PTEN by promoting oxidation of the critical Cys124 residue in the catalytic domain of PTEN and forming an intramolecular disulfide bond with Cys71 (Lee et al. 2002; Leslie et al. 2003). This inhibition is reversed by thioredoxin (Fig. 5.1). This regulation process might occur under physiological conditions. Accordingly, it has been proposed that cell stimulation by EGF triggers PI3Kinase activation that induces NOXs activation. The resulting ROS inactivate PTEN leading to further accumulation of PIP3 to complete a positive feedback loop (Kwon et al. 2004). Binding of thioredoxin-1 to PTEN Cys212 of the C2 domain of PTEN inhibits PTEN membrane translocation and activation (Meuillet et al. 2004).

Hypoxia, a hallmark of tumours, promotes transcriptional inhibition of AIF (tumour apoptosis-inducing factor) through HIF-1 (hypoxia induced factor-1), resulting in oxidative inactivation of PTEN and epithelial–mesenchymal transition of colorectal cancer (Xiong et al. 2016).

Oxidation of PTEN-binding partners can also affect PTEN activity. For example, the oncogene DJ-1 binds to PTEN and reduces its catalytic activity. Oxidation of DJ-1 increases its affinity for PTEN, resulting in more profound decreases in PTEN activity (Kim et al. 2009). ROS might also affect PTEN indirectly via pro-inflammatory redox-sensitive pathways, such as NF-κB.

Scavengers of ROS, such as sodium pyruvate, which reacts with H_2O_2 to yield sodium acetate, carbon dioxide and water, and anti-inflammatory agents therefore constitute approaches to restore PTEN activity.

5.2.4.3 S-Nitrosylation

Ischemia, superoxide anion, hydrogen peroxide and nitric oxide (NO) can trigger S-nitrosylation of protein cysteine residues. It was reported that low NO concentrations lead to S-nitrosylation of Cys-83 leading to PTEN inactivation (Numajiri et al. 2011). The NO scavenger c-PTIO efficiently prevents PTEN S-nitrosylation.

5.2.4.4 Acetylation

The Histone Acetylase (PCAF)/lysine acetyltraansferase 2B (KAT2B) has been reported to promote PTEN acetylation on Lys125 and Lys128 in response to growth factor stimulation (Okumura et al., 2006). As these residues are within the catalytic pocket, acetylation negatively regulates its enzymatic activity. PTEN is also acetylated on Lys402, which is located within the C-terminal PDZ-domain-binding motif of PTEN, by CREB-binding protein (CREBBP) favouring

PTEN interaction with proteins with PDZ domains (Ikenoue et al. 2008). CREBBP and the sirtuin SIRT1 have been identified as the main PTEN acetyltransferase and deacetylase, respectively. Interestingly, PCAF forms a complex with CREBBP.

It has also been recently demonstrated that non-selective Histone Deacetylase (HDAC) or HDAC6-specific inhibitors switch PTEN into an open conformation and induce its membrane translocation through acetylation at Lys163, resulting in the inhibition of cell proliferation, migration and invasion, as well as xenograft tumour growth in athymic mice (Meng et al. 2016). Such inhibitors may be clinically relevant to treat tumours with wild-type PTEN.

5.2.4.5 Mono/Polyubiquitinylation Proteasome

PTEN is regulated by ligation of the protein modifiers ubiquitin (76 amino acids) on the Lys amino-acid residues 13 and 289. NEDD4 was identified as an E3 ligase that ubiquitylates PTEN (Wang et al. 2007). Other E3 ligases have also been reported to target PTEN, including X-linked inhibitor of apoptosis protein (XIAP) and WWP2. Polyubiquitination of PTEN leads to its degradation by the proteasome complex, whereas monoubiquitylation is essential for PTEN nuclear import (Wang et al. 2007, Trotman et al. 2007 (Fig. 5.1). Although NEDD4 proved to be overexpressed in colorectal cancer (Kim et al. 2008), its effect on the growth and morphology of human colonic cell lines seems to be independent of PTEN (Eide et al. 2013).

The monoubiquitylation of PTEN and its nuclear compartmentalization, is reversed by the deubiquitylase USP7 (Song et al. 2008). Nuclear exclusion of PTEN has been associated with cancer progression. Some inhibitors of USP7 are under development in several bio-pharmaceutical companies (Zhou et al. 2018).

5.2.4.6 Sumoylation

PTEN can be modified by the small ubiquitin-like modifier (SUMO) on Lys254 and Lys266 in the C2 domain. SUMOylation, principally at Lys266, in the CBR3 loop, which plays a major role in PTEN membrane association, was shown to promote binding to the plasma membrane via electrostatic interactions (Huang et al. 2012). This leads to decreased PI3K/AKT signalling, suppression of anchorage-independent cell growth and tumour growth *in vivo*. Subsequently it was shown that SUMOylation of Lys254 controls PTEN nuclear localization (Fig. 5.1). Following cell exposure to either γ-irradiation or DNA-damaging chemotherapeutic agents, SUMO conjugated PTEN was excluded from the nucleus in an ATM protein kinase manner. Cells lacking nuclear PTEN were hypersensitive to DNA damage.

Several other studies have shown that there may be competition between SUMOylation and ubiquitination. Gonzalez-Santamaria et al. (2012), showed that Lys289 can also be SUMOylated. As Lys289 is also a major site for PTEN monoubiquitination, which drives nuclear import, competition for modification on this site would be predicted to affect nucleocytoplasmic partitioning. In another study, PTEN SUMOylation was shown to be enhanced by the SUMO E3 ligase PIASxα, resulting in reduced PTEN polyubiquitination and increased stability, culminating in negative regulation of the PI3K/AKT pathway, cell proliferation inhibition and tumour suppression (Wang et al. 2014).

5.2.4.7 Ribosylation

PTEN can also be ribosylated by tankyrases (TNKS1 and TNKS2) on Glu40/Glu150 in the phosphatase domain and Asp326 in the C2 domain. This promotes the recognition of PTEN by an E3 ubiquitin ligase, RNF146, leading to subsequent PTEN ubiquitination and degradation. Knockdown of TNKS1/2 in colorectal cancer cell lines resulted in the inhibition of tumour growth in PTEN-expressing cells but not in PTEN-depleted cells. This indicates that targeting TNKS in tumour cells may only be effective in wild-type PTEN contexts. Interestingly, expression of tankyrases was found to be negatively correlated with PTEN levels in human colon carcinomas. Combined, all these findings support the rationale to explore the development of tankyrase inhibitors as potential anti-cancer agents.

5.2.4.8 Other Postranslational Modifications of PTEN

PTEN is also subject to S-sulfydration on both Cys71 and Cys124, which has been proposed to prevent the S-nitrosylation associated with inhibition of PTEN catalytic activity. PTEN can also be methylated on Lys313 by the oncogenic protein methyltransferase SET and MYND domain containing 2 (SMYD2), which has been proposed to result in negative regulation of PTEN activity and increased PI3K/AKT signalling (Nakakido et al. 2015).

5.2.5 Protein-Protein Interactions

PTEN interacts with many effector systems through its different protein domains (lipid binding, catalytic, C2 domain and the PDZ binding motif in the C-terminus), which are crucial for its localization as well as for the organization of a variety of sub-membranous complexes associated with cell signal mediators, including ion channels, transmembrane receptors and regulatory enzymes (Harris and Lim 2001; Kotelevets et al. 2005, Chastre et al. 2009, Lima-Fernandes et al. 2011).

The cellular activity of PTEN is thus commonly modulated via inclusion in multiprotein signalosomes (Fig. 5.1).

Modulating protein–protein interactions involved in disease pathways is an attractive strategy for developing drugs, but remains a challenge to achieve. One approach is to target certain domains within proteins that mediate these interactions (Berg 2003; Arkin et al. 2004, 2014). One example of such a domain is the PDZ domain (Dev 2004). Proteins with PDZ domains usually encompass a series of such domains, alone or combined with other protein-protein interaction domains, and act as scaffolding molecules allowing the organization of effector proteins as signalosomes and their targeting to selective cellular subdomains.

A series of proteins with PDZ domains interact with the C-terminus of PTEN, these include MAGI-1/2/3, NHERF, MAST3, hDLG1. We demonstrated that PTEN was recruited to E-cadherin junctional complexes through the interaction with the 2nd PDZ domain of MAGI-1, whereas the C-terminus of β-catenin interacts with PDZ5. The colocalization of PTEN and PI3K and their antagonistic activities on PIP3 levels allows the subtle regulation of junctional complex activities (Kotelevets et al. 2001, 2005; Chastre et al. 2009). Subsequently, we identified by yeast two-hybrid analysis human DLG, a protein with multiple PDZ domains, as a binding partner for the PTEN PDZ-BD and demonstrated Dlg1-PTEN interaction in colonic HT-29 epithelial cells (Kotelevets, unpublished data).

Recently, Zaric *et al* identified MAGI-1 as a celecoxib-induced inhibitor of Wnt/β-catenin signalling with tumour- and metastasis-suppressive activity in colon cancer cells. They reported that this Cox-2 inhibitor upregulated MAGI-1 in human colonic cell lines and that MAGI-1 overexpression attenuated primary tumour growth and spontaneous lung metastasis in an orthotopic model of colorectal cancer (Zaric et al. 2012). One interesting point that was not addressed in their study concerns the role of PTEN in this process. Interestingly, another study reported that celecoxib promoted the membrane translocation of PTEN and the inactivation of Akt (Zhang and Gan 2017). Taken together, these data suggest that inhibition of Cox-2 leads to increased expression of MAGI-1 and the subsequent targeting of PTEN to the plasma membrane.

The widespread occurrence of PDZ domains as organizers of signalling pathways makes them an important subject for biological studies. Changes in the expression of several PDZ domain-containing proteins have been associated with cancers (Nagayama et al. 2004; Park et al. 2006). The therapeutic usefulness of inhibiting PDZ-based protein-protein interactions has been clearly demonstrated by using peptide and non-peptide small molecules (Aarts et al. 2002; LeBlanc et al. 2010). Because PDZ domains have well-defined binding sites, they are promising targets for drug discovery. However, there is still much to learn about the function of these domains before drugs targeting PDZ interactions can become a reality. The first cell-permeable inhibi-

tor of a PDZ interaction was reported in a study that described how the interaction between the PDZ domain of MAGI and the PDZ motif of PTEN was irreversibly blocked by a low-molecular-mass compound. The interaction between MAGI and PTEN is thought to regulate the activity of the kinase Akt/PKB. Compound treatment of HCT116 cells expressing endogenous PTEN, MAGI and Akt/PKB showed enhanced AKT activity (Fujii et al. 2003). By creating analogue libraries, the structure of the compound was suggested to be a useful starting point for finding class- and domain-selective inhibitors. Further chemical optimization could render the compound useful as a tool for exploring the effects and side effects of inhibiting PDZ interactions *in vivo* (Fujii et al. 2007).

The multifunctional scaffolding proteins β-arrestins (β-arrs) control distinct functional outputs of PTEN to regulate cell proliferation, migration and multicellular assembly. β-arr binding to PTEN increases its lipid phosphatase activity and inhibits cell proliferation. However, during cell migration of glioma cells, β-arr binds the C2 domain of PTEN to inhibit its lipid phosphatase-independent anti-migratory function (Lima-Fernandes et al. 2011). β-arr1 also binds the C2 domain of PTEN as part of a membrane-associated regulatory complex incorporating the Cdc42 GTPase-activating protein ARHGAP21 and Cdc42 (Fig. 5.1). This complex controls Cdc42-dependent mitotic spindle formation and lumen formation in 3D cultures of colorectal cancer cells. Disruption of the complex provokes mitotic spindle misorientation and abnormal multilumen formation that are evocative of colorectal cancer (Javadi et al. 2017).

PTEN interacts *via* its phosphatase domain with homodimers of the p85α regulatory subunit of the PI3K (PIK3R1). Importantly, this interaction positively regulates the lipid phosphatase activity of PTEN and impairs PTEN degradation by competing with the E3 ligase WWP2 (Rabinovsky et al. 2009; Chagpar et al. 2010; Cheung et al. 2015). Thus, PIK3CA overexpression or PIK3R1 mutations could lead to PI3K pathway hyperactivation by decreasing PTEN expression and activity.

Many disparate proteins interact with PTEN and negatively regulate its tumour suppressing activity by a wide variety of mechanisms. These proteins include DJ-1, α-mannosidase 2C1 (Man2C1), shank-interacting protein–like 1 (SIPL1), and PI(3,4,5)P3-dependent RAC exchange factor2a (PREX2a) (He et al. 2010, 2011; Fine et al. 2009). Man2C1, PREX2a and SIPL1 bind directly to PTEN and inhibit its lipid phosphatase activity.

Furthermore, paxillin, an important adaptor protein of focal adhesions, was identified as an interaction partner of PTEN (Herlevsen et al. 2007). A recent study showed that PTEN downregulates paxillin expression in human colon cancer tissues via the PI3K/AKT/NF-κB pathway and that paxillin expression contributes to colon tumourigenesis (Zhang et al. 2015).

Greater understanding of the pathophysiological relevance *in vitro* and *in vivo* will be critical in strategies for developing drugs toward modulating protein-protein interactions.

5.2.6 Controlling PTEN Conformation and Subcellular Localization

In addition to the bioactive small molecules targeting positive or negative PTEN regulators mentioned above, the development of alternative strategies to control PTEN conformation and subcellular localization might constitute powerful approaches to restore or enhance PTEN tumour suppressor activity. As a proof of concept, Nguyen et al. screened a library of randomly mutated human PTEN and identified mutations that increase its recruitment to the plasma membrane. This enhanced PTEN (ePTEN) exhibited an eightfold increase in ability to suppress PIP3 signalling (Nguyen et al. 2014). These findings open up interesting new perspectives on pharmacological strategies that could therefore be harnessed to achieve enhanced

forms of PTEN using small molecule conformational activators/stabilisers. In relation to this, an intramolecular bioluminescence resonance energy transfer (BRET)-based biosensor of PTEN with PTEN sandwiched between the energy donor Renilla luciferase (Rluc) and the energy acceptor yellow fluorescent protein (YFP) was recently described that can report signal-dependent conformational changes of PTEN in live cells (Lima-Fernandes et al. 2014; Misticone et al. 2016). The PTEN biosensor could therefore potentially be used as conformational readout in high-throughput screens to identify small molecules that enhance or restore PTEN function.

Another interesting point concerns the balance in the subcellular localisation of PTEN at the plasma membrane, in the cytosol, mitochondria, endoplasmic reticulum and nucleus. Some strategies succeeded in targeting PTEN to the plasma membrane (Meng et al. 2016; Zhang and Gan 2017) to exert tumour suppressor activity. Whether the nuclear pool of PTEN is affected remains an interesting question. Enhancing PTEN targeting to the endoplasmic reticulum may promote Ca2+ release and sensitivity to apoptosis. As stated above promoting PTEN nuclear exclusion should sensitize cancer cells to genotoxic agents. Based on the observation that cancer cells are more prone to export nuclear PTEN, further studies are required to delineate the benefit to induce pharmacological nuclear exclusion of PTEN and the impact on neighbouring non-transformed cells.

Another level of complexity resides in the heterogeneous PTEN distribution at the plasma membrane and its contribution as a member of molecular signalling complexes. During chemotaxis, PTEN and PI3K exhibit a reciprocal pattern of localization, PI3K being located at the leading edge and PTEN at the rear (Li et al. 2005). PTEN is also recruited to E-cadherin junctional domains and likewise PTEN is also recruited to the plasma membrane with β-arr2 following GPCR stimulation. Complementary approaches, such as the development of permeant bi-functional nanobodies might allow targeting PTEN in specific subdomains.

5.3 Toward Targeted Therapies

Experimental models of carcinogenesis using transgenic mice reveal that PTEN inactivation cooperates with the main genetic alterations identified in human CRC, including KRAS activation, and APC and TGFBR2 inactivation, to promote cancer progression (Davies et al. 2014; Shao et al. 2007; Marsh et al. 2008; Yu et al. 2014). Restoring PTEN expression/activity should therefore benefit all patients with colorectal cancer, regardless of the subtype. Nevertheless, particular attention should be devoted in the case of activation of downstream effectors controlled by PTEN, such as Akt1 mutation (Carpten et al. 2007).

It should be underlined that as for other targeted therapies, the strategy proposed here will require the identification of patients who are most likely to respond to the treatment and to define the appropriate and personalized approach to restore PTEN activity. Due to the higher rate of PTEN mutations in the subgroup of patients with CRC high microsatellite instability (15% of CRC), the MSI-h status of the tumour will direct to PTEN sequencing. Typing of CRC for MSI and analysis of gene mutations, e.g. *KRAS* are performed routinely in clinical practice. In the case of PTEN deletion or mutation, (since genome editing is far away from being used in the clinic) an alternative approach would be to take advantage of PTEN deficiency-related defects in homologous recombination. This defect sensitizes tumour cells to inhibitors of polyadenosine diphosphate ribose polymerase (PARP), involved in the repair of DNA double-strand breaks (Dillon and Miller 2014). Five clinical trials are evaluating the efficiency of PARP inhibitors in connection with PTEN status (NCT02286687, NCT02401347, NCT03207347, NCT03016338, NCT02576444).

In cases where PTEN is wild-type, immunohistological analysis of PTEN accumulation, subcellular localisation, and activation of downstream PI3K targets (Akt, S6K) could be monitored to provide information on the level of dysregulation (transcriptional, post-transcriptional, post-translational).

Ex vivo testing of organotypic CRC slices cultured on porous membrane supports would permit to simultanenously screen a series of selected compounds, based on the level of PTEN dysregulation identified by immunochemistry and to assess the restoration of the activity of the tumour suppressor. A series of permeant fluorescent labeled probes are now available to monitor *in situ* tissue response to treatment: live or dead cells, enzyme activities (*e.g.* caspases). Proof of concept to test individual tumour responses to anti-cancer drugs was recently provided using a 96-well plate-based microfluidic device that allows to expose organotypic slices to multiple compounds either simultaneously or sequentially (Chang et al. 2014).

It is also conceivable to optimize the identification of PTEN defects in these organotypic slices using fluorescently-labelled permeant nanobodies targeting selective PTEN epitopes and/or downstream effector systems. FRET could then be used as readout to report changes in PTEN conformation or subcellular localisation, or upon molecular assembly of signalosomes, *e.g.* TORC1 complex.

5.4 Conclusions and Prospects

This review illustrates the diversity and complexity of the mechanisms that can downregulate PTEN function during carcinogenesis. Restoring/enhancing PTEN activity in colonic cancer cells may represent a promising therapeutic approach, since it would be predicted to directly impact cell growth, trigger apoptosis, but also increase tumour cell sensitivity to therapeutic agents. This is a critical issue, since anti-cancer treatments have dose-limiting toxicities.

Further studies are required to elucidate the cross-talk between PTEN and other (anti-) oncogene pathways during carcinogenesis, and their significance in terms of resistance to chemotherapies. Nevertheless, knowledge gleaned in how PTEN signalling is regulated will provide the basis to explore the potential of a personalized approach to restore/enhance PTEN activity in cancer.

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6 Wnt Signalling-Targeted Therapy in the CMS2 Tumour Subtype: A New Paradigm in CRC Treatment?

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Abstract

Colorectal cancers (CRC) belonging to the consensus molecular subtype 2 (CMS2) have the highest incidence rate, affect mainly the distal colon and rectum, and are characterized by marked Wnt/β-catenin/Transcription Factor 7-Like 2 (TCF7L2) pathway activation and also by activation of epidermal growth factor receptor (EGFR) signalling. Despite having the highest overall survival, CMS2 tumours are often diagnosed at stage III when an adjuvant chemotherapy-based regimen is recommended. Nevertheless, colorectal cancer stem cells (CSCs) and circulating tumour cells may still evade the current therapeutic options and metastasize, stressing the need to develop more tailored therapeutic strategies. For example, activation of EGFR signalling is being used as a target for tailored therapy, however, therapy resistance is frequently observed. Therefore, targeting the Wnt signalling axis represents an additional therapeutic strategy, considering that CMS2 tumours are "Wnt-addicted". Several efforts have been made to identify Wnt antagonists, either of synthetic or natural origin. However, an inverse gradient of Wnt/β-catenin/TCF7L2 signalling activity during CRC progression has been suggested, with early stage and metastatic tumours displaying high and low Wnt signalling activities, respectively, which lead us to revisit the "just-right" signalling model. This may pinpoint the use of Wnt signalling agonists instead of antagonists for treatment of metastatic stages, in a context-dependent fashion. Moreover, the poor immunogenicity of these tumours challenges the use of recently emerged immunotherapies. This chapter makes a journey about CMS2 tumour characterization, their conventional treatment, and how modulation of Wnt signalling or immune response may be applied to CRC therapy. It describes the newest findings in this field and indicates where more research is required.

Keywords

CMS2 therapy · Immunomodulation · Just-right signalling · Nutraceuticals · Wnt antagonists/agonists · Wnt signalling

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6.1 CMS2 – A Coin of Two Faces – The Highest Response to Chemotherapy *vs.* the Lowest Immunogenicity

6.1.1 Introducing the CMS2 Tumour Subtype

CMS2 (canonical) tumours display an epithelial-like gene expression profile compatible with the activation of canonical pathways involved in colorectal tumorigenesis. Accordingly, they are characterized by marked activation of Wnt and MYC pathways and up-regulation of downstream target genes associated with CRC. They also show the activation of other signalling pathways like EGFR and vascular endothelial growth factor (VEGF). This subtype of tumours has the highest incidence rate (approximately 37%) among all CMS and is localised mainly in the distal colon and rectum, in contrast with other CMS. These tumours are microsatellite stable (MSS) and characterized by high chromosomal instability (CIN), presenting the highest number of copy number gains in oncogenes and copy number losses in tumour suppressor genes compared with the other CMS. Other characteristic features are high frequency of mutations in the *adenomatous polyposis coli* (*APC*) and in the tumour protein p53 (*TP53*) genes, a lack of CpG island methylation phenotype (CIMP), high expression of the oncogenes *EGFR*, erb-b2 receptor tyrosine kinase 2 (*ERBB2*, also known as *HER2*), insulin-like growth factor 2 (*IGF2*), insulin receptor substrate 2 (*IRS2*) and transcription factor hepatocyte nuclear factor 4α (*HNF4A*), as well as cyclins. Patients with these tumours display the highest overall survival, although they are often diagnosed at stage III (Guinney et al. 2015; Inamura 2018). Five-year overall survival for all CMS2 stages are the highest of any subtype, reaching 77%, compared with 73%, 75% and 62% for CMS1, 3 and 4, respectively, and presented higher survival rates after relapse (35 months) (Guinney et al. 2015; Thanki et al. 2017).

The EGFR signalling pathway plays a crucial role in the regulation of the cellular response to growth signals and its constitutive activation in CRC promotes growth and proliferation through the Kirsten rat sarcoma viral oncogene homolog (KRAS)/RAF/mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) axes (Wee and Wang 2017). In CMS2, activation of EGFR signalling is mainly at the expense of *KRAS* mutations. Mutations in the B-Raf proto-oncogene, serine/threonine kinase (*BRAF*) gene are rare, presenting the lowest mutation frequency among all CMS subtypes, although *KRAS* mutations are relatively frequent among all tumour subtypes. Of note, the poor prognosis of *KRAS* mutations applies only to MSS tumours with CMS2/CMS3 epithelial-like gene expression profiles. Accordingly, *KRAS* mutations had the strongest prognostic value in epithelial (CMS2/3) MSS tumours, with statistical significance only in CMS2. Indeed, patients with *KRAS*-mutated CMS2 and MSS tumours had an overall survival rate of 59%, significantly lower than the corresponding 75% survival rate for wildtype-*KRAS* patients (Smeby et al. 2018). Contributing to this poorer prognosis may be the recent association of *KRAS* mutations with suppressed cytotoxic immunity of T helper (Th) 1 cells in CRC, and hence reduced immune reactivity, irrespective of mismatch repair (MMR) status, tumour location and transcriptional subtype, particularly in CMS2 and CMS3 tumours (Lal et al. 2018).

6.1.2 Current Chemo- and Targeted Therapies in CMS2 Tumours: Where Are We?

Approximately 39% of CMS2 cancers are stage III at the time of diagnosis and standard adjuvant chemotherapy is recommended at this stage (Thanki et al. 2017). In high-risk stage II and III CRC, a combination of fluoropyrimidine based therapies such as 5-fluorouracil (5-FU), leucovorin and oxaliplatin (FOLFOX) or capecitabine with oxaliplatin (XELOX) are generally used (Labianca et al. 2013).

Interestingly, treatment of the CMS2 subgroup showed to be sensitive to either 5-FU and/or

oxaliplatin. Indeed, evaluation of *in vivo* efficacy of this chemotherapy in patient-derived xenograft (PDX) models led to the observation of a delay in outgrowth of CMS2 (Linnekamp et al. 2018). Recently, it was shown that these CRCs benefitted significantly from adjuvant chemotherapy treatment in both stage II and III. Additionally, Isella and colleagues sub-stratified tumours to identify specific tumour-intrinsic traits associated with response to standard-of-care treatment, using a combination of transcriptional subtyping based on gene expression from the tumour epithelial cells only and independent of stromal-derived signals combined with CD8 immunohistochemistry. CMS2 tumours were divided in 3 subgroups with a common background of high Wnt signalling: (i) intrinsic subtypes [CRIS] C (elevated EGFR/ERBB signalling, sensitivity to EGFR inhibitors, often *KRAS wild-type*), (ii) D (Wnt activation- leucine rich repeat-containing G protein coupled receptor 5 (LGR5) stem cell signature, *IGF2* gene overexpression and amplification, often *TP53* wild-type) and (iii) E (Wnt-producing Paneth cell-like phenotype, often *TP53* and *KRAS* mutated). Notably, only the CRIS-C subtype significantly benefitted from adjuvant chemotherapy in stage II and III, while CRIS-D significantly benefitted in stage III only. CRIS-C patients with low levels of CD8+ tumour-infiltrating lymphocytes (TILs) were most at risk of relapse in both stages and should be treated with adjuvant chemotherapy. These results are particularly relevant to identify within the CMS2 group those patients who can benefit from adjuvant standard-of-care chemotherapy (Allen et al. 2018; Isella et al. 2017).

Notwithstanding, one cannot overlook that in patients undergoing chemotherapy prior to surgery this neoadjuvant regimen may modulate the gene expression signatures/profiles of tumours, in some cases towards a more mesenchymal-like phenotype with a poorer prognosis, thus influencing their accurate CMS connotation (Trumpi et al. 2017).

An increased understanding of colorectal carcinogenesis pathways involved in tumour invasion and metastasis has led to the development of monoclonal antibodies (mAbs) to EGFR, namely cetuximab or panitumumab, to block this receptor, thereby preventing activation of signal transduction pathways involving RAS, PI3K/AKT and SRC kinase (Zhao et al. 2017a). The effectiveness of these mAbs has been confirmed by phase II and III clinical trials. Similarly, blocking VEGF/VEGFR signalling proved to be effective across different treatment lines in metastatic CRC (mCRC) and contributed greatly to improve patients' survival in recent years (Hopirtean and Nagy 2018).

Extensive molecular characterization of CRC and correlation with response to therapy identified *KRAS* and *NRAS* mutations as negative predictive markers of response to anti-EGFR mAbs (Allegra et al. 2016; Amado et al. 2008; Karapetis et al. 2008; Van Cutsem et al. 2011; Zhao et al. 2017a). These mutations are the only ones approved by the Food and Drug Administration (FDA) as molecular biomarkers of response to therapy in CRC. In the case of VEGF signalling and tyrosine kinase inhibitors (TKIs), biomarker discovery has proved to be more problematic as they have multiple targets.

Considering the abovementioned, treatment of mCRC follows in general a combinatory therapy in first and second line based on FOLFOX or 5-FU/leucovorin/irinotecan (FOLFIRI) (de Gramont et al. 2000; Grothey et al. 2004), which may include combinations with (at least) an anti-EGFR mAb for RAS wild-type patients and/or an anti-VEGF compound (e.g. the mAbs bevacizumab and ramucirumab, the recombinant fusion protein aflibercept, or the multi-kinase inhibitor regorafenib) (Van Cutsem et al. 2016).

Of note, the abovementioned activation of EGFR and VEGF signalling characteristic of CMS2 makes this subgroup attractive to be tackled by therapies targeting these pathways. Accordingly, this tumour subtype was particular sensitive to anti-EGFR mAbs in preclinical models, especially in comparison with CMS1 and CMS4 (Linnekamp et al. 2018; Sveen et al. 2018). Indeed, distal carcinomas, particularly of CMS2 phenotype, may also carry *EGFR* and *IRS2* amplifications, which are markers of cetuximab sensitivity. Strikingly, a retrospective study involving CRC patients from two clinical trials

(CRYSTAL and FIRE-3) concluded that the survival benefit of the anti-EGFR therapy was restricted to patients diagnosed with a distal primary tumour. Patients with distal tumours also had an improved rate of overall survival following first-line therapy with FOLFIRI plus cetuximab vs FOLFIRI alone or FOLFIRI plus bevacizumab (Tejpar et al. 2016).

The introduction of next-generation sequencing of clinical samples and the inclusion of circulating tumour DNA (ctDNA) analyses, together with the development of preclinical models, led to the identification of additional mutational events, although none of them is used yet as predictive marker of response to therapy. Among these are *EGFR*, *ERBB2* and MET proto-oncogene, receptor tyrosine kinase (*MET*) amplification, and mutations in mitogen-activated protein kinase kinase 1 (*MAP2K1*, also known as *MEK1*), *ERBB2*, fibroblast growth factor receptor 1 (*FGFR1*), *EGFR* and platelet derived growth factor receptor alpha (*PDGFRA*) genes (Dienstmann et al. 2017). Interestingly, *EGFR* mutations are the only alterations detected exclusively after treatment with EGFR inhibitors, but not prior to this treatment. Recently, tumours with *EGFR* copy-number below 4.0 appeared also to be as refractory to anti-EGFR treatment as tumours with mutation in any of the RAS/RAF/PIK3CA pathway genes (Algars et al. 2017). In contrast, quadruple wild-type tumours (*KRAS*, *NRAS*, *BRAF* and *PIK3CA*) have been reported as sensitive to dual EGFR targeting, including TKIs combined with anti-EGFR mAbs, both of which inhibit EGFR through different mechanisms (Weickhardt et al. 2012).

Making use of *ERBB2* amplification for targeted therapy, in a heavily pre-treated *KRAS* wild-type subgroup of patients with advanced-stage CRC, substantial clinical benefit was recently reported with a dual HER2-targeted regimen, trastuzumab (HER2-inhibitor), in combination with the TKI lapatinib (Sartore-Bianchi et al. 2016), or with anti-EGFR mAbs, resulting in tumour regression in PDXs (Kavuri et al. 2015). It has also been reported a substantial clinical benefit in patients treated with biomarker-driven HER2-targeted therapies, with response rates and duration of response that compared closely with those observed in immunotherapy (Hurwitz et al. 2017). Alongside with the observed sensitivity to anti-EGFR therapy, CMS2 tumours also showed to respond to HER2 inhibitors (Sveen et al. 2018), which indicates *HER2* as a driver oncogene in CRC and a potential biomarker for targeted treatment, particularly in CMS2.

Moreover, as actionable *ERBB2* and *IGF2* copy number gains, that potentially drive resistance to anti-EGFR mAbs in patients with wild-type *KRAS/NRAS* genotype, are enriched in CMS2 tumours, the CMS2 subtype appears to be the most appealing to test combinations of pan-ERBB and IGF1R inhibitors (Sveen et al. 2018).

Altogether, these findings mark the emergence of the 'multi-gene, multi-drug' paradigm of precision medicine and liquid biopsies upsurge as a powerful tool to provide information about primary therapy resistance or evasion mechanisms to guide clinical decisions. Indeed, mutations in the *RAS/RAF* genes emerged in a large proportion of tumour/liquid biopsy/ctDNA samples from patients whose tumours were initially diagnosed as wild-type for *KRAS* pre-treatment. In one third of cases, multiple events coexist in the same sample (Sawada et al. 2018). Notably, ctDNA analyses in different time-points along treatment have shown that the percentage of *KRAS*-mutated alleles increased on anti-EGFR treatment and declined after drug withdrawal (Dienstmann et al. 2017).

Despite the partial success of conventional chemotherapeutic regimens (FOLFIRI or FOLFOX) and of targeted therapies in the treatment of colorectal tumours and patient survival (Cunningham et al. 2004; Hurwitz et al. 2004; Tournigand et al. 2004), cells with a metastatic/stemness-like signature eventually evade therapy in a great proportion of CRCs (Dasari et al. 2013; Dylla et al. 2008; Mertins 2014; Zhao et al. 2017b). Indeed, up to 30–50% of mCRC patients exhibit intrinsic tumour chemoresistance and almost all who are responsive at the beginning may eventually acquire chemoresistance, stressing the need for novel first-line mCRC therapeutic approaches and biological markers towards a more personalized medicine (Anderson et al.

2011; de Gramont et al. 2000; Douillard et al. 2000; O'Connell et al. 2008; Patil et al. 2017). Therefore, an alternative to intermittent administration of targeted therapies may be drugging common genomic alterations besides those of MAPK pathway, such as mutations in the Wnt/β--catenin axis, as previously suggested (Dienstmann et al. 2017). This is particularly valid in the CMS2 subtype considering the "Wnt-addiction" of these tumours. Therefore, Wnt signalling targeted therapy will be the recipient of the major focus of this chapter, including immunotherapy that despite the low immunogenicity of CMS2 subtype deserves a special highlight.

6.1.3 Harnessing the Full Potential of Immunotherapy in the Low Immunogenic CMS2 Subtype

Immunotherapy has increasingly proven to be a key treatment modality that can make a significant impact on the lives of many cancer patients. Immune checkpoint inhibitors targeting the programmed cell death protein 1 (PD-1) pathway have led to remarkable clinical benefits in various cancers. Currently, immune checkpoint inhibitors already approved for cancer treatment include ipilimumab (anti-cytotoxic T-lymphocyte-associated protein 4 (anti-CTLA4)), nivolumab, pembrolizumab (anti-programmed death-1 (anti-PD-1)), and atezolizumab (anti-programmed death ligand-1 (anti-PD-L1)) (Pai et al. 2017). Le and colleagues reported the results of a phase II trial with the anti-PD-1 immune checkpoint inhibitor pembrolizumab and established a positive correlation between somatic mutation prevalence and the clinical success in PD-1 axis blockade (Le et al. 2015). This becomes extremely relevant for CRC, which presents higher rates of somatic mutations in comparison to other solid tumours (Network 2012). However, compared to CMS1, CMS2 cancers have a low mutation rate (defined as non-hypermutated, or <8 mutations per 10^6 bases (Thanki et al. 2017), predicting low immunogenicity. This was recently confirmed when Becht and colleagues (Becht et al. 2016) conducted transcriptomic analyses of the immune, fibroblastic and angiogenic microenvironment, using 1388 CRC samples from three independent discovery and validation cohorts. With the aim to integrate the immune score previously defined by Galon and colleagues (histology-based analysis of the cancer's invasive margin or central location of memory and cytotoxic TILs) (Galon et al. 2006, 2007) and the inflammatory microenvironment data within the CMS subtypes, they revealed that the CMS2 group displays low immune and inflammatory signatures and are typically PDL1-negative. This low immune signature was extended to low expression of markers of cytotoxic lymphocytes, low densities of CD8 T cells and CD68 macrophages (Becht et al. 2016), lack of TILs and immunoregulatory cytokines in the microenvironment, which suggests that these tumours are poorly immunogenic (Colangelo et al. 2017; Dienstmann et al. 2017). Although increased expression of major histocompatibility complex, class I (MHC-1) genes, E (*HLA-E*) and G (*HLA-G*), primarily observed in CMS1 tumours, is also found in a subset of CMS2, immunosuppressive genes are weakly expressed in CMS2 tumours. This suggests a different mechanism of immune escape in these subtypes, such as the involvement of other oncogenic pathways leading to complete immune avoidance by exclusion of T cells from the tumour site (Roelands et al. 2017). For instance, the Wnt/β--catenin signalling pathway, which is activated in most epithelial tumours, correlates with T cell exclusion across solid tumours and cause suppression of *CCL4* gene transcription (Roelands et al. 2017) required for recruitment of dendritic cells (DCs). Hence, this immune escape mechanism might contribute to the low immunogenicity observed in CMS2 tumours.

Like the remaining CMS, CMS2 exhibited significant enrichment of innate immune cells (macrophages M0 and M1, activated mast cells and neutrophils) and significant depletion of plasma cells, resting mast cells and resting DCs. Frequent enrichment was also observed for regulatory T cells (Tregs) and memory activated Th and frequent depletion of cytotoxic T lymphocytes

(CTLs), memory resting Th and macrophages M2 (Karpinski et al. 2017).

CMS2 displayed the highest enrichment in Th cells (naive and memory activated) and memory B cells. In contrast, they were characterised by low number of cancer-associated fibroblasts (CAFs) and endothelial cells and by significantly lower influx of leukocytes in their active states, with exception of anti-tumour memory B cells and active T CD4 memory cells, and reduced immune activation (Karpinski et al. 2017). Nonetheless, the use of check-point inhibitors to modulate immunogenicity in CRC treatment was not yet very promising, except for the MSI/CMS1 group (Le et al. 2015; Singh et al. 2015b). Therefore, for the poorly immunogenic CMS2 tumours, other approaches such as adoptive T cell therapies or cancer vaccines with DCs to stimulate tumour infiltration with antigen-specific CTLs130 have been proposed (Becht et al. 2016; Jackie Oh et al. 2016).

Strikingly, another strategy under investigation is the combination of immune modulators and anti-EGFR therapy in a *RAS* wild-type population, reflecting the notion that the immune system contributes to the therapeutic effects of mAbs. The mechanism is based on the recently discovered association of *KRAS* mutations with suppressed Th1/cytotoxic immunity in CRC, and hence reduced immune reactivity (Lal et al. 2018). Considering this for CMS2 tumours, one possible combination might be anti-EGFR mAb cetuximab (used in pan-RAS wild-type CRCs with EGFR signalling activation) with anti-PD-1 antibody as immune checkpoint inhibitor, as already studied in non–small-cell lung cancer (NSCLC) (Chen et al. 2015).

Although CMS2 tumours exhibit low levels of neo-antigens, the expression of tumour-specific antigens at the cancer cell surface (e.g. mucin 1, carcinoembryonic antigen, EGFR and HER2/neu) has been positively correlated with antitumor immune responses in CRC. Their potential as targets for immune-based CRC therapies, for example using vaccines or other cellular therapy strategies was already revised elsewhere (Riley et al. 2018).

Alternatively, chemotherapeutic regimens might be also used to shape immune microenvironment of cancer liver metastases. Accordingly, multiple trials are investigating the value of combined administration of standard radio- and chemotherapies known to induce immunogenic cell death (ICD) of CRC cells with alternative agents that can increase expression of T cell chemokines and enhance T cell infiltration in a non-antigen-specific way. For example, histone deacetylase (HDAC) inhibitors (HDACis) were found to increase expression of multiple T cell chemokines in cancer cells, macrophages and T cells, resulting in enhanced T cell infiltration and PD-1 sensitivity (Zheng et al. 2016), which may assume particular relevance in CMS2 therapy as this subtype is characterised by reduced expression of T cell attracting chemokines and resistant to PD-1 inhibition (Dienstmann et al. 2017; Roelands et al. 2017). Interestingly, the combination of the HDACi romidepsin with anti-PD-1 therapy is now being tested in advanced CRC phase I trial (NCT02512172).

Another aspect to consider is that standard radio- and chemotherapies affect the patient's immune response. For example, in a cohort of mCRCs that was highly enriched for CMS2 (*TP53* and *APC* mutated, despite *KRAS* mutations) and treated with neoadjuvant chemotherapy (based on fluoropyrimidine with oxaliplatin or irinotecan), two clusters of liver metastases were identified based on high or low signalling and expression profiles of genes related with ICD (Ostrup et al. 2017). Thus, the activation of immune response in this biological scenario following neoadjuvant chemotherapy might be balanced by feedback mechanisms and immune escape, suggesting that immune therapy might be used in combination with ICD-promoting agents (e.g. conventional neoadjuvant chemotherapy) to overcome immunosuppression and improve the treatment for liver metastases for certain subgroups (Ostrup et al. 2017). However, different chemotherapeutic regimens may trigger different immune responses. For instance, FOLFOX may reduce the levels of circulating myeloid-derived suppressor cells and hence immunosuppression,

while FOLFIRI may elicit the opposite effect in advanced CRC (Kanterman et al. 2014).

Moreover, combination of oxaliplatin with the cyclophosphamide was also explored as another potential combinatory therapy to subvert a checkpoint inhibition-resistant solid tumour into a sensitive one (Pfirschke et al. 2016). Further research will be needed to ascertain oxaliplatin's role in activation of immune responses and its application in the context of CRC immunotherapy (Riley et al. 2018).

As above mentioned, several pieces of evidence have supported that radiotherapy can also activate the host immune system by various mechanisms, including the increased presentation of tumour neoantigens, MHC-I expression, and immune-activating chemokines and cytokines (e.g. interferon-γ) in the tumour microenvironment, hence representing a promising strategy for MSS cancers (Derer et al. 2016; Riley et al. 2018). Interestingly, enhanced antitumor responsiveness arising from the potential synergy between radiotherapy immune priming and checkpoint inhibition, with either CTLA-4– or PD-1–directed therapy alone or in combination, was already demonstrated in early phase I clinical trials (Deng et al. 2014; Twyman-Saint Victor et al. 2015). Hence, to overcome CRC cells' resistance to radiotherapy (e.g. PD-1 upregulation), it would be feasible/interesting to combine the former with PD-1-targeted therapy to achieve best clinical outcomes in CRC treatment, as suggested elsewhere (Riley et al. 2018). Accordingly, a phase II trial combining nivolumab, ipilimumab and radiation therapy in MSS and MSI-high CRC and pancreatic cancer (NCT03104439), as well as combination of anti-PDL-1/PD-1 therapy with radiotherapy or modified FOLFOX, are currently on-going (NCT02437071; NCT02375672) (Riley et al. 2018; Roelands et al. 2017).

Although 5-FU has demonstrated synergy with radiotherapy *in vitro*, one should be careful considering that low-dose fractionated radiotherapy combined with 5-FU may inhibit the immune-priming effects of radiotherapy. These observations would have serious consequences for patients with CMS2 rectal cancers because nowadays combined neoadjuvant 5-FU/radiotherapy treatment remains the gold standard option for these cases (Sauer et al. 2004).

Finally, the Wnt/β-catenin signalling pathway has been linked to modulation of immune cell infiltration in the tumour microenvironment, i.e. to immune evasion, as it has been recently associated with suppression of CD4+ T cell immunity in CRC (Fu et al. 2015; Pai et al. 2017; Spranger and Gajewski 2015; Sun et al. 2017). Moreover, tumours deprived from T cell infiltration are bad candidates for immunotherapy and have a poorer prognosis (Gajewski 2015).

According to a segregation analysis based on gene expression profiles of almost 9000 tumour samples, about one third of them was characterized as non-T cell inflamed. *APC*, *AXIN1/2* and *CTNNB1* (encoding β-catenin) mutations were associated to a non-T cell inflamed gene signature. Besides, β-catenin was inversely correlated with CD8+ T cell infiltration. Altogether, these results suggest β-catenin expression as a predictive tool to select the patient cohort that might benefit from checkpoint inhibition therapy, as reviewed elsewhere (Pai et al. 2017).

Interestingly, it is plausible to hypothesize that by inhibiting the Wnt/β-catenin signalling pathway in CMS2 tumours, one could improve CD8+ T cell infiltration and priming, enabling a scenario in which immune checkpoint inhibition may be effective. Therefore, future preclinical and clinical assays should consider and explore the potential role of Wnt/β-catenin signalling modulators as possible adjuvants to immune checkpoint inhibitors (e.g. anti-PD1, anti-CTLA4, anti-PD-L1) (Pai et al. 2017).

Overall, CMS2 tumours are poorly immunogenic and lack immune cell infiltration, requiring the right combinatory therapeutic approach to convert these "cold" tumours to "hot" tumours targetable by immunotherapeutic approaches, that is, towards a CMS1-like reactive immune phenotype.

Altogether, a better patient stratification regarding the CMS group and tumour immunogenicity, a better knowledge of the cellular and molecular mechanisms behind the absence of immune cells within the tumour microenvironment, as well as the identification of novel

biomarkers for immune response will be required to achieve a more personalized therapy with the best clinical outcome (Roelands et al. 2017).

6.2 Wnt Targeted Therapy in CMS2 Tumours: Antagonizing or Agonizing, a Double-Edged Sword?

The Wnt/β-catenin pathway regulates several aspects of embryonic development and adult tissue homeostasis, controlling cell self-renewal, differentiation and apoptosis. Particularly, it participates in the control of intestinal homeostasis and maintenance of the intestinal stem cell pool/niche (Mah et al. 2016). Indeed, this signalling axis is one of the most frequently deregulated pathways in several tumour types, especially in CRC where it reaches about 90% of cases.

Briefly, under normal physiological conditions, activation of the canonical Wnt signalling begins with the binding of secreted Wnt ligands (e.g. Wnt3a and Wnt1) to a complex of the Frizzled (FDZ) receptor and low-density lipoprotein-related protein 5/6 (LRP5-6) co-receptor. Phosphorylation of LRP co-receptors by protein kinases CK1 (casein kinase 1) and GSK3β (glycogen synthase kinase 3 beta) stimulates the membrane recruitment of Dishevelled (DVL) and Axin proteins and this inhibits the assembly of the β-catenin destruction complex – mainly composed by APC, Axin-1/2, CK1 and GSK3β proteins-, thus enabling β-catenin stabilization by preventing its cytoplasmic degradation via the ubiquitin-proteasome pathway (Novellasdemunt et al. 2015; Stamos and Weis 2013; Zhan et al. 2016). This leads to cytoplasmic accumulation of β-catenin, which then is translocated into the cell nucleus where it forms a transcriptional complex with members of the TCF/LEF family of transcriptions factors (namely TCF7L2) and regulates the expression of Wnt signalling target genes that regulate important cellular processes like cell proliferation, apoptosis and stemness (MacDonald et al. 2009; Novellasdemunt et al. 2015; Zhan et al. 2016).

On the other hand, in the absence of extracellular Wnt ligands or signals from other signalling cascades, the N-terminus of β-catenin is phosphorylated within the destruction complex (by GSK3 and CK1) stimulating the recruitment of E3 ligase β-TrCP towards the complex. This promotes β-catenin ubiquitination, targeting it for proteosomal degradation, thus restraining the transcription of Wnt target genes (MacDonald et al. 2009; Novellasdemunt et al. 2015; Zhan et al. 2016).

However, the balance of this signalling may be disturbed by the existence of mutations in Wnt signalling players, mostly in *APC* and *CTNNB1*, that result in ineffective turnover/degradation of β-catenin and, ultimately, in hyperactivated signalling. Hence, in this scenario, the aberrantly stabilised intracellular β-catenin translocates into the nucleus and constitutively activates the expression of Wnt/β-catenin target genes that culminate in unrestrained cell growth, being one of the hallmarks of colorectal tumorigenesis and progression. Though, even in the presence of mutations that drive CRC onset and progression, different levels of β-catenin may exist in CRC cells, according to the "just-right" signalling model (Albuquerque et al. 2002, 2010, 2011; MacDonald et al. 2009; Novellasdemunt et al. 2015; Zhan et al. 2016).

Since the unrestrained hyperactivation of Wnt/β-catenin signalling pathway, is the hub of CMS2 tumour onset and progression, targeting this pathway represents a highly attractive path along the road to precision therapy. Strikingly, this has been recently reinforced by the finding that among adenomas, the CMS2 subtype was the most frequent subtype and at higher risk of progression, thus representing an opportunity for the development of targeted therapies to counteract Wnt/β-catenin signalling in early tumour stages. In agreement with this is the finding that *Apc* restoration in a mouse model of CRC whereby *Apc* was suppressed, induced sustained tumour regression without relapse (Dow et al. 2015). Several attempts have been made to discover and/or develop Wnt signalling inhibitors, ranging from small-molecule compounds, to

antibodies, peptides and even natural bioactive compounds, that may target this pathway through different mechanisms: "non-specific" or disruption of the ligand/receptor, the β-catenin destruction and the nuclear/transcriptional factor complexes, respectively (Krishnamurthy and Kurzrock 2018; Novellasdemunt et al. 2015; Zhan et al. 2016). Even so, most compounds targeting Wnt signalling are still in preclinical or early clinical trial phases or were withdrawn, since it seems difficult to circumvent the toxicity inherent to the inhibition of this pathway, due to its crucial role in regulation of intestinal homeostasis and the stem cell niche (Lu et al. 2016; Mah et al. 2016).

So far, Wnt signalling inhibition in colorectal tumorigenesis has only been accomplished clinically by "generic"/non-specific Wnt signalling modulators, already approved for other malignancies, so called nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin and sulindac (Krishnamurthy and Kurzrock 2018; Novellasdemunt et al. 2015). Among NSAIDs, aspirin has been reported to supress colorectal neoplasia, and has been suggested as a chemopreventive agent, as its regular use correlates with lower CRC incidence even in predisposed individuals (Burn et al. 2011; Cao et al. 2016). This NSAID may act either by inhibiting the cyclooxygenase-2/prostaglandin-E2 (COX-2/PGE2) pathway, by promoting β-catenin ubiquitination and its proteosomal degradation in a COX-2-independent manner, or by targeting the NF-KB pathway, with subsequent decrease in Wnt transcriptional activity (Gala and Chan 2015). Nonetheless, despite the effect of NSAIDs, several of these compounds are associated with numerous gastrointestinal toxicity-related side effects (Gala and Chan 2015).

Next, we will summarize some of the most well-known and new Wnt negative modulators identified so far, either synthetic or of natural origin. Then, we will relate the feasibility of Wnt antagonists in CRC therapy and discuss the application of Wnt agonists, in a tumour stage-dependent scenario.

6.2.1 Wnt Antagonists: Tailored Treatment for Downregulating Wnt Signalling

6.2.1.1 Small Molecules Targeting TCF7L2/β-Catenin Transcriptional Complex Players

Several efforts have been made towards the development of small molecules targeting the TCF7L2/β-catenin complex, either by disrupting directly the binding between these two players or indirectly by targeting β-catenin (Krishnamurthy and Kurzrock 2018; Novellasdemunt et al. 2015; Yan et al. 2017). PKF115-584, ICG-001 (PRI-724), PKF118-310, CGP049090 and NCB-0846 are some of the small-molecule inhibitors of this transcriptional complex that have been often cited (e.g. by disrupting β-catenin interaction with TCF7L2, LEF-1 or APC, or TCF/DNA), despite the widespread biological side-effects that this inhibition might have (Krishnamurthy and Kurzrock 2018; Novellasdemunt et al. 2015; Yan et al. 2017). Among these, ICG-001 binds selectively to the N-terminus of CREB binding protein (CBP) and blocks the interaction between this coactivator and β-catenin, restraining TCF7L2/β-catenin transcriptional activity (Emami et al. 2004). Although it reached the clinical trial phase in combination with chemotherapy and bevacizumab, it was withdrawn without results due to drug supply issues (NCT02413853).

Additionally, several other TCF/β-Catenin complex inhibitors have been identified among synthetic compound libraries by *in silico* virtual screening, high throughput screening or structural optimization of lead compounds, based on the crystal structure of these signalling players and other biochemical assays (Fang et al. 2016; Masuda et al. 2016; Tian et al. 2012; Yan et al. 2017). For instance, BC21 may block TCF7L2/β--Catenin complex assembling by occupying part of the TCF7L2 binding pocket of β-catenin and blocking the establishment of hydrophobic interactions in key binding sites and TCF7L2 binding,

which correlated with downregulation of Wnt target genes, inhibition of cell viability and colony formation (without cytotoxicity on normal HEK293 and HUVEC cells) (Tian et al. 2012).

Wnt signalling may also be regulated by Traf2- and Nck-interacting kinase (TNIK) that regulate directly the Wnt transcriptional complex via phosphorylation of TCF7L2, which turns TNIK blocking into an appealing strategy to supress this pathway (Masuda et al. 2015; Shitashige et al. 2010). In this context, NCB-0846, a small molecule that occupies the ATP-binding pocket/cavity of TNIK in an inactive conformation, was identified to abrogate its kinase activity and disturb the TCF7L2/β-catenin interaction. *In vitro* this compound has shown to impair tumour-initiation ability of colorectal CSCs and to downregulate the expression of mesenchymal markers (Slug, Snail, Twist, Smad2 and Vimentin) in HCT116 cells, while *in vivo* it inhibited Wnt-driven CRC onset in $Apc^{min/+}$ mice. However, its effect might not be fully selective towards Wnt signalling targeting, since it also may interfere with other signalling pathways (Masuda et al. 2016).

Another antagonist potentially appealing for CMS2 therapy is the newly identified 4-(5-Fluoro-1 H - B e n z o [d] I m i d a z o l - 2 - y l) - N , NDimethylaniline (HI-B1) compound that, in PDX studies, showed a selective anticancer effect against tumours showing high expression levels of nuclear β-catenin relatively to tumours presenting low nuclear expression levels of this protein. Therefore, HI-B1 anticancer effect appears to depend on high levels of Wnt/β-catenin signalling that are typical of CMS2 tumours (Shin et al. 2017). HI-B1 inhibits Wnt transcriptional complex by inhibiting the interaction between TCF7L2 and β-catenin by directly targeting the latter, which correlated with its ability to downregulate β-catenin levels, to induce apoptosis and to decrease cell growth *in vitro,* in either anchorage-dependent or -independent conditions, and *in vivo,* in PDX tumours and Apc^{min} mouse models (Shin et al. 2017).

Due to signalling pathway crosstalk, peroxisome proliferator–activated receptor isotype y (PPARy) assembles with TCF7L2 and interacts with β-catenin (Jansson et al. 2005). In agreement with this, treatment of HCT116 cells with FG535 blocked the recruitment of the coactivators β-catenin and glutamate receptor-interacting protein 1 (GRIP1) to PPARy, suggesting that this compound may impair the assembly of a complex containing these players and binding to DNA at TCF/LEF-binding sites, inhibiting target gene expression (Handeli and Simon 2008). More relevant results on the biological effects of FG535 in CRC were reported recently, showing its potential to downregulate the expression of invasiveness-related (matrix metallopeptidase-7/9 (*MMP7*/*MMP9*), snail family transcriptional repressor 1 (*SNAI1*) and Vimentin) and Wnt target genes (including those encoding for Axin-2 and cyclin D1), which correlated with impaired cell proliferation, stemness and migratory and invasion skills in HT29 and SW480 cells, as well as with lower tumour xenograft growth *in vivo* (Chen et al. 2017a).

6.2.1.2 Small Molecules Targeting the APC/β-Catenin/Axin Destruction Complex

The multi-subunit destruction complex – involving APC, Axin-1/2, the kinases GSK3β and CK1, PAD2 (peptidyl arginine deiminase 2) and the E3-ubiquitin ligase β-TrCP – acts as the key gatekeeper for regulation of β-catenin levels and, hence, β-catenin-mediated gene transcription (Novellasdemunt et al. 2015; Stamos and Weis 2013; Zhan et al. 2016), making its players important targets for modulation of Wnt signalling.

One of the most well described mechanism is the stabilization of Axin activity by inhibition of Tankyrase-1 and -2 (TNKS1 and TNKS2) activity that belong to the poly(ADP-ribose) polymerases (PARPs) family. TNKS have been described as modulators of protein stability and turnover of components of the degradation complex, including the cellular levels of β-catenin (Lehtio et al. 2013; Mariotti et al. 2017). Indeed, it was demonstrated that these enzymes may participate in modulation of Axin stability by binding at its N-terminus (close to the binding site of APC) and performing poly-ADP-ribose modifications (PARsylation) in this protein, which elicits its polyubiquitination and further degradation via

ubiquitin-proteosome pathway (Huang et al. 2009; Morrone et al. 2012). More recently, it was shown that TNKS play a crucial role in the formation of the β-catenin destruction complex by being recruited by either APC2 or Axin into degradasomes (cytoplasmic puncta), thus regulating β-catenin degradation (Croy et al. 2016; Martino-Echarri et al. 2016; Thorvaldsen et al. 2015). Hence, TNKS inhibitors (TNKSi) may act by stabilizing the interaction and promoting the assembly of TNKS and AXIN in degradasomes in which β-catenin is degraded (Croy et al. 2016; Martino-Echarri et al. 2016).

Several small-molecule TNKSi towards CRC therapy have been identified in the last years, including XAV939 (Huang et al. 2009), IWR-1 (Chen et al. 2009), JW55 (Waaler et al. 2012), JW74 (Waaler et al. 2011) and its analogue G007-LK (Lau et al. 2013), NVP-TNKS656 (Arques et al. 2016), G-631 (Zhong et al. 2016), K-756 (Okada-Iwasaki et al. 2016) and AZ1366 (Quackenbush et al. 2016). They revealed anticancer activity in CRC cells, however, the study of their biological effects is still in its infancy.

Moreover, a recent report has highlighted the feasibility of synergizing TNKS inhibition with conventional chemotherapeutic agents. For example, XAV939 was combined with 5-FU and/or Cisplatin with overall results showing significant increase in apoptosis, upregulation of Axin and downregulation of β-catenin and CSCs markers in SW480 and SW620 cells (Wu et al. 2016). Also, AZ1366 combination with irinotecan displayed improved ability to impair tumour growth in explants, although its mechanism might not be via modulation of Wnt signalling (Quackenbush et al. 2016). Additionally, potential synergy between NVP-TNKS656 and PI3K/AKT signalling pathway inhibitors (triciribine and 4-hydroxytamoxifen) and MEK inhibitors (AZD6244) has also been suggested (Arques et al. 2016; Schoumacher et al. 2014).

Interestingly, Tanaka and colleagues have shown that short-form APC mutant proteins lacking all the β-catenin-downregulating motifs (20 amino acid repeats, 20AARs) might be used as potential predictive biomarkers for CRC sensitivity to TNKSi, while cells expressing "long" APC mutant protein that retain two or more of these motifs might be resistant (Tanaka et al. 2017). In their study, induced downregulation of long-form APCs with two 20AARs led to increased Wnt/β--catenin signalling levels and upregulated expression of its target gene *AXIN2*, and turned cells sensitive to treatment with TNKSi (Tanaka et al. 2017). This is extremely relevant according to the "just-right" signalling model (Albuquerque et al. 2002, 2010, 2011) and CMS2 "Wnt-addiction", mostly driven by *APC* mutations, as abovementioned. Accordingly, it is known that *APC* mutant genotypes that lead to truncated proteins retaining 1 or, less frequently, none of the 20AARs, are more frequent in MSS distal tumours (Albuquerque et al. 2010, 2011; Christie et al. 2013) which are highly represented in CMS2 tumours. This results in higher β-catenin signalling levels than that obtained from truncated proteins retaining 2 or 3 20AARs, which are more frequent in the proximal colon and consequently in other CMS subtypes. Hence, it is plausible to suggest that TNKSi may be promising Wnt signalling modulators specially to counteract CMS2 tumorigenesis. One possible application might be to circumvent radio-chemoresistance mediated by hyperactivation of Wnt signalling in rectal tumours.

Despite none of the identified Wnt signalling antagonists have been applied so far in CRC therapy, an antagonistic effect was recently reported for nitazoxanide, an already FDA-approved drug for antiparasitic purposes, bringing closer the Wnt antagonists to the clinical practice. Indeed, nitazoxanide can inhibit this pathway in a GSK3β- and APC-independent manner by directly targeting and stabilizing PAD2 that catalyses protein citrullination/deamination (a post-translational modification that converts an arginine residue in citrulline). In this context, treatment with this drug demonstrated an increase in citrullination and degradation of β-catenin (dependent on the proteasome but not on ubiquitination) in HCT116 and SW480 cells, disrupting its transcriptional activity, measured as lower expression levels of Wnt target genes, impaired colony-formation ability *in vitro,* and lower occurrence of intestinal adenomas *in vivo* (Qu et al. 2018).

6.2.1.3 Small-Molecule Inhibitors of Upstream Signalling Players: Targeting Ligands and Receptors

Lastly, the ligand-receptor interaction has been one of the most well explored signalling interfaces, with several on-going clinical trials, to block hyperactivated Wnt signalling, using either small molecules or antibodies designed against Wnt ligands or FZD receptors to disrupt ligand/receptor interaction at the cell surface (Gurney et al. 2012; Krishnamurthy and Kurzrock 2018; Lu et al. 2016). Some FZD receptors have been associated with CRC cells survival, invasiveness and metastasis, and in the case of FZD-7 its overexpression might also be associated with a poor prognosis (Ueno et al. 2009).

However, as emphasized in a recent review, targeting the ligand/receptor interface might be of limited efficiency, because the receptor signals upstream of *APC* that is found mutated in about 80–90% of CRCs (Phesse et al. 2016), in particular in the CMS2 subtype. Nevertheless, considering the "just-right" signalling model, the targeting of Wnt receptors might still decrease Wnt signalling in some *APC* mutant tumours since not all *APC* mutations have the same impact on signalling activity and can retain some functionality (Albuquerque et al. 2002, 2010, 2011; Phesse et al. 2016). Accordingly, CRCs harbouring *APC* mutations still responded to FZD7 inhibitors although the precise mechanism remains to be clarified (Phesse et al. 2016; Vincan et al. 2005).

In this context, the antibody Vanituctumab (OMP-18R5) has shown the ability to target FZD-1, -2, -5, -7 and -8 receptors in several solid tumours by binding directly to a conserved epitope within their extracellular domain, and its use in a CRC PDX model correlated with inhibition of tumour growth and tumour-initiating cell frequency, besides displaying synergetic effects with the conventional drug irinotecan (Gurney et al. 2012). However, this antibody did not show anticancer effect in *APC* or *CTNNB1* mutated CRCs, contrary to *APC* and *CTNNB1* wild-type tumours (Gurney et al. 2012). This suggests that Vanituctumab may not have successful outcome/effectiveness in the context of CMS2 therapy, however this might also be due to its non-specific and wide spectrum of action among FZD receptors.

Although several small FZD inhibitors with potential anticancer activity in CRC have been explored so far, much remains to be understood about the diversity of Wnt ligands and FZD receptors and the biological effects of Wnt-FZD interactions (Kikuchi et al. 2011).

Additionally, other small-molecule Wnt modulators targeting the co-receptor LRP5/6 extracellular domain have been identified (Jackson et al. 2016; Lee et al. 2018). GSK3178022 is an antibody against LRP6 and was shown to block stimulation by both Wnt and R-Spondin (RSPO) ligands, possibly by disturbing LRP6 turnover or by blocking more downstream signalling events. By hindering LRP6 stimulation, GSK3178022 was shown to impair the expression of Wnt target genes in *in vitro* preclinical assays using either CRC cell lines or PDX models (Jackson et al. 2016).

Another strategy is neutralizing Wnt ligands directly or blocking their secretion by the so-called Porcupine (PORCN) inhibitors (Krishnamurthy and Kurzrock 2018). PORCN is an enzyme that catalyses post-translational palmitoylation of Wnt ligands that is necessary for their extracellular secretion (Takada et al. 2006). Several PORCN inhibitors have been identified for CRC and other solid tumours therapy, namely PORCN inhibitors LGK974 (Liu et al. 2013; van de Wetering et al. 2015) and ETC-1922159. Both were already subject to Phase I/II trials in CRC and/or other solid tumours, but conclusive results are still unavailable (NCT02278133; NCT01351103; NCT02521844).

Finally, in the category of Wnt ligand neutralization, OMP-131R10 stands out as an anti-RSPO3 antibody targeting RSPO3-positive mCRCs and is currently in phase I trial in combination with FOLFIRI regimen, still waiting for results (NCT02482441). Also in phase I trial for mCRC (NCT02655952) is Foxy-5, which mimics the Wnt5a peptide, a Wnt antagonist that has been found silenced in CRCs by promoter methylation and whose expression has been shown to impair β-catenin activity and suppress cell proliferation, migration and invasion (Zhu et al. 2014).

However not all CRC patients may respond in the same way to upstream signalling antagonists. They may carry mutations in downstream players and a substantial part would be refractory due to *APC* mutations. Further research will be needed to establish a stratification of the patients that might best respond to this type of antagonists, namely against FZD7, taking into account the *APC*/*CTNNB1* mutation status and the "just-right" signalling model (Albuquerque et al. 2002, 2010, 2011; Phesse et al. 2016).

6.2.1.4 Nutraceuticals Inhibiting Wnt Signalling: How Nature Can Help Shaping CRC Therapy

As discussed so far, there is a plethora of chemically engineered compounds designed to modulate the Wnt signalling pathway, most of them by antagonizing its main effectors. Notwithstanding, Nature also offers several natural compounds that may antagonize CRC onset and progression. The strong evidence from epidemiological studies, supporting a positive correlation between a high intake of fruits and vegetables with a lower risk of CRC incidence, has been drawing attention for the application of natural compounds in CRC chemoprevention and therapy (Lanou and Svenson 2011; Pericleous et al. 2013; Song et al. 2015). Due to their presence in plants, especially fruits and vegetables, and their bioactivity in prevention and treatment of several human pathologies, such as cancer, these bioactive compounds have been termed phytochemicals and/or nutraceuticals. Nutraceuticals comprise several natural compounds with different structure and botanical origin, including phenolic compounds (e.g polyphenols, like flavonoids, curcuminoids, stibenes and tannins), terpenoids (e.g. carotenoids and antioxidative vitamins), and glucosinolates (precursors of isothiocyanates, ITCs), among others (Kuppusamy et al. 2014; Liu 2004; Pan et al. 2011).

Interestingly, the anticancer potential of nutraceuticals relies on their multi-target mechanism of action, either by exerting apoptotic, antioxidant, antiangiogenic or antiproliferative activities, but also by modulating different signalling pathways involved in cancer stemness, metastasis and drug resistance (Chang and Yu 2016; Kuppusamy et al. 2014; Liu 2004; Pan et al. 2011; Pistollato et al. 2015; Priyadarsini and Nagini 2012). In this section, the most recent evidence of nutraceutical roles in CRC prevention and therapy will be addressed, focusing on specific nutraceuticals with ability to modulate the Wnt signalling pathway and consequently to target CMS2 tumour subtype.

A study carried out by our group, using a 3D model of HT29 CRC cell spheroids, has demonstrated that ITCs and ITCs-enriched natural extracts derived from different cruciferous vegetables may differentially target the Wnt signalling pathway. For instance, watercress extract and its main ITC phenethyl isothiocyanate (PEITC), decreased β-catenin mRNA levels, whereas broccoli extract and its main ITC sulforaphane, downregulated *TCF7L2* and *AXIN2* expression. Both ITCs and extracts had the ability to reduce cell proliferation, stemness and metastatic potential of CRC cells (Pereira et al. 2017). Notwithstanding, further studies will be required to scrutinize the primary mechanisms of ITC action in complex cellular scenarios.

Regarding phenolic compounds, another study performed by our group has demonstrated the effect of polymethoxyflavones (PMFs)-enriched orange peel extract in the same 3D CRC cell model with downregulation of the expression of the Wnt target gene and known CRC stemness marker *LGR5*, in a dose-dependent manner, an effect mainly attributable to tangeretin in this extract (Pereira 2016; Pereira et al. unpublished data).

In addition, epigallocatechin-3-gallate (EGCG), a green tea-derived catechin, has been shown to modulate Wnt/β-catenin/TCF signalling pathway by downregulating β-catenin expression in two mouse models of intestinal tumorigenesis, preventing intermediate and advanced stages of CRC (Orner et al. 2002). Moreover, EGCG has demonstrated to induce the shuttling of β-catenin from the nucleus towards cytoplasm and cell membrane in HT29 cells, while increasing the expression of E-cadherin and decreasing the expression of Wnt target genes, c-Myc and cyclin D1. *In vivo* assays with the $Apc^{min/+}$ mouse model of intestinal tumorigen-

esis, demonstrated that oral uptake of this reduced aberrant nuclear β-catenin and the levels of activated, phosphorylated ERK1/2 and AKT (at Ser473) proteins (Ju et al. 2005). More recently, using CSC-enriched cell spheroids generated with DLD-1 and SW480 cells, Chen and coworkers demonstrated the ability of EGCG to target colorectal CSC subpopulations by suppression of the Wnt/β-catenin signalling pathway. They observed upregulated expression of GSK3β and decreased levels of phosphorylated GSK3β (Ser9), alongside with a decrease in β-catenin and c-Myc levels. Besides its antiproliferative effect and ability to induce apoptosis, exposure to this nutraceutical also impaired significantly spheroid formation (in number and size) together with downregulation of stemness markers (Nanog, Oct-4, CD44, CD133 and ALDHA1) in a dose-dependent manner (Chen et al. 2017b).

Resveratrol, another polyphenol mainly found in peel of red grapes (Singh et al. 2015a), has also shown promising effects in Wnt signalling including impaired β-catenin nuclear translocation, leading to lower c-Myc and MMP-7 expression, impaired cell proliferation, invasion and migration (Ji et al. 2013). Another study reported it to induce mitochondrial-mediated apoptosis, to impair cell proliferation, sphere formation and translocation of nuclear β-catenin in human colorectal CSCs *in vitro*, along with downregulation of Wnt targets (c-Myc and Cyclin-D1), and to diminish tumour incidence *in vivo* (Reddivari et al. 2016).

Another compound that modulates Wnt signalling is curcumin, a phytochemical extracted from *Curcuma longa* (root of turmeric). In a study using SW620 cells, treatment with this nutraceutical led to downregulation of Wnt signalling players (β-catenin and TCF7L2), Vimentin and CXCR4, in parallel with upregulation of E-cadherin, Axin and NKD2 (the latter two acting as negative regulators of Wnt signalling). This supports the role of curcumin in suppression of Wnt signalling, EMT, and, therefore, in CRC invasion and metastasis (Zhang et al. 2016). Similar results obtained for SW480 cells suggest that curcumin may also induce its antiproliferative effect by modulating Wnt signalling via suppression of miR-130a expression (Dou et al. 2017).

The flavonoid apigenin has also been shown to modulate CRC cell proliferation, migration, invasion and organoid growth *in vitro* in a dose-dependent manner by the ability to inhibit β-catenin nuclear translocation (Xu et al. 2016). Apigenin may also decrease the levels of cytosolic and nuclear β-catenin through induction of its autophagy-mediated lysosomal degradation, as a consequence of AKT/mTOR signalling inhibition (Lin et al. 2017).

Aside, phytochemicals may also target the β-catenin/TCF7L2 transcriptional complex. For instance, resveratrol may also act downstream of GSK3β by destabilizing directly the interaction between β-catenin and TCF7L2 in a dose-dependent manner, leading to a lower transcriptional activation of Wnt-responsive genes and impaired cell growth in both Wnt-stimulated and *APC*-mutated CRC cells (Chen et al. 2012). Also, 11α, 12α-epoxyleukamenin E (EPLE), an ent-kaurane diterpenoid extracted from *Salvia cavaleriei*, has shown to selectively exert antiproliferative effect in CRC cells by occupying and disrupting the binding interface of β-catenin/TCF7L2 complex and, hence, its ability to act as a transcriptional activator, as corroborated by the downregulation of the expression of Wnt signalling target genes, co-immunoprecipitation and X-ray crystal structure results (Ye et al. 2015).

Epigenetic regulation is another mechanism of action of nutraceuticals to modulate Wnt signalling and cancer cell proliferation and growth, mostly by restoring Wnt antagonists' function. In addition to modulating β-catenin levels, genistein may also be involved in the upregulation of Dickkopf-related protein 1 (DKK1), another Wnt antagonist, by inducing histone H3 acetylation of the DKK1 promoter (Wang et al. 2012). PEITC may also alter the epigenetic profile of CRC cells through modulation of histone epigenetic regulators, induction of different DNA methylation patterns (e.g. hypomethylation of Polycomb-group complex target genes) and suppression of HDAC binding to euchromatin, along with upregulation of apoptosis-related genes (Park et al. 2017).

Notwithstanding, one of the most appealing and promising aspects of nutraceuticals' application in CRC therapy in a near future might be their potential combination with chemotherapeutic regimens to avail possible anticancer synergies, thus enabling lowering chemotherapy dosage. Consequently, this would allow to decrease toxicity and side-effects of conventional chemotherapeutic drugs and, hence, an uninterrupted therapy regimen (Redondo-Blanco et al. 2017). Last but not least, by combining chemotherapy with nutraceuticals with known anti-stemness and anti-metastatic potential, this combinatory regimen may also allow to prevent tumour metastasis and recurrence by circumventing CSC-associated chemoresistance, for instance to 5-FU, the gold standard chemotherapeutic drug in CRC therapy as demonstrated for EGCG (Toden et al. 2016), resveratrol (Buhrmann et al. 2018), curcumin (Bachmeier et al. 2018; Jalili-Nik et al. 2018; Shakibaei et al. 2015; Toden et al. 2015; Wei et al. 2018) and PMFs-enriched orange peel extract (Pereira 2016; Pereira et al. unpublished data).

Accordingly, a phase I clinical trial of curcumin in combination with FOLFOX in 12 CRC patients with inoperable liver metastases has been conducted and the dose escalation study showed that curcumin was a safe and well-tolerated adjunct to FOLFOX chemotherapy in patients at doses up to 2 g daily (James et al. 2015). Another randomized control clinical trial combining curcumin with FOLFOX has been conducted in 33 CRC patients with inoperable liver metastases to determine a target dose, side effects and antitumor efficacy, but results are still unwarranted (Irving et al. 2015). Also promising is the recent finding that curcumin may revert oxaliplatin-acquired resistance in CRC cell lines (Ruiz de Porras et al. 2016).

Although, several clinical trials have been carried out to screen the potential use of phytochemicals in CRC prevention and therapy (Alam et al. 2018) – as the ones for curcumin (Carroll et al. 2011), resveratrol (Nguyen et al. 2009; Tome-Carneiro et al. 2013), black raspberry phytochemicals (Kresty et al. 2016; Wang et al. 2014a) and ITCs (NCT03034603; NCT00968461; NCT01228084) – there is still a long way to go for phytochemical acceptance in CRC treatment, even as an adjuvant in combinatory treatment. For example, studies with nutraceuticals shed doubt on whether 'more is better', on their bioavailability and on the attainment of a therapeutic dosage *in vivo*. Solutions might include using special delivery systems (e.g. micro- and/or nanoparticles) to entrap phytochemicals and release them in a targeted and controlled way (Nair et al. 2010; Priyadarsini and Nagini 2012; Wang et al. 2014b). Therefore, it is feasible to cogitate the combination of nutraceuticals-loaded delivery systems with conventional chemotherapeutic drugs, namely 5-FU, in CRC therapy, as reported previously for chitosan-coated cinnamon/oregano-loaded solid lipid nanoparticles (Kamel et al. 2017) and for curcumin-loaded N,O-carboxymethyl chitosan nanoparticles (Anitha et al. 2014). Moreover, specific delivery to tumour cells would circumvent the side effects associated with the inhibition of Wnt signalling in normal cells that need physiological activation for their homeostasis, as previously mentioned.

6.2.2 Wnt Agonists: Are We Counteracting the "Just-Right" Amount of Wnt Signalling Driving CRC Metastasis?

As discussed so far, current efforts have been mostly spent on the development of Wnt signalling antagonists aiming at Wnt signalling down-regulation, disruption or attenuation, and for some the clinical outcome is still to be determined. Thus far, clinical trials involving Wnt signalling inhibitors have not been succeeded (Lu et al. 2016). One of the reasons, as mentioned above, may rely on the fact that when counteracting activated Wnt signalling in colorectal tumours, physiological Wnt signalling is also being inhibited in normal cells, for which a specific threshold is crucial for cell homeostasis.

Notably, antagonists may not be counteracting the "just-right" amount of Wnt signalling that drives CRC metastasis. In agreement, a paradox

appears to exist regarding Wnt signalling hyperactivation and CRC progression. Varnat *et al.* have demonstrated that, contrary to non-mCRCs displaying a high Wnt/TCF signalling signature, mCRCs show downregulation of Wnt/TCF signalling in parallel with increased Sonic hedgehog (SHH) signalling. In this work, the occurrence of an inverse gradient of Wnt and SHH signalling in patient-derived CRCs and in their $CD133^+$ colorectal CSCs subpopulation was observed, indicating a metastatic transition characterized by a shift from a high-to-low Wnt signalling accompanied by a low-to-high shift in SHH signalling. Hence, these results suggest that although the use of Wnt/TCF signalling inhibitors may be beneficial in the treatment of early stage CRCs, the same may not apply to advanced or mCRCs, as observed by enhancement of tumour metastasis after TCF blockage *in vivo* (Varnat et al. 2010). Additionally and contrary to early stages, advanced tumours and metastases have also revealed downregulation of the Wnt/TCF gene expression program in patient tumour samples, probably due to methylation of CSC-associated target genes (de Sousa et al. 2011; Varnat et al. 2010). This calls for a revisitation of the "just-right" signalling model according to which specific Wnt signalling levels are selected for during tumour formation (Albuquerque et al. 2002). This theory was also demonstrated in several *Apc* mutant mouse models displaying CRC phenotypes that diverged in a β-catenin signalling dose-dependent manner (Gaspar and Fodde 2004).

More recently, Seth *et al*. demonstrated that xenograft growth and metastases are not enhanced by increased Wnt signalling. Moreover, they proposed that partial downregulation of TCF function enhances CRC metastasis to distant organs and that endogenous Wnt/TCF signalling generally counteracts tumour progression, since blockage of TCF function via expression of a dominant-negative TCF7L2 (dnTCF7L2) boosted metastatic progression (Seth and Ruiz i Altaba 2016). Therefore, in parallel with the selection for different "just-right" signalling levels for tumour formation between the proximal and distal colon (Albuquerque et al. 2010, 2011) and between upper gastrointestinal tract and colonic tumours, optimal Wnt signalling levels may also differ between tumour initiation and metastasis.

Indeed, advanced tumours and metastases have revealed a downregulation of the Wnt/TCF gene expression program in patient tumour samples and partial downregulation of TCF function, accompanied by a low-to-high shift in SHH signalling, was associated with enhanced CRC metastasis to distant organs (de Sousa et al. 2011; Seth and Ruiz i Altaba 2016; Varnat et al. 2010). These findings support the rational that the best strategy to counteract the metastatic transition in CRC may not be the blockage of Wnt signalling (Seth and Ruiz i Altaba 2016; Varnat et al. 2010), but instead the use of Sonic Hedgehog (SHH) signalling inhibitors, or even Wnt/TCF signalling agonists. Hence, an important take-home message is that use of Wnt antagonists or agonists should be context-dependent, that is, they should be applied carefully considering the tumour stage, as well as the limitations of the conventional treatments. For instance, CRC patients with synchronous metastases or undergoing neoadjuvant chemoradiotherapy regimens before surgical resection, in which cells with a metastatic/stemness-like signature may evade conventional therapies and metastasise to other organs (Dasari et al. 2013; Mertins 2014; Zhao et al. 2017b), may benefit more from SHH inhibitors or Wnt agonists to counteract cells' metastatic signature. However, the context and tumour stage, in which these compounds may be used still needs intensive research before clinical application and, similarly to what happens with the use of Wnt antagonists, efficient drug delivery systems to target preferentially tumour cells would be needed to avoid side-effects in normal cells.

Considering Wnt signalling hyperactivation in tumorigenesis, very few Wnt agonists in CRC have been explored so far compared to antagonistic drugs (Lu et al. 2016). According to a recent review based on patent and patent applications databases for targeting Wnt signalling in cancer, most agonists have been designed to act at the extracellular ligand-receptor interface and to inhibit DKK or to act as Wnt ligands (Lu et al. 2016).

Notwithstanding, deregulation of the Wnt/β-catenin signalling pathway has also been associated with epigenetic gene silencing driven by histone methylation/deacetylation or DNA methylation of promoters of Wnt signalling players (Serman et al. 2014). Interestingly, these epigenetic alterations can be modulated by epigenetic agents, namely DNA methyltransferase inhibitors or HDACi (Vaiopoulos et al. 2014). Indeed, it has been suggested that upregulation of Wnt signalling and induction of apoptosis in CRC cells by several HDACis with different chemical structures – sodium butyrate; Trichostatin A (TSA) and Vorinostat (suberoylanilide hydroxamic acid, SAHA) (both hydroxamic acids) and MS275 (benzamide derivate) – is mediated by an increase in activated β-catenin via dephosphorylation of Ser-37 and Thr-41 residues initiated at the ligand level (Bordonaro et al. 2007). According to these studies, response to treatment with these compounds might be elicited by activating pro-apoptotic genes (Bordonaro et al. 2007), which would be in accordance with the "just-right" signalling model that postulated that too much Wnt/β-catenin signalling would lead to apoptosis induction (Albuquerque et al. 2002). Considering the higher activation of Wnt/β-catenin signalling, likely associated to APC truncated proteins retaining 1 or 0 20AARs, characteristic of distal MSS tumours (Albuquerque et al. 2010, 2011), the CMS2 subtype might be a good candidate for targeted therapy with Wnt agonists, in the right context and tumour stage, since the threshold level for Wnt hyperactivation and apoptosis induction would be easier to achieve, i.e. would need less increment of Wnt/β-catenin signalling, with less side-effects for normal cells.

Although the use of HDACis to boost Wnt signalling activity in CRC is not being explored so far in clinical trials, their anti-CRC activity in the context of the modulation of other biological and cellular events (e.g. thymidylate synthase-mediated resistance, apoptosis, proliferation, autophagy, anti-tumour immunity) has been studied in combination with other compounds (Di Gennaro et al. 2010; Fazzone et al. 2009; Patel et al. 2016; Wilson et al. 2010; Yang et al. 2012).

Although CRC is the disease with most trials evaluating the effects of Wnt signalling targeting, there is still no on-going clinical trial evaluating Wnt agonist drugs in this pathology (Lu et al. 2016). The only trial cited (NCT01882660) aimed to evaluate the effect of the epigenetic modulator Decitabine (5-aza-2′-deoxycytidine) in the de-repression of Wnt target genes via demethylation in CRC tumours, but it was abandoned due to slow inclusion of patients. Nevertheless, low dose of this DNA-demethylating agent has shown promising anti-cancer effects *in vivo* by enhancing PD-1 blockade-based immunotherapy.

Regarding the effect of natural bioactive compounds as Wnt agonists in CRC very few reports exist. For instance, it has been reported that derivatives of the group of indirubins (phytochemicals originally isolated from *Indigo naturalis*, a plant used in traditional Chinese medicine) act as Wnt agonists by inhibiting GSK3β, eliciting however undesired pro-tumour effects as upregulation of stemness properties and chemoresistance to 5-FU (Liu et al. 2012, 2017). As discussed before in this chapter, this effect would likely be observed if tested in early tumour stages and without Wnt hyperactivation. As also mentioned, the use of delivery systems to target drugs specifically to tumour cells would likely circumvent the side effects associated with Wnt signalling activation. On the other hand, another indirubin derivative, LDD970, was suggested as a potential therapeutic compound in CRC, according to its ability to inhibit the usually overexpressed and hyperactivated Aurora kinase A (via autophosphorylation and phosphorylation of histone H3 at Ser10 residue) and to impair migration and growth of HT29 cells (Ndolo et al. 2017).

In summary, considering some controversial effects of Wnt agonistic compounds, further studies will be needed to ascertain their effect in CRC therapy, according to tumour molecular stratification, Wnt signalling levels ("just-right" model), tumour stage (early vs. advanced) and possible combinatory therapeutic regimens.

6.3 Conclusions

Despite the benefit in current clinical practice of treating CMS2 tumours with conventional chemo- and targeted therapies (FOLFOX or FOLFIRI plus anti-EGFR, anti-VEGF), and also the scientific advances in this field, cells with a metastatic signature are still able to evade therapy and metastasise to other organs in a great proportion of treated CRC patients (see Fig. 6.1). Moreover, attention should be given to clear differences within the same group of tumours between distal and proximal location in the colon with respect to prognosis and therapy response. In order to consider emergent immunotherapy options, the low immunogenicity of this subtype may be increased by combination with radio/chemo or specific targeted therapies like anti-EGFR (see Fig. 6.1a), and this is included already in certain clinical trials of Wnt antagonists.

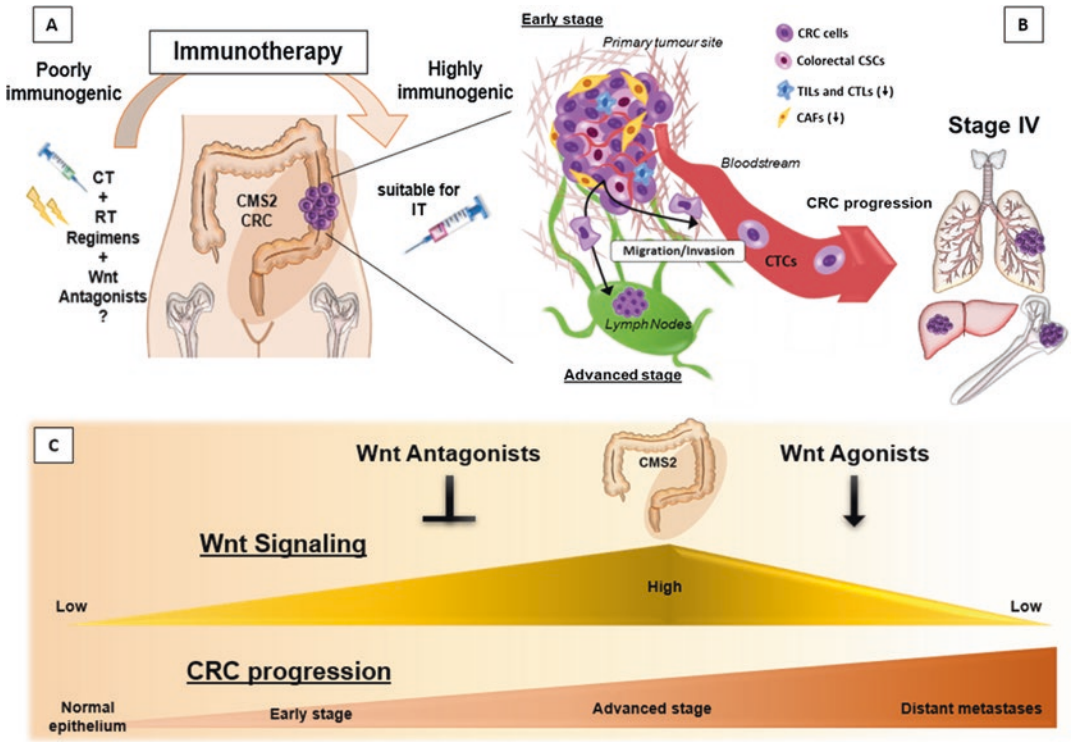

Fig. 6.1 Targeted therapy of the CMS2 subtype of CRCs according to tumour biology, Wnt signalling activity and tumour stage. (**a**) CMS2 tumours affect mainly the distal colon and rectum, are characterized by poor immunogenicity, low infiltration of immune cells (e.g. tumour-infiltrating lymphocytes, TILs) and CAFs, hyperactivation of Wnt/β-catenin signalling pathway and are often diagnosed at an advanced stage (III), in which an adjuvant treatment based on chemo- (CT) or chemoradiotherapy (CT + RT) is applied. Though, the poorly immunogenic "cold" tumours might be switched to highly "hot" immunogenic tumours by the administration of conventional CT and/or RT or Wnt antagonists along with the application of immune checkpoint modulators, making them more responsive to immunotherapy (IT). (**b**) In a great proportion of treated CRC patients cells are still able to evade therapy and metastasise to other organs. (**c**) Wnt signalling hyperactivation is a hallmark of colorectal tumorigenesis, and tumours from the CMS2 subtype are known to be "Wnt-addicted". Still, despite increasing along tumour progression, Wnt signalling activity becomes attenuated in the transition towards a metastatic stage/signature while other signalling pathways, like SHH, are enhanced. This leads us to revisit the "just-right" signalling model. Therefore, a proposal for future targeted therapy approaches would be the treatment of early CMS2 tumours with Wnt antagonists, whereas more advanced or metastatic CMS2 tumours may benefit more from Wnt agonists to prevent tumour progression towards distant organs

Notably, targeting the Wnt signalling axis may also become a potential therapeutic strategy, considering CMS2 "addiction" to this signalling pathway (see Fig. 6.1a and 6.1c). Several efforts have been made to identify compounds with Wnt antagonistic activity, either of synthetic or natural origin. Accordingly, we highlighted compounds targeting the high Wnt/β-catenin signalling levels observed in CMS2: (i) at the ligand/receptor interface; (ii) at the β-catenin destruction complex and, (iii) at the β-catenin/TCF7L2 transcriptional complex levels. In this context, the application of tankyrase inhibitors might be one of the most promising because CMS2 tumours in distal colon carrying short-form APC mutant proteins are highly sensitive to these compounds. For example, this could circumvent radiochemoresistance in rectal tumours mediated by hyperactivation of Wnt signalling. Regarding nutraceuticals, these have a more transversal mechanism of action, being able to target and restrain this pathway at different steps of the signalling cascade. Though, one should beware that inhibition of this pathway may unleash cytotoxic effects since it regulates intestinal homeostasis, especially the stem cell niche, reason why application of such antagonists may struggle to reach clinical benefit. More importantly, the high-to-low gradient of Wnt/β-catenin/TCF7L2 signalling intensity during the progression from early to advanced CRC stages (based on the selection of different β-catenin levels during tumorigenesis in accordance with the "just-right" signalling model), might indicate the application of Wnt signalling agonists to be more advantageous and clinically relevant for the treatment of metastatic CRC. Thus, the use of positive and negative Wnt pathway modulators requires context-dependent decisions (see Fig. 6.1c). In CMS2 tumours, in particular those localised distally, the application of agonists could be relevant even in early stage tumours considering their high levels of Wnt/β--catenin signalling and the potential induction of apoptosis by certain Wnt signalling agonists in this context. Unfortunately, there is still a long way to go until the consequences of antagonist and agonist applications be unveiled and accepted towards better clinical outcomes.

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7 Impact of the Microenvironment on Tumour Budding in Colorectal Cancer

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Abstract

Tumour Budding (TB) is recognized as an adverse prognostic factor in colorectal cancer (CRC). TB is the detachment of isolated cancer cells or small clusters of such cells mainly at the invasion front. One question that arises is of the role of the tumour stroma regarding the permissiveness of the formation and progression of TB. In this review, we will examine potential factors affecting TB, in particular we will analyse the potential effect of inflammation, hypoxia, extracellular matrix and Cancer-Associated Fibroblasts (CAFs).

Keywords

Budding · Cancer-associated fibroblast · Epithelial-mesenchymal transition · Migration · Tumor-environment

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7.1 Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in the world, and the fourth principal cause of cancer deaths worldwide (Jemal et al. 2011). The prognosis and therapy strongly depend on the UICC (Union for International Cancer Control) tumour stage. Nevertheless, it is well known that a certain proportion of stage I/II cancers develop an aggressive clinical course. However, approximately 40% of stage III cancers show a favourable outcome despite the occurrence of regional lymph node metastases (Armin et al. 2017). Therefore, alternative or additional prognostic factors, including new histopathological features or molecular targets, are now studied to improve both prognostic estimation and therapeutic stratification. One such histomorphological feature is TB; it reflects a detachment of tumour cells at the invasion front of epithelial cancers into single cells or small cell clusters. Based on well-designed retrospective studies, TB has been linked to adverse outcome of CRC patients in several clinical scenarios. Budding takes place in the tumour stroma; a type I collagen rich environment produced by Cancer-Associated Fibroblast (CAFs). CAFs are important contributors to invasion and metastasis of cancer cells in CRC (De Wever et al. 2004; De Boeck et al. 2013b; Calon et al. 2014) and their presence is negatively associated with disease outcome (Tsujino et al. 2007).

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This minireview provides an overview of the tumour microenvironment leading to tumour budding and stromal invasion.

7.2 Tumour Budding

7.2.1 Definition of Tumour Buds

TB is a histomorphological phenomenon found in epithelial cancers, and corresponds to the detachment of isolated cancer cells or small clusters of such cells at the invasion front, infiltrating the surrounding stroma (Fig. 7.1). The process through which cancer cells undergo epigenetic and molecular changes and gain invasive stromal characteristics is referred to as epithelial-mesenchymal transition (EMT; Kalluri and Weinberg 2010).

In 2016, consensus was reached to define tumour buds as a single cancer cell or a cell cluster consisting of maximum 4 cancer cells (Lugli et al. 2017).

7.2.2 Diagnostic and Prognostic Impact/Clinical Scenarios

TB is recognised as an unfavourable prognostic factor in CRC independent of disease stage. Indeed, the correlation between the presence of TB within the stroma of CRC and lymph node metastasis, distant metastasis, local and distant recurrence and cancer-related death at 5 years is now well established (Rogers et al. 2016).

The 'International Tumor Budding Consensus Conference' (ITBCC) suggests the application of

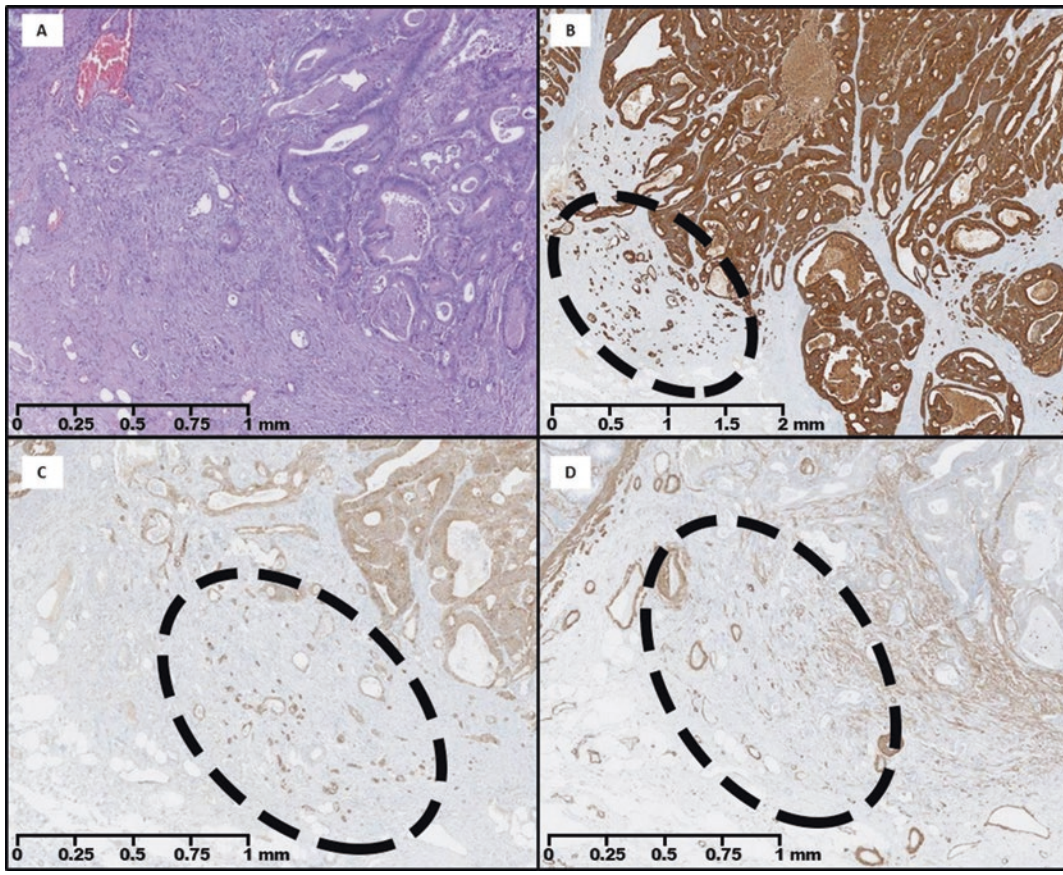

Fig. 7.1 Representative illustrations of tumor budding (TB) at the invasion front of colorectal cancer; stained in Haematoxylin & Eosin (**a**), cytokeratin (**b**), epithelial-cadherin (**c**) and α-Smooth Muscle Actin (**d**)

TB as an additional prognostic factor in 3 clinical situations to facilitate management of patients with CRC (Lugli et al. 2017):

- TB predicts lymph node metastasis in endoscopically resected pT1 tumours. The presence of TB at the invasion front hence allows identification of patients with a high risk to present lymph node metastasis; they can be selected for radical surgery.
- TB in stage II colorectal cancers is associated with a higher risk of recurrence; therefore, stage II CRC patients with high-grade TB can be selected for adjuvant therapy.

These two scenarios are very well described in existing literature with abundant studies showing consistent results. However, a third scenario was discussed, that of TB within biopsies and impact on the management in the preoperative setting.

The prognostic importance of TB in preoperative biopsies in CRC was first described in 1989 by Morodomi and colleagues, who suggested that the degree of TB within biopsy material could be used to evaluate the probability of lymphatic invasion and nodal metastases (Morodomi et al. 1989). Recently, this concept of 'intratumoural budding' (ITB; Lugli et al. 2011) was further elaborated and found to be highly correlated with the presence of 'peritumoural budding' (PTB) at the invasion front. Moreover, its presence correlates with aggressive tumour features and worse clinical outcome (Zlobec et al. 2014). This finding suggests that important tumour features can be assessed in biopsies, which rarely sample the invasive margin, and thus potentially add decisive tumour-related information to the management of patients in the preoperative setting. Indeed, a strong correlation between ITB and nodal metastases and poor long-term prognosis has been shown (Rogers et al. 2014; Giger et al. 2012). However, ITB in pre-treatment rectal cancer biopsies is associated with non-response to neo-adjuvant chemo-radiotherapy (Rogers et al. 2014). Patients who are likely not to present a complete pathological response to neo-adjuvant therapy must be managed accordingly, and could qualify for an escalation of therapy. Further inter-laboratory studies will demonstrate the uniform reporting of TB scores, a necessary step before widespread clinical implementation.

7.2.3 Genetics: Consensus Molecular Subtyping

In 2015, the colorectal cancer subtyping consortium was able to unify six independent molecular classification systems for CRC into four distinct groups, based on their gene expression, which are known as Consensus Molecular Subtype (CMS; Guinney et al. 2015). The aim of this novel classification is to better inform the clinician of prognosis and of therapeutic response in the heterogeneous disease collectively known as CRC. TB is an adverse prognostic factor across all CRC stages and is associated with the mesenchymal CMS4 phenotype. *KRAS/BRAF* mutations are strongly correlated with tumor budding suggesting their involvement in regulation of this process (Trinh et al. in press 2018).

This classification distinguished 4 subtypes of CRC cancer cells:

7.2.3.1 CMS1

The CMS1 subtype is characterised by defective DNA mismatch repair system consistent with microsatellite instability (MSI). Furthermore, they are characterised by a strong immune infiltration (De Smedt et al. 2017) along with strong activation of immune evasion pathways (Guinney et al. 2015). TB is infrequently found in patients with MSI tumours (Jass 2002). It is hypothesised that the inherent immune cell infiltrate leads to the destruction of the TBs (Lugli et al. 2009).

7.2.3.2 CMS2

Cancer cells of this subtype show epithelial differentiation and are characterised by chromosomal instability, which leads to chromosomic rearrangements with losses and/or gains of large segments of chromosomes, loss-of-heterozygosity and aneuploidy. They also show a marked activation of Wnt-β-catenin and Myc signalling pathways. This subtype is associated with superior survival after relapse,

and larger proportion of long-term survivors in this subset.

7.2.3.3 CMS3

In contrast to the other CMS groups, CMS3 is more ambiguous and shows an enrichment for multiple metabolic signatures (Guinney et al. 2015). Tumours of the CMS 3 subtype seem to be genetically stable but metabolically active with little involvement in frequently occurring oncogenic pathways or in immune cell infiltration.

7.2.3.4 CMS4

Cancer cells of the CMS4 subtype show a marked activation of the TGF-β pathway and Wnt-signalling pathway in addition of markers of EMT and angiogenesis. This subtype shows the worst overall survival, worst 5-year survival and relapse-free survival among all subtypes (Thanki et al. 2017). The reversible process through which epithelial cells gain mesenchymal traits enabling them to infiltrate the stroma is called EMT (Kalluri and Weinberg 2010), an ubiquitous phenomenon during embryonic development, wound healing and cancer progression (Li et al. 2016). In this process, epithelial cells lose their polarity and modify their protein expression, enabling them to gain migratory capacity, invasiveness and elevated resistance to apoptosis (Kalluri and Weinberg 2010)**.**

The underlying mechanism of the EMT process involves genetic and epigenetic changes, modifying the cellular protein make-up leading to enhanced mobility. Some cancer cells only lose their epithelial phenotype partially, so they show both epithelial and mesenchymal properties; this is referred to as 'partial EMT' (Grigore et al. 2016). Evidence supports the existence of pan-cytokeratin (epithelial marker) and vimentin (mesenchymal marker) double-positivity in tumour buds (Meyer et al. 2016).

Different subtypes can coexist within the same tumour. A shift from CMS2 (epithelial) to CMS4 (mesenchymal) was observed as tumour cells transit from the tumour bulk to the budding regions (De Smedt et al. 2017). Aligning their results with the new CMS classification ascribed to CRC they showed that CRC undergo a switch from the epithelial CMS2 in the centre of the tumours to the mesenchymal subtype CMS4 in the TBs. Single cell microdissection followed by single cell genomics has recently shown the relationship between breast ductal carcinoma in situ and invasive ductal carcinoma (Casasent et al. 2018). This strategy would also enable to evaluate the true implication of EMT in TB and map the relationship between TB in the centre vs invasion front.

Furthermore, a tight link between stromal positivity for markers such as TWIST1, ZEB1 and SNAIL (considered 'classical' markers of EMT) and a high-grade TB phenotype has been demonstrated (De Smedt et al. 2017). Interestingly, these EMT markers are often limited to the so-called CAFs, and not the TBs themselves. This arises the question whether the stroma plays a role in the formation of TB by providing a more or less conductive surrounding for the budding process.

7.2.4 Is TB Considered an Invasive Process and Is It Driven by EMT?

Studies on serial section of CRC which allowed the creation of 3D reconstructions of the tumour-stroma interface (Bronsert et al. 2014) showed that the tumour mass projects finger-like extensions into the stroma. These extensions may give the false impression to be detached structures in 2D sections, even though they would remain connected to the tumour mass. It should be mentioned however, that the conclusion of absent single cell migration raises concerns due to the low sample size (n = 3) and the selection of very early cancers (i.e., those that would typically show the least amount of TB).

Cells migrating through the stroma can either migrate as isolated cells, based on cytoskeletal rearrangements and without cell-cell adhesions, or as an adhesive group in clusters, based on coordinated collective cytoskeletal activity while maintaining cell-cell junctions to their neighbouring epithelial cells as well as to the extracellular matrix (te Boekhorst et al. 2016). TB adhere

to the mesenchymal cells of the stroma. The disconnection of TBs from the tumour body is marked by loss of E-cadherin, a transmembranous protein which plays a vital role in the adherens junctions between epithelial cells (Karamitopoulou et al. 2010). These cell migration modes underlie distinct molecular programs, which define the specificity, mechanical strength and consequences of cell-cell and cell-matrix interactions.

Collectively moving cells have the ability to switch to single cell migration, and vice versa. Indeed, migrating clusters of cells can detach into individual cells while moving through loosely organised stroma (te Boekhorst et al. 2016). This phenomenon is consistent with downregulation of cell-cell junctions and is known as 'unjamming'.

The transcriptional downregulation of adherens junctions as well as of tight junctions and desmosomes occurs when cells undergo an epithelial-mesenchymal transition (EMT; te Boekhorst et al. 2016). With fewer intercellular attachments, cells can detach from the surrounding epithelium, or migrating clusters, and migrate through the stroma individually.

Detachment of small multicellular groups of cells from tumour lesions is followed by intravasation and entry into the circulation as a multicellular cluster, which in sequence constitute important and efficient steps toward metastatic organ colonisation. In this respect, TB have been hypothesized as a cause of local and distant metastasis and a source of circulating tumour cells. Isolating these cells and applying next-generation sequencing techniques reveals that the DNA profile of tumour buds is identical to the main tumour. These findings underline furthermore that TB are not derived from a more aggressive subclone of the tumour (Centeno et al. 2017).

Recent genomic studies on metastases, describing the polyclonal nature of cancer cells in metastasis, (Ulintz et al. 2018) challenge the conventional conception that distant metastases must arise from single cells disseminated for polyclonal seeding as a major mechanism for metastatic spread. In fact, cancer cells can disseminate collectively through the bloodstream to colonise distant organs (Cheung et al. 2016).

7.3 Impact of the Microenvironment on TB

Cancer cells exist within a complex ecosystem consisting of an ECM scaffold populated by carcinoma-associated fibroblasts (CAFs), endothelial cells and immune cells. Studies have shown that not only the cancer cells' genetic and molecular changes play a role in EMT, but so does the tumour microenvironment. We will discuss the roles of the following factors:

7.3.1 Inflammation

The expression of neoantigens, in consequence of genetic mutations and stromal invasion of the cancer cells, induce an inflammatory reaction, a defence mechanism of the host to stop the vicious proliferation and invasion (Li et al. 2016). On one hand, the inflammatory cells do have a host-protecting function, which can destroy the invading cancer cells. On the other hand, the local production of pro-inflammatory cytokines, such as TNF-α (by activating the NF-kB pathway), IL-6 and IL-1β produced by macrophages can stimulate tumorigenic pathways and thus stimulate growth and survival of cancer cells. TNF-α and IL-1β stimulate the expression of TGF-β, which promotes EMT. IL-6 also activates the TGF-β signalling pathway, but furthermore stimulates downregulation of E-cadherin and upregulation of vimentin through the JAK/STAT3/Snail pathway in head and neck cancer (Li et al. 2016).

The presence of tumour-infiltrating lymphocytes indicates a favourable outcome and a decreased metastatic potential. The absence of intratumoural lymphocytic inflammation is an independent predictive factor of lymph node metastasis (Mlecnik et al. 2016).

The severity of TB is inversely correlated to the presence of peritumoural lymphocytic inflammation at the invasion front. The type and frequency of immune cells within densest TB regions shows that among other cells, CD8+ T-lymphocytes are markedly over-represented and Lugli and colleagues identified the CD8+ T-lymphocytes/TB index as an independent prog-

nostic factor (Lugli et al. 2009). Indeed, the ratio of invading TBs and defending CD8+ T-lymphocytes, is more prognostic than either feature alone.

Patients with marked inflammation, even with high-grade budding tumours, have a significantly better outcome, compared to patients with mild inflammation and high-grade budding. Outcome of patients with high TB tumours however is still worse than for patients with low TB (Max et al. 2016). In high-grade TB CRC, a marked inflammation showed to be a predictor of favourable progression-free survival and cancer-specific survival, independent from other variables (T and N stage, grade, lymphatic and venous invasion).

CD8+ T-lymphocyte infiltration can also be assessed in endoscopic biopsies of rectal cancer and correlates with the absence of lymph node metastasis (Koelzer et al. 2014).

7.3.2 Hypoxia

It is well known that patients with hypoxic tumours have worse outcome. Tumour cell proliferation strongly increases oxygen consumption. This induces a local microenvironment that is relatively hypoxic, inducing over-expression of HIF-1, which acts as a transcription factor for functions in cancer progression, including proliferation and survival, motility, cytoskeletal structure, angiogenesis, ECM metabolisms and drug resistance (Laura D'Ignazio et al. 2017; Fan et al. 2013; Yang et al. 2008). HIF-1 cooperates with the transcription factor TWIST, an EMT inducer**.**

Significant relationship between high-grade TB at the invasive edge (PTB) of CRC and HIF-1 expression (Righi et al. 2015). Furthermore, they showed the existence of an 'hypoxic tumour phenotype' in CRC showing TB: CRC with high-grade TB showed higher HIF-1 expression than CRC with low-grade TB.

7.3.3 Extracellular Matrix and Desmoplasia

During stroma infiltration, cancer cells travel through pre-existing gaps in the ECM or reversible or irreversible reorganize the ECM through respectively collagen realignment or matrix metallo-proteinases (MMPs). In addition, matrix reorganisation is induced by production and deposition of ECM molecules. In addition to cancer cells, stromal CAFs play a major role in ECM reorganization and accumulation. This phenomenon, also called desmoplasia, influences cell mobility and leads to activation of a series of EMT-inducing signals, such as TGF-β, Wnt and Rho GTPases (Forse et al. 2017; Jansen et al. 2018).

The density of the ECM determines the invasion mode of mesenchymal tumour cells (Haeger et al. 2014). Whereas fibrillar, high porosity ECM enables single-cell dissemination, dense matrix induces cell-cell interaction, leader-follower cell behaviour and collective migration as an obligate protease-dependent process.

Multiple phenotypes of desmoplasia can be identified, such as mature—when the stroma is composed of mature collagen fibres (fine and elongated fibres into multiple layers); intermediate—when keloid-like collagen is intermingled with mature fibres; and immature—consisting of a myxoid stroma in which no mature fibres are included (Ueno 2004). It is unknown how this desmoplastic maturity influences TB.

7.3.4 Cancer Associated Fibroblasts

Tumours are chronic wounds that do not heal (Dvorak 1986; Karagiannis et al. 2012). This stromal 'wound healing'-like reaction associated with tumours promotes both the development and progression of cancer through stromal cells of fibroblastic origin that actively participate in the wound healing reaction and which are known as CAFs (De Wever et al. 2014).

CAFs are the preponderant cell population in the tumour stroma. They are derived mainly from tissue resident fibroblasts or are recruited from distant reservoir sites such as the bone marrow (Direkze et al. 2004; De Boeck et al. 2013a). CAFs are one of the most abundant cells present in the tumour stroma (Calon et al. 2014) and typically show a large spindle-like shape similar

to smooth muscle cells (Tao et al. 2017). They have been shown to support the cancer cells by releasing regulation factors into the tumour's local microenvironment, stimulating tumour growth, angiogenesis, metastasis and therapy resistance (Tao et al. 2017).

The activation of the residual Normal Fibroblasts (NF) to CAFs is induced by cytokines secreted by the cancer cells, but the underlying mechanisms remain unclear. Studies have shown a differential expression of 46 genes regulated by the TGF-β signalling pathway (Navab et al. 2011), suggesting an important role of TGF-β receptors. These identified genes were described to be paracrine local and systemic acting factors. Indeed, Bone marrow-derived mesenchymal stem cells are recruited to the tumour's microenvironment by the combined stimulation of TGF-β and CXCL12 signalling (Quante et al. 2011). Furthermore, TGF-β stimulates reactive oxygen species production and experiments point to the importance of NAD(P)H-oxidase-4 (NOX4) as potential therapeutic target to prevent CAF activation (Hanley et al. 2018). Resolution of the wound healing response is associated with enhanced fibroblast apoptosis (Iredale et al. 1998). By analogy, one may predict that CAFs can also be uniquely sensitive to proapoptotic stimuli. Indeed, BH3 (BCL-2 homology domain 3) mimetics, such as Navitoclax, are apoptosis inducers in CAFs (Mertens et al. 2013).

The presence of CAFs could represent an attempt by the host to ward off tumour cells, thereby exerting antagonistic biological forces. Alternatively, this process may benefit the tumour, by facilitating TB and neovascularisation, and impeding access to host lymphocytes, macrophages, and other immune regulator cells. This contradiction suggests that multiple spatiotemporal activities of peritumoural CAF exist which have opposing effects on cancer behaviour. A high degree of TB is significantly associated with elevated c-Met expression at the invasion front in CRC (Satoh et al. 2014) in comparison to more superficial parts of the tumour in both high- and low-grade budding tumours. c-Met is a tyrosine kinase receptor that binds scatter factor/hepatocyte growth factor (SF/HGF). These observations suggest that the production of HGF by CAFs may benefit the tumour, by facilitating TB and neovascularisation, and impeding access to host lymphocytes, macrophages, and other immune regulator cells. Indeed, CAFs secrete SF/HGF and are linked to CRC cell invasion (De Wever et al. 2004). Other soluble factors involved in the invasion process are neuregulin-1 (NRG1; (De Boeck et al. 2013b), chemokines such as CXCL12 (Orimo et al. 2005), and cytokines such as IL-6 that stimulate the NF-κB pathway (New et al. 2017) and proteinases (De Wever and Mareel 2003).

It is suggested that direct cell-cell contacts between CAF and TB can occur through heterophilic adhesion involving N-cadherin at the CAF membrane and E-cadherin at the cancer cell membrane (Labernadie et al. 2017). CAF-deposited ECM proteins provide a macromolecular structure for migration of cancer cells. CAFs exert adhesive forces to the matrix causing increased stiffness which influences a switch from collective migration to single cell migration (te Boekhorst et al. 2016).

Extracellular Vesicles (EV) are nano-sized membrane vesicles which contain bioactive proteins, lipids, and nucleic acids, and emerge as functional agents of cancer (Van Deun et al. 2017). CAFs secrete EVs and stimulate cancer invasion and metastasis (Luga et al. 2012) and could therefore be involved in the process of TB as well.

7.4 Conclusions

TB is now considered an additional prognostic factor in CRC, ranked similarly to tumour grade and recommendations for reporting tumour budding in CRC were reached during the International Tumour Budding Consensus Conference (Lugli et al. 2017). Indeed, TB is a prognostic biomarker (and potential predictive factor) that can influence the clinical management of patients with CRC (Zlobec and Lugli 2018). CAFs and the tumour environment are important factors that may stimulate or constrain TB. Incorporation of this tumour context will improve the diagnostic

rationale of TB and will allow to identify relevant therapeutic strategies. Interdisciplinary collaboration between researchers, pathologists, and clinicians will provide new opportunities and insights into cancer diagnosis and management.

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8 Anti-EGFR Therapy to Treat Metastatic Colorectal Cancer: Not for All

Marta Martins, André Mansinho, Raquel Cruz-Duarte, Soraia Lobo Martins, and Luís Costa

Abstract

The development of monoclonal antibodies (mAbs) cetuximab and panitumumab, which target the transmembrane protein epidermal growth factor receptor (EGFR), mark a major step forward in the treatment of metastatic colorectal cancer (mCRC). However, this therapeutic progress proved to be effective only in a very restricted subset of patients. Although several mechanisms of resistance, both primary and acquired, have been identified, the only established predictive tumour biomarker for the treatment of mCRC patients is the *RAS* mutational status. *RAS* activating mutations predict a lack of response to these therapies while low levels of primary resistance characterize *RAS* wild type (WT) patients (only about 15%). However, even WT patients that initially respond to anti-EGFR therapy, eventually undergo tumour progression. In this context, there is still more to be done in the search for effective predictive markers with therapeutic applicability. In this chapter, we provide an overview on the mechanisms that contribute to resistance to EGFR-targeted therapy and highlight what is still missing in our understanding of these molecular mechanisms and approaches to overcome them.

Keywords

Colorectal cancer · Epidermal growth factor receptor · Primary resistance · Secondary resistance · Targeted therapy

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8.1 Introduction

Cancer is a worldwide health problem whose incidence has been increasing every year, severely threatening human wellbeing. Colorectal cancer (CRC) ranks among the third most frequent cancer type and the fourth leading cause of cancer-related death (Ferlay et al. 2015). Although impressive advances in cancer therapy have been achieved over the last 20 years (better surgical techniques, better screening methods, improved postoperative

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care, regular follow-ups and more effective adjuvant therapies), CRC is still an illness with undesirably high mortality, mainly associated to the metastatic setting. The prognosis of patients with mCRC has been improving, and correspond now to median overall survival (OS) of over 30 months, depending on the treatment options (Heinemann et al. 2014; Venook et al. 2017).

Novel therapies targeting the epidermal growth factor receptor (EGFR) have significantly contributed to the improvement of the OS of mCRC patients. EGFR is a transmembrane receptor belonging to the ErbB tyrosine kinase family which consists of four related proteins: EGFR (ErbB1/HER1), HER2/neu (ErbB2), HER3 (ErbB3) and HER4 (ErbB4) (Hynes and Lane 2005; Wieduwilt and Moasser 2008). All family members contain an extracellular ligand-binding domain with two cysteine-rich regions, a single membrane-spanning region and a cytoplasmic tyrosine kinase domain. In healthy cells, EGFR signalling is tightly regulated by various extracellular ligands, namely EGF, Amphiregulin, Epiregulin and TGFα, which induce homo- and hetero-dimerization with other ErbB members and the subsequent activation of downstream pathways, such as RAS-RAF-MEK-MAPK, PIK3CA-AKT, the SRC family kinases, PLCγ-PKC and JAK/STATs (Fig. 8.1) (Oda et al. 2005). These pathways are involved in several essential cellular processes including proliferation, migration, invasion, survival and angiogenesis. However, in pathologic conditions, constitutive activation of EGFR or downstream effectors (by activating mutations, increased copy number and upregulations) are described as responsible for tumour development and metastasization. EGFR is expressed in various cancers including in CRC with a frequency of 60–80%, where it plays a key role in tumour development and progression (Spano et al. 2005). Therefore, its essential function together with its deregulated activity in cancer justified the rational for the development of EGFR inhibitors. To date, two monoclonal antibodies (mAbs) against EGFR were approved for the treatment of patients with mCRC. Cetuximab is a human-mouse chimeric monoclonal antibody (IgG1 subtype), whereas panitumumab is a fully human anti-EGFR monoclonal antibody ($IgG2_K$ subtype). Both antibodies recognize and bind to the extracellular domain of EGFR, not only blocking the ligand-binding region and therefore preventing its ligand-mediated activation, but also causing receptor internalization and degradation, inhibiting further signalling propagation (Ciardiello and Tortora 2008). Furthermore, cetuximab induces antibody-mediated cytotoxicity due to its ability to recruit immune effector cells such as macrophages and monocytes to the tumour, which have specific receptors to bind the antibody's constant Fc domain (Yang et al. 2013a, b). Cetuximab and panitumumab were proven to give similar benefit in terms of progression-free survival (PFS), overall survival (OS), response rate (RR), as well as quality of life, in several phase II and III clinical trials in combination with chemotherapy or as single agent (Bokemeyer et al. 2009; Van Cutsem et al. 2011; Douillard et al. 2014). Unfortunately however, only a small percentage of mCRC patients are sensitive to anti-EGFR therapy due to primary or innate resistance. And even those that initially respond, eventually acquire resistance and relapse under this therapy (secondary resistance). When used as a single agent in unselected mCRC patients, Cetuximab and panitumumab achieved only a RR of about 10–20% (Cunningham et al. 2004; Van Cutsem et al. 2007). This low RR is caused by the high frequency of genetic or epigenetic alterations in proteins involved in EGFR regulation itself and downstream pathways (such as RAS, BRAF, PI3K and PTEN) that blunt the response to mAbs targeting EGFR. The subgroup of patients with tumours wild-type for *RAS*, *BRAF*, *PIK3CA* and expressing normal levels of *PTEN* have the best response to mAbs (De Roock et al. 2010a, b; Karapetis et al. 2014). Nevertheless, still about 10% of these individuals remain resistant to anti-EGFR therapies, suggesting the existence of still unknown alternative mechanisms capable of influencing treatment effectiveness.

To date the molecular mechanisms of response to anti-EGFR mAbs are not yet completely understood. For instance, reports have shown that some patients experience benefit to cetuximab, although having undetectable levels of EGFR

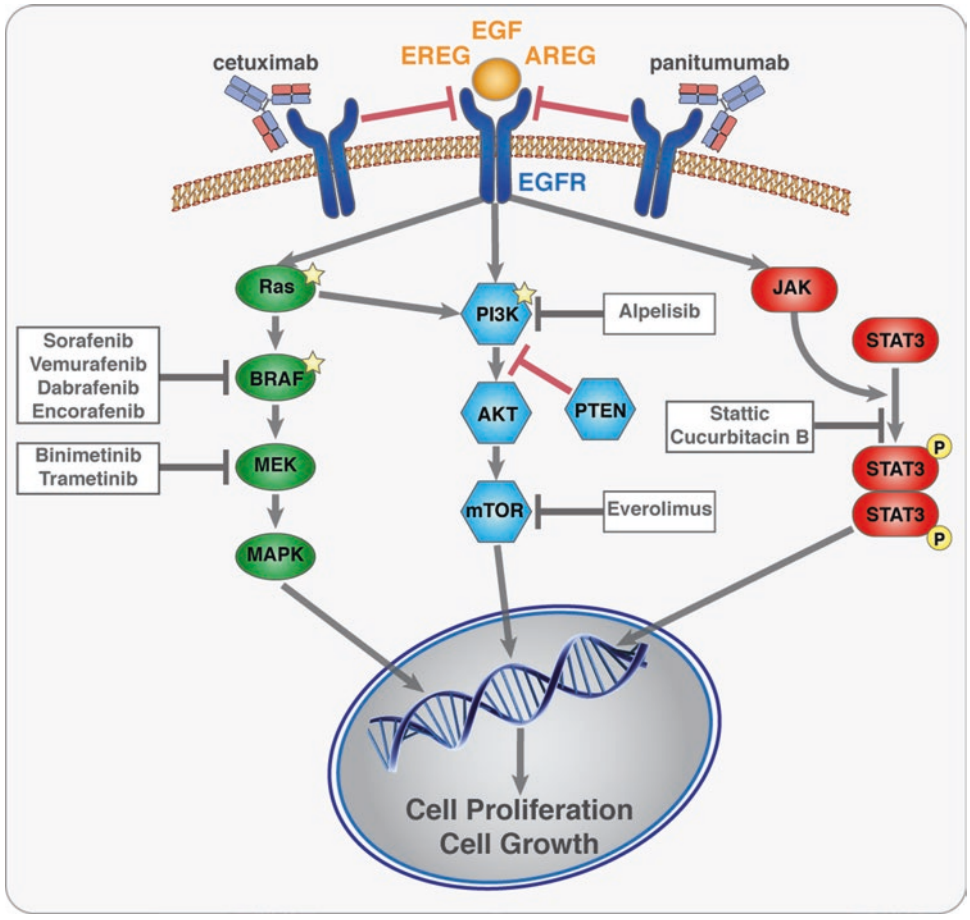

Fig. 8.1 EGFR-mediated signalling pathways and clinically available strategies for their inhibition. EGFR ligands bind to the extracellular domain of the receptor leading to its activation and downstream signal propagation, which is essential for tumour cell growth and proliferation. The antibodies cetuximab and panitumumab prevent ligand binding to EGFR, thus blocking receptor signalling. Further targeted agents are available to inhibit EGFR-stimulated downstream pathways and represent potentially valuable tools to overcome resistance to anti-EGFR treatment. Stars indicate oncogenic mutations involved in resistance of tumours to anti-EGFR therapy

(Chung et al. 2005), or occasionally patients that although harbouring *RAS* activating mutations are able to respond to these therapies (Karapetis et al. 2008; Linardou et al. 2008). Therefore, in this era of targeted medicine, clinical and translational efforts are being made to better understand the molecular complexity of mCRC tumours in order to better adapt the treatment to the molecular characteristics of the specific patient. Furthermore, the identification of clinical relevant predictive biomarkers able to effectively select patients who will likely benefit from these therapies, will not only prevent unnecessary toxicity in resistant patients, but also allow them to receive undelayed alternative treatments.

The aim of this chapter is to provide an overview on the molecular mechanisms that underlie resistance to EGFR-targeted mAbs in mCRC and to discuss possible future directions on how to overcome them.

8.2 Molecular Events That Positively Correlate with Primary Response

8.2.1 Alterations in EGFR Copy Number

Early studies conducted both in heavily pre-treated chemotherapy-refractory patients or in chemotherapy-naïve mCRC patients, have demonstrated that about 80% of unselected mCRC do not benefit from anti-EGFR therapy (Cunningham et al. 2004; Misale et al. 2012). In this context, it was hypothesized that EGFR mutations, levels of expression or levels of its specific ligands could be associated to the lack of response. Soon was realized that, contrarily to non-small-cell lung carcinoma (NSCLC) where mutations in the kinase domains of EGFR correlate with clinical responsiveness to the small molecule tyrosine kinase inhibitors (TKI) gefitinib or erlotinib (Gazdar 2009), point mutations in EGFR are extremely rare in CRC and when they do occur, they are associated with secondary resistance (Barber et al. 2004). Therefore, several studies further assessed whether levels of EGFR expression could correlate with treatment resistance, considering that trastuzumab, a mAb against human epidermal growth factor receptor 2 (HER2/neu) was most effective in patients with metastatic breast tumours overexpressing HER2/neu (Perez et al. 2014). Disappointingly, levels of expression of EGFR were found not to correlate with clinical response to cetuximab or to panitumumab (Chung et al. 2005; Jonker et al. 2007). Curiously, however, alterations in EGFR gene copy number were later confirmed in retrospective analysis of clinical trials to be associated with responses to EGFR-targeted therapy. For example, in a cohort study, about 90% of the patients, who experienced an objective response, had an increase in copy number (three- to five-fold), detected by fluorescence *in situ* hybridization (FISH) (Moroni et al. 2005). In contrast, only 5% of non-responders showed an increased EGFR copy number. Although only a modest increase in copy number was seen, correlation with response was further confirmed in another large and more homogenous cohort (Sartore-Bianchi et al. 2007). Intriguingly however, is the fact that increased EGFR gene copy number did not seem to correlate with increased expression of this protein (Cappuzzo et al. 2008; Campanella et al. 2010). Therefore, the reason why and how this amplification correlates with response is largely unknown and requires further studies. Furthermore, due to lack of technical standardization and definition of a clear and reproducible cut-off for gene amplification, the assessment of gene copy number by FISH shows high variability, which further makes this biomarker clinically unpractical. Finally, statistical correlation between the increased copy number of EGFR and response to cetuximab and panitumumab is not strong enough to allow the clinical use of this biomarker for the predictive selection of patients (Personeni et al. 2008; Sartore-Bianchi et al. 2012).

8.2.2 Alteration in EGFR-Ligands Expression

Other molecular alterations that positively associate with response are the levels of expression of the EGFR ligands Amphiregulin (AREG) and Epiregulin (EREG). In a prospective clinical trial of 110 patients with mCRC, AREG and EREG levels were higher in pre-treatment tumours from responding patients compared to non-responders (Khambata-Ford et al. 2007). A subsequent in a lager cohort of *KRAS* WT patients showed similar effects, namely that expression of higher levels of mRNA for either of these ligands was linked to sensitivity to cetuximab monotherapy, improving disease control rate and progression-free survival (Jacobs et al. 2009). Interestingly, patients with *KRAS* WT or *KRAS* mutant tumours have similar response rates when ligand expression levels are low and in both cases experience worst responses to cetuximab. It is believed that an autocrine or paracrine loop generated by the increased expression of these ligands is responsible for driving the growth of these tumours. Low levels of expression of AREG and EREG may characterize a tumour that is less dependent on EGFR and, therefore, less sensitive to its inhibition.

Similarly to EGFR gene copy number, the levels of expression of AREG and EREG have so far been difficult to assess (mRNA levels), score and reproduce. Therefore, at present, these markers cannot be used to select patients for cetuximab or panitumumab therapy.

8.3 Primary Resistance to Anti-EGFR Therapy

8.3.1 *RAS* Mutations

RAS-RAF-MAPK is the signalling pathway mostly studied in cancer given the high frequency of genetic alterations in its components, as well as, its crucial role in cell growth and differentiation. The RAS family is composed of the three genes *KRAS*, *NRAS* and *HRAS* that encode small guanosine-triphosphate (GTP) hydrolases, that act as signal transducers by cycling between a GDP and GTP-bound conformation upon cell surface receptor stimulation (Malumbres and Barbacid 2003). CRC tumours present with about 40% of *KRAS* mutations, 3–5% of *NRAS* and less than 1% of *HRAS* genetic alterations (Bos 1989; Fernández-Medarde and Santos 2011). In tumours, mutations found in RAS family members generally lead to constitutive activation of these proteins and their downstream effector pathway (MAPK pathway), independently of the upstream signalling cascade or growth factor receptor.

A large number of retrospective analyses of data from previous clinical trials over the last decade have led to the discovery that patients with *KRAS* activating mutations in codons 12 (70–80% of *KRAS* mutations) or 13 (15–20% of *KRAS* mutations) of exon 2 do not benefit from cetuximab or panitumumab agents. Clinical trials in which EGFR-targeted mAbs cetuximab or panitumumab were used to treat either chemotherapy-refractory (NCIC trial) or naïve mCRC patients (OPUS, CRYSTAL and PRIME trials), demonstrated that *KRAS* WT patients had a statistically significant improvement in OS and PFS, whereas *KRAS* mutant patients did not show any benefit in OS, in PFS or quality of life (Van Cutsem et al. 2011; Douillard et al. 2014; Van Cutsem et al. 2015a, b). The exclusion of patients with RAS mutations has allowed the identification of the subgroup of patients that is more likely to benefit from anti-EGFR therapies. Therefore, in patients with wild-type RAS genotype median OS was 25.8 months versus 20.2 months (HR = 0.77, 95% CI = 0.64–0.94, p = 0.009), in favour of the combination of panitumumab and FOLFOX (infusion of fluorouracil, leucovorin, and oxaliplatin) compared with FOLFOX alone (Douillard et al. 2014). Similar results were presented in the CRYSTAL (28.4 months vs. 20.2 months, HR 0.69, 95% CI = 0.54–0.88) and OPUS trials (ORR 58% vs. 29%; HR 3.33 [95% CI = 1.36–8.17, p = 0.0084), in which randomized patients received first-line cetuximab in combination with FOLFIRI (fluorouracil, leucovorin, and irinotecan) or FOLFOX respectively (Bokemeyer et al. 2011; Van Cutsem et al. 2011).

These results have not only shown that patients harbouring *RAS* mutations do not experience any benefit from those treatments, but also that in some cases it could even be detrimental for them. PRIME trial shows that the presence of RAS mutations was associated with inferior PFS and OS in patients receiving first line panitumumab plus FOLFOX compared with FOLFOX alone (Douillard et al. 2014). Overall, this information led the American and European health authorities in 2009 to restrict the use of panitumumab and cetuximab only to patients with *KRAS* exon 2 WT tumours.

However, later retrospective studies revealed that further mutations in *KRAS* and *NRAS* genes were also predictive of resistance to anti-EGFR therapies (Heinemann et al. 2014; Van Cutsem et al. 2015a, b). In addition to exon 2, mutations in *KRAS* exon 3 (codons 59 and 61), exon 4 (codons 117 and 146) and in the homologous codons of *NRAS* also confer resistance to anti-EGFR therapy, and are altogether called “the expanded RAS mutations” (Heinemann et al. 2014). Thus, a meta-analysis of nine randomized trials confirmed that treatment with mAbs had better efficacy reflected in PFS and OS for *RAS* WT patients when compared with the expanded *RAS* mutant group (Sorich et al. 2015). In

response to this data, EMA and FDA have updated their recommendations against the use of cetuximab e panitumumab in patients with extended RAS mutations.

Considering that patients with expanded RAS mutations constitute about 53% of all mCRC cases, several attempts have been made to inhibit RAS directly in these patients. Initial approaches have tried to inhibit RAS farnesylation (a necessary step to attach RAS proteins to the cell membrane), which have shown a potent antitumour activity in preclinical studies (Kohl et al. 1995), but this was not confirmed in clinical trials (Macdonald et al. 2005). Another interesting approach was the identification of small-molecule inhibitors that could form a disulfide bond with the cysteine residue in the G12C mutant KRAS protein (about 8% of all *KRAS* mutations in CRC) (Ostrem et al. 2013). These compounds do not affect WT KRAS but preferentially bind the G12C mutant, inhibiting its activity. Similarly, efforts have been made in identifying compounds that bind and covalently react with the GDP-bound state of KRAS G12C, trapping it in an inactive conformation (Patricelli et al. 2016). However, these studies resulted in only limited demonstration of KRAS inhibition in cells and lack demonstration of *in vivo* efficacy and specificity. Very recently, Matthew et al. have designed and characterized a promising G12C inhibitor (ARS-1620) with features necessary to achieve *in vivo* covalent targeting and inhibition of mutant allele-specific G12C cell lines and tumour models (Janes et al. 2018). This could be a promising step towards bringing KRAS mutant specific inhibitors to the clinic. Nevertheless, several other approaches have been used to target RAS: (i) blocking downstream effectors such as MEK (Yoon et al. 2011) and PI3K (Migliardi et al. 2012), (ii) identification of synthetic lethal interactions with mutant KRAS (interactions that when co-occur in a cell result in cellular death) (Costa-Cabral et al. 2016), or (iii) the use of small-molecule inhibitors of KRAS (Welsch et al. 2017). Finally, a combination therapy of inhibitors co-targeting MEK and CDK4/6 with trametinib and palbociclib, respectively, was highly efficacious in *KRAS*-mutant CRC patient-derived xenografts (Ziemke et al. 2016), but a clinical validations of this strategy is still missing.

Despite these promises, targeting RAS in cancer remains one of the most difficult assignments in cancer therapy. Our incomplete knowledge about RAS-mediated signalling, regulatory feedback loops, pathway redundancy and mechanisms by which RAS activates its downstream effectors, prevents the design of more effective therapies. It is, therefore, essential to fill the gaps of our knowledge regarding RAS-mediated processes in order to develop more effective agents for targeting RAS and its effector pathways in cancer cells.

This is even more relevant given the conflicting data that a number of patients carrying *KRAS*-mutant tumours are able to respond to either cetuximab or panitumumab. Specifically the role of codon 13 mutation in this mechanism is still controversial. DeRoock et al. studied the role of G13D mutation in response to cetuximab in chemo-refractory patients and their results showed longer OS of 7.6 months compared to 5.7 ($P = 0.005$) and longer PFS of 4.0 months compared to 1.9 months ($P = 0.004$) than in G12V mutant patients (De Roock et al. 2010a, b). Although suggesting that patients with G13D-harbouring tumours respond to cetuximab, RR were lower than in *KRAS* WT patients. The same study further showed *in vitro* and in mouse models that CRC cells with the G12V mutation were insensitive but with mutation G13D were as sensitive as the *KRAS* WT to cetuximab. In contrast, a retrospective analysis of three randomized phase III clinical trials showed that patients harbouring *KRAS* codon 13 mutations did not benefit from receiving panitumumab treatment (Peeters et al. 2013). Explanations for these contradictory results may include differences between cetuximab and panitumumab treatments or in the chemotherapy regimens between the studies. Given that these mutations represent about 19% of the *KRAS*-mutant tumours, further studies are necessary to unravel the effect of *KRAS* codon 13 mutation in resistance to anti-EGFR therapy.

8.3.2 *BRAF* Mutations

Although *RAS* mutations are effective predictive marker of resistance, not all *RAS* WT patients respond to cetuximab and panitumumab. Thus, research has turned to the serine-threonine protein kinase BRAF, the main effector of KRAS in EGFR signalling, which is mutated in 5–9% of CRC patients. Importantly, *BRAF* and *KRAS* mutations are usually mutually exclusive, therefore, do not tend to coexist in the same tumour. The activating BRAF V600E mutation represents the majority of *BRAF* mutations and confers poor prognosis to its patients (Di Nicolantonio et al. 2008). OS of mCRC patients harbouring *BRAF* mutations is about 8.8 months, compared to *KRAS* mutant of 14.4 months and *KRAS* WT 20.1 months. Furthermore, De Roock et al. showed that patients with *BRAF* V600E mutation had a significantly lower response rate to cetuximab than those with WT tumours (8.3% vs. 38.0%, OR = 0.15, P = 0.0012) in chemorefractory mCRC patients (De Roock et al. 2010a, b). Several multicentre trials and meta-analyses have further confirmed that *BRAF* V600E mutation resulted in shorter PFS and OS when compared to *BRAF* WT tumours, indicating its contribution to resistance to anti-EGFR mAbs (Pietrantonio et al. 2015, Tveit et al. 2012, Therkildsen et al. 2014).

Similar to the presence of *RAS* mutations, *BRAF* V600E mutation can effectively predict patients that are unlikely to respond to anti-EGFR therapy. It is, therefore, advisable to know both *RAS* and *BRAF* status before administering EGFR-targeted therapies.

In this context, diverse strategies have been employed to overcome BRAF-mediated resistance to anti-EGFR therapy. An *in vitro* study of adding sorafenib (a multi-target small-molecule inhibitor with high affinity for BRAF) to anti-EGFR mAbs showed that even *BRAF* mutated cells can respond to cetuximab and panitumumab therapy when both inhibitors are used simultaneously (Di Nicolantonio et al. 2008). Based on these results, the combinatory therapy of BRAF and EGFR inhibitors was administered in BRAF-mutant CRC patients and resulted in increased response rates (Al-Marrawi et al. 2013). In addition to sorafenib, other compounds targeting either BRAF (such as vemurafenib) or its downstream effectors are in clinical development and could be exploited in combination with EGFR-targeted mAbs therapy. Thus, monotherapy, doublet and triplet combinations with drugs targeting the MAPK pathway have been tested in BRAF-mutant CRC. Results from vemurafenib monotherapy were disappointing when compared to the clinical activity seen in melanoma, with a median PFS of 2.1 months and only two patients progression-free for more than six months (Kopetz et al. 2015). In contrast to melanoma, CRC express high levels of activated EGFR which reactivate the MAPK pathway after BRAF inhibitor monotherapy (Prahallad et al. 2012, Corcoran et al. 2012). Based on the observed therapy resistance via a feedback activation of EGFR signalling, the BASKET trial was amended to include the assessment of the safety and efficacy of vemurafenib when combined with cetuximab, and showed improved results (median PFS of 3.7 months and OS of 7.1 months) in a heavily pre-treated patient population (Yaeger et al. 2015). Similar results have been seen when combining other BRAF inhibitors, dabrafenib and panitumumab (PFS of 3.5 months) (Atreya et al. 2015, Van Cutsem et al. 2015a, b), as well as encorafenib and cetuximab (RR 23.1%, PFS of 3.7 months) (Gomez-Roca et al. 2014, Van Geel et al. 2017). Phase II results for the latter have been presented with a median PFS of 4.2 months and an ORR of 22% (Tabernero et al. 2016). Chemotherapy was also combined with BRAF and EGFR inhibition in a phase II trial combining irinotecan, cetuximab and vemurafenib. A total of 106 patients were enrolled and results show an increasing PFS to 4.3 months with the addition of vemurafenib compared to the control arm (2.0 months) (Kopetz et al. 2017). Finally, BRAF inhibition can not only also induce EGFR-dependent MAPK reactivation but also PI3K modulation so that triple combinations targeting these pathways have been studied and shown improved results. The MEK116833 trial combining trametinib, panitumumab and dabrafenib included 24 patients which received full dose,

with an ORR of 21% and a median PFS of 4.1 months; OS was 9.1 months (Corcoran et al. 2015). A randomised phase II trial which combined encorafenib, cetuximab and alpelisib (a PI3K inhibitor) revealed a median PFS of 5.4 months in an interim analysis with an ORR of 27% (Tabernero et al. 2016). More recently, the BEACON CRC phase 3 study assessed the safety and efficacy of the combination of the BRAF inhibitor encorafenib, plus MEK inhibitor binimetinib, plus anti-EGFR antibody cetuximab in patients with *BRAF* mutant CRC after 1 or 2 prior regimens. The confirmed ORR was 41%, with 1 complete and 11 partial responses. In addition, 9 patients had prolonged stable disease up to 9.3 months and CEA/CA19-9 decreased in 96% and 82% of these patients, respectively (Huijberts et al. 2017).

Overall, given that *KRAS* and *BRAF* mutations are usually mutually exclusive and highly frequent, together they allow the identification of the majority of non-responder patients, avoiding unnecessary exposure of these patients to ineffective treatments and selecting them for alternative therapeutic options.

8.3.3 Other Putative Players

8.3.3.1 *PIK3CA* Gene and PTEN Expression

KRAS and *BRAF* WT status is not enough to define all anti-EGFR-sensitive patients. The EGFR receptor also signals through the PI3K-AKT pathway resulting in tumour cell proliferation and survival (Rommel and Fruman 2014). *PIK3CA* gene encodes the p110α protein kinase, which is the catalytic subunit of class I PI3Ks. Furthermore, besides direct activation of the PI3K-AKT pathway by EGFR, activated KRAS protein can further bind and directly activate the p110α PI3K protein. Mutations in *PIK3CA* are reported in approximately 10–18% of mCRC patients and can coexist with either *KRAS* and *BRAF* mutations (Nosho et al. 2008). Therefore, several studies have evaluated the predictive value of *PIK3CA* mutations in resistance to anti-EGFR therapies. Retrospective studies of cetuximab treatments in chemo-refractory mCRC patients have revealed that, in *KRAS* WT patients, *PIK3CA* mutations in exon 20 lead to worse outcome shown by lower response rates (0.0% *vs*. 36.8%; 95% CI 0.00–0.89; P = 0.029) than *PIK3CA* WT patients (De Roock et al. 2010a, b). Interestingly, mutations in exon 9 (60–65% of *PIK3CA* mutations) of *PIK3CA* had no effect on response rates, survival and prognosis. In two further meta-analysis studies on retrospective cohorts, *PIK3CA* exon 20 mutations, but not exon 9, were associated with absence of response, lower PFS and OS to anti-EGFR mAbs (Sartore-Bianchi et al. 2009; Mao et al. 2012). *In vitro* studies unravelled different intracellular mechanisms of action: exon 9 mutations release p110α from p85-induced inhibition in a KRAS-GTP dependent way, whereas exon 20 mutations activate the kinase domain, independently of interactions with KRAS. This fact may justify different effects of both mutations in responses to mAbs (Zhao and Vogt 2008; Zhao and Vogt 2010).

Overall, without larger prospective studies, it is still difficult to evaluate the precise role of *PIK3CA* mutations with respect to the response to EGFR-targeted therapies, especially given that they are mostly found co-occurring with *KRAS* or *BRAF* mutations.

PTEN (phosphatase and tensin homologue) is another potential marker of response to anti-EGFR therapy, given its negative role on the PI3K-AKT signalling pathway. PTEN inhibits the PI3K pathway through its lipid phosphatase activity, behaving in this way as a tumour suppressor protein (Cully et al. 2006). In mCRC, PTEN activity is reduced in about 20–40% of tumours through either *PTEN* gene silencing (via promoter hypermethylation or loss of heterozygosity) or mutations (Molinari and Frattini 2014). This loss of PTEN activity resulted in constitutive activation of the PI3K-AKT signalling pathway leading to tumour cell proliferation and survival. Studies on the association between the PTEN status and the response to mAbs are controversial and inconclusive. Frattini et al. have studied a cohort of cetuximab and irinotecan-treated patients and found that lower levels of PTEN were predictive of resistance (Frattini

et al. 2007), whereas Laurent-Puig et al. showed no significant differences in terms of RR, PFS and OS in a larger cohort of patients (Laurent-Puig et al. 2009). Moreover, in the NCIC trial, where 572 patients with pretreated mCRC were randomly assigned to receive cetuximab or best supportive care, no statistical significance was found with respect to loss of PTEN and the clinical outcome of patients treated with cetuximab (Karapetis et al. 2014). Nevertheless, two other studies corroborate the fact that loss of PTEN expression (measured by immunohistochemistry) is associated with decreased RR, PFS and OS in mCRC patients treated with anti-EGFR therapy (Loupakis et al. 2009; Sartore-Bianchi et al. 2009). Finally, a recent meta-analysis confirmed that PTEN loss was significantly associated with lack of benefit to mAbs treatment in *RAS* WT patients. However, this study concluded that the predictive power of *BRAF* and *PIK3CA* mutations were stronger than that of PTEN levels (Yang et al. 2013a, b).

Overall, given that technically the assessment of PTEN expression levels by immunohistochemical methods lack standardization, that PTEN alterations co-occur with *RAS* mutations, and that discordant levels of PTEN expression are seen between primary tumour and metastasis, the loss of PTEN expression cannot be seen at present as a reliable predictive biomarker of response to EGFR-targeted mAbs.

Nevertheless, targeted treatments against PI3K or its downstream effectors such as mTOR and AKT in preclinical models suggest great therapeutic potential when combined with receptor tyrosine kinase inhibitors (Kim et al. 2017). A clinical trial evaluating the combination of mTOR inhibitor everolimus with panitumumab and irinotecan in second-line mCRC patients showed better RR when compared to the treatment without everolimus, in RAS WT patients (Townsend et al. 2018). Another combination that is presently exploited in clinical trials is that of PIK3CA/mTOR inhibitors and MEK inhibitors (Andersen et al. 2015; Temraz et al. 2015).

Despite these promising results, larger prospective studies are needed before the role of *PIK3CA* mutations and PTEN expression levels in the mechanism of resistance, and their potential predictive value in anti-EGFR therapies can be concluded.

8.3.3.2 JAK/STAT Signalling Pathway

The Janus family of tyrosine kinases (JAK) and the signal transducer and activator of transcription (STAT) family are involved in cytokine receptor signalling as important mediators of cell survival, proliferation, differentiation, and apoptosis (Rawlings 2004). There have been pieces of evidence supporting a role of STAT family member STAT3 in resistance to the EGFR kinase inhibitor gefitinib in cells (Li et al. 2015). Furthermore, this work has shown that inhibition of STAT3 activity by Stattic (STAT3-inhibitor) sensitizes CRC cells to gefitinib treatments. In an independent work, co-treatments of gefitinib with the JAK/STAT3 inhibitor cucurbitacin B led to increased antitumour activity in CRC cells (Yar Saglam et al. 2015). These results indicate that blocking EGFR signalling is more effective in combination with inhibitors of JAK/STAT3, suggesting a putative role of this pathway in the mechanism of resistance to anti-EGFR therapies. However, further studies are required to fully confirm the role of STAT3 in the mechanism of resistance to mAbs targeting EGFR.

8.3.3.3 Others Components

Other mechanisms have been implicated in the resistance to EGFR-targeted therapy in mCRC. Expression of vascular endothelial growth factor 1 (VEGF-1) or its receptor (VEGFR) has been associated to resistance to cetuximab in preclinical models and in patients with mCRC (Bianco et al. 2008). Furthermore, inflammatory markers such as interleukin-8 (IL8) and cyclooxygenases-2 (COX2), as well as the cell cycle regulator cyclin D1 were also shown important for the outcome of patients receiving anti-EGFR therapy. Vallböhmer et al. reported that a combination of low levels of COX2, EGFR and IL8 was a good prognostic marker for patients when compared to high levels of expression of these three genes, with an OS of 13.5 months vs. 2.3 months, respectively (Vallböhmer et al. 2005). Nuclear translocation

of EGFR was also identified as a possible marker for resistance dependent on Src family kinases (Li et al. 2009). Nuclear EGFR is associated with transcription of cyclin D1 and consequently proliferation of tumour cells. Expression of the transcription factor nuclear factor κB has also been linked with resistance to cetuximab (Scartozzi et al. 2007). Finally, in *in vitro* models epithelial-to-mesenchymal transition (EMT) has also been pointed out as a mechanism involved in resistance to anti-EGFR inhibitors, as CRC mesenchymal-like cells were found sevenfold more resistant than epithelial-like cells (Buck et al. 2007). Although interesting, data regarding the previously mentioned proteins is limited and lack further validation. A comprehensive understanding of their contribution to mechanisms involved in cetuximab and panitumumab resistance is desirable and holds the promise for the generation of novel therapeutic opportunities for the treatment of CRC.

8.4 Acquired Resistance to Anti-EGFR Therapy

8.4.1 EGFR Mutations

EGFR mutations are extremely rare in CRC but have been described associated with acquired resistance to mAb treatment (approximately in 20% of patients treated with cetuximab and 1% of patients treated with panitumumab). Montagut et al. have identified EGFR S492R mutation in cell lines that acquired resistance to cetuximab and confirmed these data in patients who relapsed after cetuximab treatment (Montagut et al. 2012). This mutation is located in the extracellular domain of the receptor and prevents binding of the cetuximab antibody, however, does not seem to affect panitumumab binding. Indeed, one patient who had relapsed under cetuximab and harboured the S492R mutation, responded to panitumumab, suggesting a clinical option to overcome cetuximab resistance in these patients. Other mutations occurring in the extracellular domain of EGFR (R451C, S464L, G465R, I491M and K467T) were identified in patients who had relapsed under cetuximab treatment or in cell lines that acquired resistance to cetuximab (Arena et al. 2015). From these mutations, R451C and K467T do not prevent binding of panitumumab to the receptor. This fact resulted in the generation of new EGFR inhibitors consisting of a mixture of more than one mAb that target different epitopes located in the extracellular domain of EGFR. Sym004 (mixture of two different mAbs) and MM-151 (mixture of three fully human IgG1 antibodies) are new treatment options presently under clinical evaluation (Pedersen et al. 2010, Kearns et al. 2015). Phase I clinical trials of both compounds demonstrated their safety. In 42 mCRC patients who had acquired resistance to anti-EGFR therapy, Sym004 treatments induced about 44% of tumour shrinkage and partial response or stable disease for the other patients. In a similar way, MM-151 also showed long-lasting disease control of patients treated with MM-151 in combination with irinotecan.

8.4.2 RAS/RAF Signalling Pathway

As one of the most important signalling pathways downstream of EGFR, the RAS-RAF-MAPK cascade is also one of the most important mechanisms associated with secondary resistance to mAbs (50–80% of cases). Thus, pre-clinical and clinical studies have identified the occurrence of *KRAS* mutations in metastases that acquired resistance to EGFR inhibitors. Bouchahda et al. reported the first case of CRC liver metastasis harbouring *KRAS* mutations in a patient who had progressed under cetuximab therapy, although primary and metastatic tumours were *KRAS* WT before treatment (Bouchahda et al. 2010). In a further study, Misale et al. showed that six out of ten patients that were *KRAS* WT before the treatment were detected with *KRAS* mutations in their plasma samples during cetuximab treatment (Misale et al. 2012). The same study, also showed one case of *KRAS* amplification (an infrequent event in CRC) in a patient who relapsed after cetuximab treatment, showing that either mutations or amplifications could be associated with

acquired resistance to mAbs. In addition to the *KRAS* gene, alterations in *NRAS* and *BRAF* were also associated with secondary resistance to EGFR-targeted therapies in pre-clinical models (Misale et al. 2012). Altogether, the occurrence of *RAS* mutations in relapsed tumours was found to derive from an expansion of pre-existing clones that propagated under the selection pressure of anti-EGFR treatment, rather than from novel spontaneous mutations (Diaz et al. 2012).

8.4.3 HER2/HER3 Expression

Amplification of other receptor tyrosine kinases of the ErbB family has been described as an acquired resistance mechanism to anti-EGFR therapies. Bertotti et al. showed that HER2 gene amplification was correlated with responses to cetuximab in a patient-derived xenografts mouse model (Bertotti et al. 2011). The authors observed that HER2 amplification was only present in 2–3% of *KRAS* WT patients before treatment, however, in 36% of resistant tumours after cetuximab treatments. They further showed that combination of lapatinib (a small molecule inhibitor of both EGFR and HER2 receptors) with cetuximab or pertuzumab (a monoclonal antibody that inhibits the dimerization of HER2 with other HER receptors) was efficient in a subset of cetuximab-resistant HER2-amplified mCRC xenografts. Based on these findings, the HERACLES phase II was designed to assess the RR of trastuzumab (mAbs targeting HER2) combined with either lapatinib or pertuzumab, in *KRAS* exon 2 WT and HER2 amplified mCRC patients (Sartore-Bianchi et al. 2016). The initial results concerning the trastuzumab and lapatinib combination showed that 30% of patients achieved an objective response with a median duration of response of 38 weeks. Median PFS was 21 weeks and median OS was 46 weeks. Importantly, these results indicate that HER2 is a good druggable target in mCRC.

It should be noted that HER2 gene amplification was also associated with intrinsic resistance to anti-EGFR therapy, however, given its extremely low frequency in CRC (about 2% of cases), its role in primary resistance seems to be minor (Hynes and Lane 2005).

Additionally to HER2, HER3 has also been described to have a role in the resistance mechanism to EGFR-targeted therapies. In a cohort of mCRC patients treated with irinotecan and cetuximab, HER3 overexpression was associated with lower PFS and OS (Scartozzi et al. 2011). Moreover, HER3 is found mutated in 11% of CRC patients and owns oncogenic activity (Jaiswal et al. 2013). MEHD7945A is a humanized IgG1 mAbs with dual HER3/EGFR activity. This compound has achieved promising results in a phase I trial in patients with pretreated mCRC. However, phase II randomized trial showed no benefit for MEHD7945A plus FOLFIRI when compared to cetuximab plus FOLFIRI in *KRAS* WT chemo-refractory patients (Van Cutsem et al. 2014).

8.4.4 MET Receptor Expresssion

MET is a tyrosine kinase receptor for the ligand Hepatocyte Growth Factor (HGF), which upon activation leads to several cellular processes such as cell proliferation, invasion, apoptosis and survival (Organ and Tsao 2011). Several pieces of evidence suggest an involvement of the MET pathway (through MET amplification or increased HGF expression) in the mechanism of both primary and secondary resistance to EGFR mAbs in *KRAS* WT patients (Krumbach et al. 2011). Although in primary samples amplification of MET was only reported in 2% of cases, interaction between EGFR and MET was seen upon activation with TGF-alpha and correlated with acquired resistance to cetuximab in cells (Troiani et al. 2013). Treatment of those cells with MET inhibitor restored cetuximab sensitivity. Furthermore, *in vivo* studies showed an increased level of MET amplification in cetuximab-resistant patients WT for *RAS*, *BRAF*, *PIK3CA* and HER2, whereas amplification had not been seen in pre-treatment tumours (Bardelli et al. 2013). Finally, in a randomized phase II clinical trial of chemo-refractory *KRAS* WT anti-EGFR naïve patients, the combination of

anti-HGF mAbs and panitumumab led to higher response rates and a trend for better outcome in the patient population with MET overexpression (Van Cutsem et al. 2014). A phase I trial assessing safety of cabozantinib (a small molecule MET inhibitor) plus panitumumab in chemorefractory *KRAS* WT patients is ongoing (Jia et al. 2018).

8.5 Conclusions

The high complexity of mechanisms of resistance to anti-EGFR mAbs, make this therapy only efficient in a restricted CRC patient population. Presently, resistance to EGFR-targeted therapies is known to be mediated by constitutive activation of EGFR signalling cascades through deregulation of the receptor itself or downstream components of the RAS/RAF, PI3K/PTEN and JAK/STAT pathways, as well as, from the activation of alternative tyrosine kinase receptor such HER2 and MET. Despite intensive research done over the last 10 years, *RAS* mutations are effectively the only approved biomarker of response in clinical practice. More clinical and translational studies are required in order to increase our knowledge on the mechanisms behind anti-EGFR therapy resistance.

Recent efforts to segregate CRC tumours into subgroups based on their biology and gene expression patterns resulted in an unified classification which categorizes the majority of tumours into four groups called consensus molecular subtypes (CMS1–4) (Guinney et al. 2015). CMS1 (immune, 14% of cases) is enriched for microsatellite instable (MSI) tumours that display BRAF mutations, hypermethylation of CpG islands (CIMP), and immune infiltration and activation. CMS2 (canonical, 37% of cases) reflects the classical adenoma-to-carcinoma sequence, encompassing typical WNT/MYC-driven tumours with epithelial characteristics and high somatic copy number alterations (SCNA), whereas CMS3 (metabolic, 13% of cases) is enriched for KRAS-mutated tumours (although KRAS mutations are present in all CMS subtypes) with activation of metabolic pathways. Finally, CMS4 (mesenchymal, 23% of cases) has mesenchymal features, shows high SCNA, stromal content and activation of TGF-β and VEGFR pathways. Clear clinical distinction is also evident with poor prognosis for CMS4 and a relatively good prognosis for CMS1 (Thanki et al. 2017). In this context, Sveen et al. have lately shown that the CMS2 subtype is predicted to respond to EGFR inhibition, whereas tumours with a metabolic and mesenchymal-like phenotype seem strongly resistant, independently of *KRAS* and *BRAF* mutation status (Sveen et al. 2018).

Overall, the field is moving towards a more comprehensive picture of the processes involved in therapy resistance, which will certainly lead to the recognition of alternative or combinatory treatments, providing more benefit to patients and sparing unnecessary treatments.

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9 miRNAs as Modulators of EGFR Therapy in Colorectal Cancer

Diane M. Pereira and Cecília M. P. Rodrigues

Abstract

Drug resistance is a serious impediment to the treatment of cancer. The use of anti-epidermal growth factor receptor (EGFR) monoclonal antibody therapies in patients with metastatic colorectal cancer is guided by the presence of activating point mutations in *KRAS* and *NRAS* genes in the primary tumour. However, *RAS* wild-type status is still not sufficient to guarantee response to cetuximab and panitumumab, with response rates limited to 70% for combinations with multidrug chemotherapy. Therefore, additional mechanisms contributing to resistance are currently under investigation, and include genetic alterations and epigenetic mechanisms of resistance. In this regard, deregulation of miRNA expression profiles holds potential to unveil resistance and fuel the development of miRNA-based strategies to overcome EGFR-directed therapy limitations. We discuss current understanding of miRNA impact as modulators of EGFR therapy in patients with metastatic colorectal cancer and the future challenge of miRNAs in circulation as powerful non-invasive tools to monitor anti-EGFR therapy response and predict resistance.

Keywords

Anti-EGFR therapy · Epidermal growth factor receptor · Prognostic marker · miRNA · Therapy resistance

9.1 Introduction

The growing body of knowledge on the molecular mechanisms driving tumour initiation and progression has paved the way for the introduction of targeted therapies in clinical practice. In particular, overexpression of epidermal growth factor receptor (EGFR) was shown to be a frequent event in human cancer, among which colorectal cancer (Spano et al. 2005), providing the rationale for the development of therapeutic approaches directed to this receptor. In this framework, two different monoclonal antibodies raised against the extracellular domain of EGFR have been approved for the treatment of metastatic colorectal cancer: cetuximab, a chimeric monoclonal antibody (Harding and Burtness 2005), and panitumumab, a fully humanized monoclonal antibody (Cohenuram and Saif 2007). Treatment with these antibodies prevents ligand-induced EGFR tyrosine kinase activation, thereby suppressing downstream signalling pathways (Martinelli et al. 2009). These include the RAS-RAF-MEK-ERK and the PI3K-AKT axes, that in turn promote tumour cell proliferation, survival and invasive properties (Lemmon and

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Schlessinger 2010; Witsch et al. 2010). Given its chimeric origin (IgG1 subtype), cetuximab also triggers antibody-dependent cellular cytotoxicity (ADCC), an immune mechanism whereby effector cells recognize and eliminate antibody-coated tumour cells, adding to the therapeutic effects of this anti-EGFR agent (Kimura et al. 2007; Mellor et al. 2013).

Although EGFR-targeted therapies represent an undeniable step forward in the management of metastatic colorectal cancer, it quickly became evident that only a small group of patients would benefit from treatment with these antibodies (Bardelli and Siena 2010). Moreover, even the subset of patients who initially respond to anti-EGFR-based regimens will ultimately become refractory within 3 to 12 months after initiating treatment (Cunningham et al. 2004; Van Cutsem et al. 2007). Mechanisms of primary and acquired resistance towards EGFR blockade have been mainly attributed to the activation of signalling pathways operating downstream of EGFR (Zhao et al. 2017). Mutations in *KRAS* exon 2 (codons 12 and 13) were the first to be validated as having a negative predictive value for EGFR-directed therapy (Amado et al. 2008; Bokemeyer et al. 2009; Douillard et al. 2010; Karapetis et al. 2008; Lievre et al. 2006). Since then, less frequent *KRAS* and *NRAS* alterations were also recognized as biomarkers of resistance to cetuximab and panitumumab (Douillard et al. 2013; Stintzing et al. 2016). Accordingly, the administration of these targeted therapies is now restricted to wild-type *KRAS* and *NRAS* tumours, as recommended by all major international guidelines (Waring et al. 2016). However, *RAS* wild-type status is still not sufficient to guarantee response to cetuximab and panitumumab, with response rates as low as 20% for anti-EGFR monotherapy (Price et al. 2016), and limited to 70% for combinations with multidrug chemotherapy (Heinemann et al. 2016). Therefore, additional mechanisms contributing to resistance are currently under investigation, and include genetic alterations in *BRAF*, *PIK3CA*, *MET*, and *PTEN* (Bardelli et al. 2013; De Roock et al. 2010a; Frattini et al. 2007; Sartore-Bianchi et al. 2009). Alternatively, non-genetic mechanisms of resistance are also emerging, a scenario where deregulation of microRNA (miRNA) expression profiles has attracted remarkable attention (Perkins et al. 2014). Owing to their dynamic and potentially reversible nature (Biswas and Rao 2017; Salgia and Kulkarni 2018), knowledge on these epigenetic alterations holds promise of being translated into new clues to unveil resistance and alternative strategies to overcome anti-EGFR therapy limitations.

9.2 MicroRNAs

9.2.1 miRNA Structure and Mechanism of Action

miRNAs are a class of endogenous small non-coding RNAs that post-transcriptionally regulate gene expression (Bartel 2004; Lin and Gregory 2015). These molecules have been predicted to control up to 60% of all protein-coding genes (Friedman et al. 2009), participating in the modulation of a myriad of crucial cellular processes such as proliferation, motility, differentiation and apoptosis (Vidigal and Ventura 2015). miRNA biogenesis involves a collection of tightly regulated bioprocessing steps until the mature and biologically active miRNA – a ~22-nucleotide single-stranded RNA – is incorporated in the miRNA-induced silencing complex (miRISC). Following complex assembly, the mature miRNA guides the miRISC to target mRNAs, leading to gene silencing via translational repression and/or mRNA deadenylation and degradation. Target recognition proceeds predominantly through miRNA-mRNA incomplete base pairing, but involves high sequence complementarity between the 5′ end of the miRNA, known as the seed region, and the 3′-untranslated region (3’-UTR) of the mRNA (Krol et al. 2010; Pasquinelli 2012). This imperfect match and relatively short binding sites (6–8 base pairs) confer miRNAs with the ability to regulate the expression of multiple genes, while retaining their specificity. In turn, the multiplicity of targets allows for the widespread action of a given miRNA on different signalling pathways, or leads to intensified miRNA suppressing effects when controlling multiple

components of the same cascade. The functional significance of these molecules is further illustrated by the fact that different miRNAs may target the same mRNA, creating an even tighter network of gene expression regulation (Friedman et al. 2009). In the rare cases of nearly perfect miRNA-mRNA complementarity, the target mRNA may also undergo endonucleolytic cleavage (Krol et al. 2010).

9.2.2 Deregulation of miRNAs in Colorectal Cancer and Therapy Resistance

Abnormal miRNA expression, processing and function is involved in almost every aspect of tumour aetiology and became a well-established feature of colorectal cancer (Schetter et al. 2012). According to their functions in the regulation of cancer-related pathways, miRNAs have been shown to behave as either tumour suppressors or oncogenes. Downregulation of tumour-suppressive miRNAs and upregulation of miRNAs with oncogenic functions triggers the activation of multiple signalling pathways that facilitate tumour initiation, progression and metastasis (Svoronos et al. 2016). Increased and/or decreased miRNA expression in tumour cells is often the result of mutations, genomic rearrangements, epigenetic modifications, alterations in transcriptional control, and defects in miRNA biogenesis machinery (Lin and Gregory 2015; Rupaimoole et al. 2016). In addition to the impact of both genetic and epigenetic alterations on mature miRNA expression patterns, miRNA proper function can also be impaired by single-nucleotide polymorphisms or mutations within target mRNA 3'-UTR sequences (Sethupathy and Collins 2008). Importantly, these modifications are responsible for the loss of miRNA recognition elements, limiting miRNA-mediated regulation and providing tumour cells with the ability to circumvent miRNA-mRNA binding.

In colorectal cancer, miRNAs have been reported to contribute to tumour development, following distinct patterns of expression between normal colonic mucosa, adenomas and adenocarcinomas (Oberg et al. 2011; Slattery et al. 2016b). The profiles of miRNA deregulation might further help in the classification of colorectal cancer molecular subtypes. Indeed, miRNAs are differentially expressed in microsatellite stable and unstable tumours (Earle et al. 2010; Lanza et al. 2007; Sarver et al. 2009; Schepeler et al. 2008), and correlate with CpG island methylator phenotypes (Slattery et al. 2011, 2016a). Moreover, *KRAS*-, *BRAF*- and *TP53*-mutant tumours carry altered miRNA expression signatures when compared with wild-type counterparts (Mosakhani et al. 2012b; Slattery et al. 2011, 2016a). Ultimately, aberrant miRNA function was found to contribute to every colorectal cancer hallmark, and to affect virtually all fundamental signalling pathways driving tumorigenesis, including EGFR and its downstream signalling network (Mlcochova et al. 2013).

Apart from their role in colorectal cancer initiation and progression, miRNA deregulation might also contribute to anti-cancer therapy resistance, being associated with impaired response rates and worse survival outcomes. In this regard, distinct sets of miRNAs have been shown to correlate with either tumour response or tumour resistance to chemotherapy and chemoradiotherapy (Boisen et al. 2014; Della Vittoria Scarpati et al. 2012; Kjersem et al. 2014; Svoboda et al. 2012; Zhang et al. 2014; Zhu et al. 2017). Moreover, and consistent with the hypothesis that miRNAs might control anti-EGFR therapy response, miRNA profiling was demonstrated to efficiently predict the clinical benefits of EGFR blockade (Cappuzzo et al. 2014; Mlcochova et al. 2015; Mosakhani et al. 2012a). Also, colon cancer cell lines with different sensitivities to cetuximab express distinct sets of miRNAs following treatment (Ragusa et al. 2010). This chapter provides an overview of the main findings regarding the relationship between miRNAs and tumour cell response to EGFR-targeted antibodies in colorectal cancer. Focus will be given to the potential uses of miRNAs as innovative therapeutic options to overcome resistance, and to the clinical application of these small non-coding RNAs for the selection of patients who might benefit from cetuximab and panitumumab treatment.

9.3 miRNAs as Modulators of Anti-EGFR Therapy Response in Colon Cancer

The deregulation of miRNA expression profiles in cancer introduced an additional layer of complexity to the mechanisms promoting drug resistance. Particularly, miRNAs were shown to control tumour cell response to EGFR-targeted agents by directly regulating the expression of several components within the EGFR cascade, and to sustain resistance by activating both downstream and parallel compensatory signals (Adem et al. 2016; Migliore and Giordano 2013). The significance of this liaison is further emphasized by the demonstration that modulation of resistance-associated miRNAs is able to restore colon cancer cell response to cetuximab and panitumumab (Table 9.1). In the era of personalized medicine, this increasing knowledge regarding the impact of miRNAs in tumour cell response to EGFR-directed therapies will undoubtedly fuel the development of miRNA-based strategies to overcome resistance.

9.3.1 miRNAs and Anti-EGFR Therapy Resistance

The interplay between miRNAs and EGFR signalling has been widely studied in cancer. Growth factor-induced signalling is known to control miRNA expression profiles, and miRNAs can reciprocally regulate multiple tiers of the EGFR cascade (Kedmi et al. 2015). In this regard, miR-7 has been shown to simultaneously target EGFR and RAF1 (Suto et al. 2015), and might itself be regulated at the transcriptional level by EGFR-triggered signalling (Chou et al. 2010). In turn, miR-7 overexpression sensitized human colon cancer cell lines harbouring a mutant *KRAS* to cetuximab treatment (Suto et al. 2015). Similarly, the tumour suppressor miR-133b was reported to directly regulate EGFR expression, while enhancing the growth inhibitory effects of cetuximab in wild-type and mutant *KRAS* colon cancer cells (Zhou et al. 2015).

A regulatory loop involving EGFR and the miR-143/miR-145 cluster has also been described. Both miR-143 and miR-145 are recognized for

Table 9.1 miRNAs involved in EGFR-targeted therapy response in colorectal cancer

miRNAs	Target mRNAs	Impact of therapeutic modulation	References
miRNAs promoting response to anti-EGFR therapy			
miR-7	EGFR, RAF1	Overexpression of miR-7 precursor sensitized $KRAS^{MUT}$ and $BRAF^{MUT}$ colon cancer cells to cetuximab	Suto et al. (2015)
miR-133b	EGFR	Combination with miR-133b mimics improved the growth-inhibitory effects of cetuximab in $KRAS^{WT}$ and $KRAS^{MUT}$ colon cancer cells	Zhou et al. (2015)
miR-143 miR-145	KRAS, BRAF, MEK2, ERK5, RREB1	Stable miR-143 or miR-145 overexpression increased cetuximab-mediated ADCC effected by PBMCs in $KRAS^{WT}$ and $KRAS^{MUT}$ colon cancer cells	Gomes et al. (2016)
miRNAs associated with resistance to anti-EGFR therapy			
miR-199a-5p	PHLPP1, CTGF, PIK3CA	miR-199a-5p and miR-375 inhibitors sensitized $KRAS^{MUT}$ colon cancer cells to cetuximab	Mussnich et al. (2015)
miR-375		Overexpression of miR-375 precursor increases cetuximab-induced cell death in $KRAS^{MUT}$ colon cancer cells	Alam et al. (2017)
miR-100 miR-125	DKK1, DKK3, ZNRF3, RNF43, APC2	Bicistron sponges for miR-100 and miR-125 restored cetuximab response in resistant $KRAS/NRAS/BRAF^{WT}$ colon cancer cell lines and mouse xenografts	Lu et al. (2017)

Abbreviations: *ADCC* antibody-dependent cellular cytotoxicity, *MUT* mutant, *PBMCs* peripheral blood mononuclear cells, *WT* wild-type

their tumour-suppressive role in colon cancer, acting through direct repression of KRAS, BRAF, and several downstream members of the MAPK family (Akao et al. 2006b; Chen et al. 2009; Ibrahim et al. 2011; Kent et al. 2013; Pagliuca et al. 2013; Pekow et al. 2012). In line with this notion, miR-143/miR-145 overexpression strongly suppressed EGFR-induced colon cancer cell growth (Zhu et al. 2011). Conversely, EGFR signals have been shown to negatively regulate miR-143 and miR-145 expression in murine colonic cells (Zhu et al. 2011). In colon cancer cell lines, oncogenic KRAS impaired miR-143/miR-145 transcription by activating the RAS-responsive element-binding protein 1 (RREB1) (Kent et al. 2013). RREB1 is in turn targeted by miR-145 itself (Kent et al. 2010), narrowing the feedback regulatory circuit. Finally, re-introduction of miR-143 and miR-145 in human colon cancer cells increased cetuximab-mediated ADCC and apoptosis independently of *KRAS* status (Gomes et al. 2016), further suggesting that combined administration of tumour-suppressive miRNAs with systemic therapy may be an elegant strategy to overcome resistance to anti-EGFR therapies.

Using a miRNA microarray profiling platform, miR-199a-5p and miR-375 were found to be upregulated in cetuximab-resistant colon cancer cells. In turn, inhibition of either miR-199a-5p or miR-375 sensitized colon cancer cells to EGFR-targeted cetuximab treatment. Mechanistically, these miRNAs have been proposed to promote cetuximab resistance by directly targeting PHLPP1, which in turn inhibits the AKT pathway (Mussnich et al. 2015). Nevertheless, contradictory results have shown that miR-375 suppresses the expression of CTGF, a ligand of EGFR, and synergizes with cetuximab treatment (Alam et al. 2017). Moreover, the catalytic subunit of PI3K has also been identified as a target of miR-375 (Wang et al. 2014), further suggesting that this miRNA might have context-dependent opposing roles in colon cancer and, particularly, in EGFR signalling modulation.

Apart from the direct regulation of EGFR signalling components, the influence of miRNAs on alternative oncogenic pathways may also have significant effects on anti-EGFR therapy response. The long non-coding RNA MIR100HG and its embedded miRNAs, miR-100 and miR-125b, were found to be upregulated in cetuximab-resistant three-dimensional cultures of colon cancer cells, and in tumours from colorectal cancer patients who progressed on anti-EGFR therapy. Interestingly, this phenomenon correlated with the degree of cetuximab resistance and was associated with both *de novo* and acquired resistance, regardless of *KRAS/BRAF* mutational status. In return, simultaneous inhibition of miR-100 and miR-125b in cetuximab-resistant cells using bivalent miRNA sponges restored cetuximab responsiveness *in vitro* and *in vivo*, revealing a model in which these miRNAs cooperate to modulate cetuximab response (Lu et al. 2017). In this study, miR-100 and miR-125b were shown to contribute to cetuximab resistance by repressing five different Wnt/β-catenin negative regulators, leading to increased Wnt signalling (Lu et al. 2017). Nonetheless, a crosstalk between Wnt and EGFR signalling in cancer has already been demonstrated (Hu and Li 2010) and may, at least in part, account for the impact of MIR100HG and its product miRNAs on cetuximab-resistant phenotype.

9.3.2 miRNA-Based Strategies to Overcome Resistance to Anti-EGFR Therapy

Therapeutic strategies currently in preclinical development include anti-miRNA constructs designed to repress oncogenic miRNAs, as well as miRNA mimics intended to replace miRNAs with tumour-suppressive functions (Rupaimoole and Slack 2017). Interestingly, the same rationale could be used in combination with anti-EGFR therapies, targeting miRNAs that contribute to resistance (e.g. miR-21 (Gong et al. 2011) and miR-221 (Garofalo et al. 2011)), and re-establishing the expression of miRNAs capable of restoring sensitivity (e.g. miR-133b (Zhou et al. 2015), miR-143 and miR-145 (Gomes et al. 2016)). Still, several examples in which specific miRNAs, depending on the context, behave as

either tumour suppressors or oncogenes have already been identified (Svoronos et al. 2016). Of note, miR-7 (Nakagawa et al. 2015; Zhang et al. 2013), miR-199a, miR-375 (Alam et al. 2017; Chao et al. 2017; Mussnich et al. 2015), miR-100, and miR-125b (Chen et al. 2014b; Lu et al. 2017; Zhang et al. 2017) are all likely to fit in this scenario, so care must be taken when designing strategies for the modulation of these dichotomous miRNAs.

The rational combination of miRNA-based therapeutics with current cytotoxic and targeted agents provides an unprecedented opportunity to counteract tumour resistance and tailor cancer treatment. In this context, one of the most significant advantages of miRNA therapeutics stems from their unique ability to coordinately regulate multiple effectors within parallel signalling pathways (Lam et al. 2015), outperforming single-target approaches by minimizing the risk of acquired resistance. On the other hand, this inherent multi-target nature of miRNAs also translates into the need for an extensive characterization of a given miRNA targetome in order to predict potential side-effects and ensure the desired outcome before it can be considered for therapeutic intervention. Moreover, additional challenges delaying the translation of miRNA-based therapeutics into the clinic include *in vivo* stability and tissue-specific targeting of miRNA mimics/inhibitors. Still, significant advances in RNA chemistry and systemic delivery technologies have already been made, providing new formulations with low toxicity profiles and capable of targeted payload delivery (Pereira et al. 2013). Given the optimistic perspective on the field, several miRNA-based agents have now reached clinical trials for the treatment of cancer (Chakraborty et al. 2017). Those include synthetic mimics of miR-34 (MRX34; phase I, terminated) and miR-16 (MesomiR-1; phase I, ongoing) for the treatment of multiple solid tumours, and an antisense inhibitor of miR-155 for patients with cutaneous T-cell lymphoma (MRG-106; phase I, ongoing) (Rupaimoole and Slack 2017). The data arising from these studies will hopefully provide the first clues to the benefits of miRNA modulation in clinical practice, and encourage new miRNA-based therapeutics to progress into clinical development as sensitizers to anticancer drugs.

9.4 miRNAs as Predictive Biomarkers for Anti-EGFR Therapy in Colon Cancer

In addition to their direct role in regulating response to EGFR-directed therapy, miRNAs have shown great promise as biomarkers of drug sensitivity (Allen and Weiss 2010) (Table 9.2). Owing to their small size and remarkable stability, miRNAs can be reliably detected in a variety of human specimens, ranging from frozen and formalin-fixed paraffin-embedded tissues (Xi et al. 2007), to the majority of body fluids, such as plasma (Mitchell et al. 2008), serum (Chen et al. 2008), saliva (Park et al. 2009) and urine (Hanke et al. 2010). Remarkably, these molecules were shown to remain stable even after multiple freeze-thaw cycles and prolonged exposure to room temperatures (Chen et al. 2008; Mitchell et al. 2008). Moreover, a variety of miRNA detection methods has been developed, and include quantitative RT-PCR, microarray and high-throughput sequencing platforms (Ferracin and Negrini 2015). In this framework, current research has been building to support the hypothesis that miRNA expression profiles can be used to select patients who will benefit from a specific pharmacological therapy, and to avoid unnecessary treatment and collateral side effects on those who are predicted to be non-responders.

9.4.1 Tissue miRNAs as Biomarkers

The presence of activating *RAS* mutations is recognized as a major predictor of resistance to cetuximab and panitumumab (Waring et al. 2016). However, *RAS* mutational status only accounts for a portion of nonresponsive cases, and about 30% of metastatic colorectal cancer patients with wild-type *RAS* tumours also do not benefit from chemotherapeutic regimens incorporating anti-EGFR monoclonal antibodies (Heinemann et al. 2016). Interestingly, several

Table 9.2 miRNA biomarkers for anti-EGFR therapy response in metastatic colorectal cancer

miRNAs	Therapy regimen	*n*	Source	Method	Clinical observation	References
Wild-type KRAS						
miR-31	Anti-EGFR mAbs *(alone or with irinotecan)*	33	FFPE tissue	Microarray	Negative correlation with therapy response	Mosakhani et al. (2012a)
	Anti-EGFR mAbs *(alone or combined)*	132	FFPE; frozen tissue	Microarray	Positive correlation with risk of progression	Manceau et al. (2014)
	Anti-EGFR mAbs *(monotherapy)*	88	FFPE tissue	qRT-PCR	Negative relation with PFS (*RAS/BRAF*^{WT})	Igarashi et al. (2015)
	Anti-EGFR mAbs *(alone or combined)*	69	FFPE tissue	Microarray	Negative correlation with TTP (*RAS*^{WT})	Mlcochova et al. (2015)
miR-592	Anti-EGFR mAbs *(alone or with irinotecan)*	33	FFPE tissue	Microarray	Positive association with therapy response	Mosakhani et al. (2012a)
miR-99a	Anti-EGFR mAbs *(alone or combined)*	110	FFPE tissue	Microarray	Positive correlation with PFS and OS	Cappuzzo et al. (2014)
let-7c						
miR-125b						
miR-181a	Anti-EGFR mAbs *(alone or combined)*	54	FFPE tissue	qRT-PCR	Positive association with PFS	Pichler et al. (2014)
miR-345	Cetuximab (plus irinotecan)	138	Whole blood	Microarray	Negative correlation with response and PFS	Schou et al. (2014)
Mutant KRAS						
let-7a	Cetuximab *(plus irinotecan)*	59	FFPE tissue	qRT-PCR	Positive correlation with PFS and OS	Ruzzo et al. (2012)
miR-200b	Cetuximab *(plus 5-FU, oxaliplatin and bevacizumab)*	15	FFPE tissue	qRT-PCR	Positive correlation with PFS	Mekenkamp et al. (2012)
miR-143	Cetuximab *(plus 5-FU, oxaliplatin and bevacizumab)*	15	FFPE tissue	qRT-PCR	Negative correlation with PFS	Mekenkamp et al. (2012)
miR-193a	Anti-EGFR mAbs *(combined therapy)*	45	FFPE tissue	Microarray; qRT-PCR	Positive association with PFS (*KRAS/BRAF*$^{WT/MUT}$)	Takahashi et al. (2017)
Unknown KRAS status						
miR-155	Cetuximab *(plus 5-FU and oxaliplatin)*	15	Serum	qRT-PCR	Increased in non-responders	Chen et al. (2014a)

Abbreviations: *5-FU* 5-fluorouracil, *FFPE* formalin-fixed paraffin-embedded, *mAbs* monoclonal antibodies, *OS* overall survival, *PFS* progression free survival, *qRT-PCR* quantitative RT-PCR, *TTP* time to progression, *WT* wild-type

studies have addressed this issue, suggesting miRNAs as candidate biomarkers of response to EGFR-targeted agents in a wild-type *KRAS* background. In this context, upregulation of miR-31-3p and/or miR-31-5p was found to be associated with poor response (Mosakhani et al. 2012a) and shorter progression-free survival (PFS) (Igarashi et al. 2015; Manceau et al. 2014) in metastatic colorectal patients treated with cetuximab or panitumumab. A negative correlation between miR-31-3p/5p expression levels and time-to-progression (TTP) after cetuximab treatment has also been demonstrated (Mlcochova et al. 2015). Finally, a follow-up to these studies recently confirmed the negative predictive value of miR-31-3p in patients receiving cetuximab-based chemotherapy for operable colorectal liver metastases (Pugh et al. 2017), adding proof to the strength of miR-31 in the identification of wild-type *KRAS* patients who are unlikely to respond to anti-EGFR therapy. On the other hand, increased expression of the miRNAs from the miR-99a/let-7c/miR-125b cluster, together or individually, correlated with significantly longer

PFS and overall survival (OS) rates in wild-type *KRAS* metastatic colorectal patients receiving anti-EGFR monoclonal antibodies (Cappuzzo et al. 2014). Similarly, patients with high miR-181a expression levels had better PFS than those from the low expression group, suggesting that this miRNA may also be predictive of benefit to cetuximab and panitumumab treatment in the wild-type *KRAS* population (Pichler et al. 2014).

Conversely, although wild-type *KRAS* status has been established as a necessary condition for anti-EGFR treatment indication, a small fraction of chemotherapy-refractory patients carrying *KRAS* mutations might still benefit from this therapy (De Roock et al. 2010b). In this framework, mechanisms of post-transcriptional inhibition of mutant KRAS could be of clinical relevance, introducing an additional scenario where miRNAs may become interesting tools to guide treatment choice. The human *KRAS* 3'UTR contains multiple let-7 complementary sites, subjecting *KRAS* mRNA to let-7-mediated regulation (Akao et al. 2006a). In turn, let-7 has been suggested to provide survival advantages by targeting mutant KRAS under anti-EGFR therapy. Indeed, in mutant *KRAS* metastatic colorectal patients receiving cetuximab-based chemotherapy, upregulation of let-7a correlated with improved PFS and OS (Ruzzo et al. 2012). Still, the presence of a specific 3'UTR polymorphism known to compromise let-7 binding to *KRAS* mRNA (LCS6) failed to correlate with survival outcomes in patients treated with anti-EGFR therapy (Sha et al. 2014). As for let-7a, increased expression of miR-200b (Mekenkamp et al. 2012) or miR-193a-3p (Takahashi et al. 2017) was found to be associated with significant benefits in terms of PFS in mutant *KRAS* tumours treated with cetuximab. Interestingly, miR-200 and miR-193 families are also known to regulate KRAS (Kopp et al. 2014; Seviour et al. 2017), reinforcing the idea that miRNAs interfering with KRAS-signalling may have predictive value in the mutant population. However, improved PFS has further been described to correlate with downregulation of the KRAS-targeting miR-143 (Chen et al. 2009; Mekenkamp et al. 2012), a contradicting observation that clearly reflects the complexity of mechanisms dictating EGFR-targeted therapy response.

Although the analysis of miRNA expression profiles directly from tissues has greatly improved our knowledge on the factors predicting therapy response and survival outcomes, the invasive nature of the procedures required for sample collection limits its application. Interestingly, the discovery of circulating miRNAs may offer an opportunity to replace tissue biopsies by easily obtainable blood products, and translate miRNA biomarkers into clinical decision-making.

9.4.2 Circulating miRNAs as Biomarkers

Circulating miRNAs possess a unique set of features providing the basis for their development as non-invasive cancer biomarkers. First of all, cell-free miRNA molecules are remarkably stable in blood flow. In this regard, extracellular vesicles (Hunter et al. 2008; Valadi et al. 2007), lipoproteins (Vickers et al. 2011) and ribonucleoprotein complexes (Arroyo et al. 2011) have all been found to shield miRNAs from RNases in circulation. Moreover, extracellular miRNA expression profiles were shown to mirror the patterns of deregulation observed in corresponding tumour tissues (El Sharawy et al. 2016), providing accessible material to follow miRNA dynamics in cancer and to evaluate the effects of therapeutic interventions. Finally, these miRNA candidates are also expected to offer improved specificity and sensitivity over currently available protein markers and screening tests (Schwarzenbach et al. 2014; Sun et al. 2012).

The first reports addressing the correlation between tumour-associated circulating miRNAs and response to anti-EGFR therapies in colorectal cancer are now emerging. For instance, serum levels of miR-155 were upregulated in metastatic disease, and declined to values found in healthy controls after surgery and cetuximab-based adjuvant chemotherapy in patients who responded to therapy, but remained elevated in non-responsive cases (Chen et al. 2014a). Also, high levels of circulating miR-345 in whole blood were found to

be associated with poor response and decreased PFS in wild-type *KRAS* metastatic colorectal cancer patients receiving third-line cetuximab plus irinotecan therapy (Schou et al. 2014). Together, these studies support the notion that miRNAs in circulation may become powerful non-invasive tools to monitor anti-EGFR therapy response and predict resistance. However, lack of specificity, reproducibility and consensus for data normalization remain as challenges to overcome, encouraging the implementation of accurate and standardized guidelines for miRNA isolation and quantitation in serum and plasma (Shigeyasu et al. 2017; Witwer 2015). Despite these apparent limitations, a surprising number of clinical trials using miRNAs as candidate biomarkers in their protocols are already undergoing, and include studies aiming to evaluate miRNA levels during exposure to targeted agents, as well as studies assessing circulating miRNAs as indicators of therapy response (Ferracin and Negrini 2015).

9.5 Concluding Remarks

Since the initial discovery of miRNA deregulation in colorectal cancer, it became clear that these small non-coding RNAs might participate in the underlying mechanisms of anti-EGFR therapy resistance. In this context, rational combination of EGFR-targeted agents with miRNA therapeutics emerged as a promising strategy to maximize treatment efficacy. However, one of the main obstacles for the development of miRNA-based therapeutic strategies remains the identification of context-relevant miRNA candidates. Indeed, current treatment options for metastatic colorectal cancer heavily rely on combination protocols, whereas most of the studies here reviewed have been designed to evaluate the impact of miRNA modulation on anti-EGFR monotherapy. Thereby, and before considering the incorporation of miRNA-based therapeutics in colorectal cancer treatment, it will be crucial to understand the contribution of miRNAs in the response to multidrug regimens, a scenario where additional mechanisms of resistance are expected to arise. Ultimately, miRNAs with targets in more than one resistance-associated pathway will be the best candidates to progress into clinical evaluation. It is also important to emphasize that tumour cell response to cetuximab and panitumumab appears to be predominantly influenced by miRNAs with validated targets within the EGFR signalling network. Still, further studies will be required to better characterize the array of miRNAs controlling anti-EGFR therapy response, increasing the probabilities of finding valid targets for miRNA modulation in cancer treatment. Finally, the comprehensive characterization of the repertoire of oncogenes and tumour suppressors targeted by any potential miRNA candidate will be crucial to minimize the risk of toxicity and unwanted side-effects.

In addition, miRNAs are attractive candidates for the identification of biomarkers outperforming *RAS* mutation status in predicting response to EGFR blockade. Particularly, special focus has been given to the potential of circulating miRNAs as minimally invasive biomarkers of response. However, the expression of cancer-specific miRNAs in serum/plasma can be masked by miRNAs from unrelated inflammatory states and different tissue origins (e.g. blood, endothelial and liver cells), hampering the interpretation of single miRNA biomarkers. Combination of panels of multiple miRNAs should be considered to enhance sensitivity and specificity in selecting patients who will benefit from cetuximab- or panitumumab-based treatments. Even more promising will be the identification of tumour signatures integrating miRNA expression profiles with clinicopathological features.

Remarkably, *in vitro* models capable of recapitulating tumour heterogeneity and microenvironment are being developed for the identification of mechanisms leading to anti-EGFR therapy resistance (Luraghi et al. 2018), holding incredible promise to enhance the precision with which personalized medicine is delivered in metastatic colorectal cancer. As more miRNAs are tested in large-scale validation studies and clinical trials, either as biomarkers or cancer therapeutics, the clinical expectations for these tiny molecules will likely continue to grow.

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