Chapter 4

BRAF Mutation in Colorectal Cancer

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Additional information is available at the end of the chapter

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Abstract

The *BRAF* mutant colorectal cancer subgroup is a small population with unique clinicopathological and molecular features. This subgroup has been associated with particularly poor prognosis and advanced disease. The poor response of these patients to available treatments has driven much of the effort in trialling combination targeted treatments involving *BRAF* and *MEK* inhibitors. Most recently, an observed survival benefit with intensive triplet chemotherapy agents would encourage its use as first-line treatment in suitable candidates given that few of these patients proceed to second- or third-line treatments.

Keywords: BRAF, colorectal cancer, dabrafenib, trametenib, FOLFOXIRI

1. Introduction

The *BRAF* mutant (MT) colorectal cancer (CRC) population is a small and unique subgroup noted for its association with poor prognosis and survival. *BRAF* mutation occurs in approximately 10% (range, 5–22%) [1, 2] of the unselected CRC population and consistently has inferior median survival outcomes ranging from 8 to 14 months [3, 4]. Failure to achieve good survival outcomes through standard doublet chemotherapy agents in this population has ignited efforts to combine multiple target therapies, aiming for breakthroughs. In this chapter, the *BRAF* gene and its signalling pathway are explored in detail. *BRAF* gene mutation frequency and its impact on clinical presentation as well as its prognostic and predictive significance are also discussed. Updates on the current and latest management strategies as well as novel investigational treatments in this subgroup are also presented.

2. BRAF and the RAS/RAF/MEK/ERK signalling pathway

V-raf murine sarcoma viral oncogene homologue (RAF) is one of the most intensively researched mammalian effectors of RAS in the RAS/RAF/MEK/ERK signalling pathway (**Figure 1**) [5, 6]. The RAF protein itself is made up of three conserved regions: CR1, CR2, and CR3. CR1 and CR2 are situated in the N terminus. CR1 acts as the main binding domain for RAS. CR2 is the regulatory domain. CR3 is situated at the C terminus and functions as the catalytic kinase domain [7].

When GTP bound, RAS recruits RAF protein to the cell membrane and binds to it. This binding process activates RAF kinase by the phosphorylation of two amino acids (T599 and S602 of BRAF) situated in the activation segment of the kinase domain. RAF then phosphorylates its downstream effectors MEK1, MEK2, ERK1, and ERK2, leading to the activation of cellular proliferation, differentiation, and transcriptional regulation (**Figure 1**) [7].

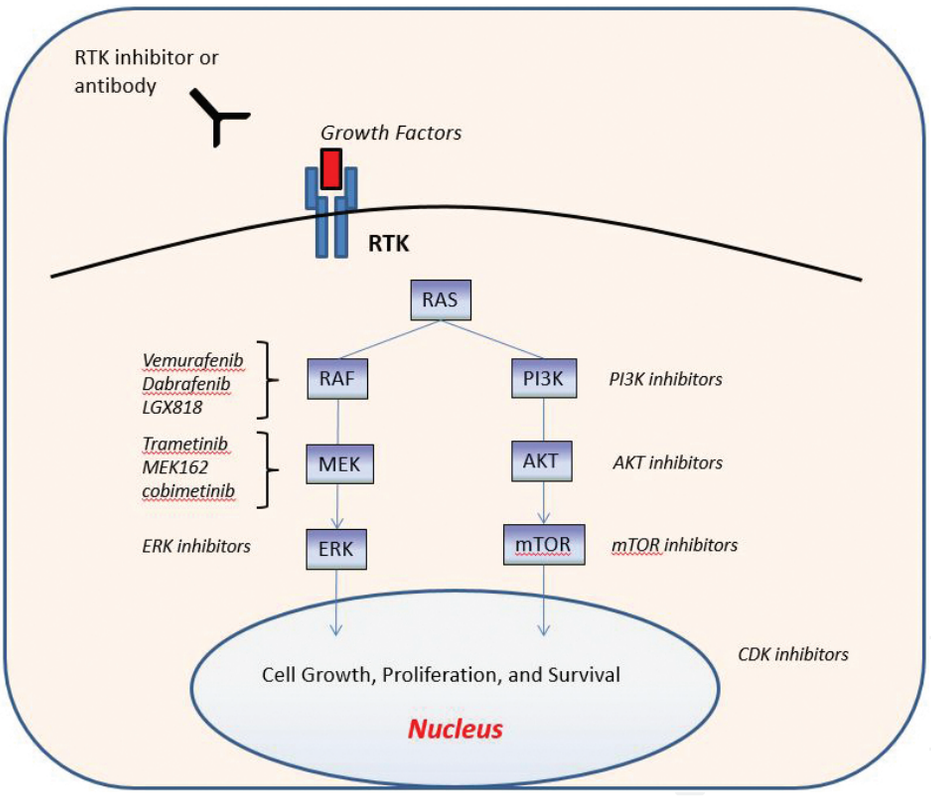

Figure 1. RAS and PIK3CA signalling pathways.

B-RAF (*BRAF*) together with A-RAF and C-RAF are the members of the RAF kinase family [8]. These three RAF isoforms are homologous in sequence and substrate specificity but do differ in their biological functions and regulations. Of these, BRAF remains the most potent activator of MEK [9].

The *BRAF* gene is a proto-oncogene located on chromosome arm 7q34, composed of 18 exons. There are more than 30 different *BRAF* mutations [10]. The most common activating mutation,

BRAF V600E (p.Val600Glu/c.1799T>A), accounts for 90% of all activating *BRAF* mutations and is found in exon 15 at nucleotide position 1799 [11]. The thymine-to-adenine transversion within codon 600 leads to the substitution of valine by glutamate at the amino acid level. This mutation occurs in the activating segment of the kinase domain, resulting in increased basal kinase activity. Compared to wild-type (WT) *BRAF*, *BRAF* V600E demonstrates an almost 500-fold increase in endogenous kinase activity [10, 12].

In solid tumours, the highest incidence of *BRAF* mutations is in malignant melanoma (27–70%), CRC (5–22%), and serous ovarian cancer (~30%) and less (1–3%) in non-small cell lung carcinoma (NSCLC) [13–15]. In colonic cell lines, the oncogenic effects of *BRAF* V600E include cell proliferation and inhibition of apoptosis [16]. Although dependent on continued BRAF activity for tumourigenic growth, *BRAF* MT cells did not require an upstream RAS function for proliferation [17].

2.1. *BRAF* mutation detection methods

CRC *BRAF* mutations can be identified using first- and second-generation direct sequencing, immunohistochemistry (IHC), and, potentially, circulating tumour cells (CTC).

Sanger sequencing is the earliest form of first-generation direct sequencing. Sanger sequencing was developed in 1975 and relies on the chain-termination sequencing of amplified DNA by polymerase chain reaction (PCR) and detection through electrophoresis. It requires approximately 18 to 19 h to process and is also 10 times less sensitive than pyrosequencing. Sanger sequencing method also cannot detect the changes in chromosomal copy number and translocations [18].

Next-generation sequencing (NGS) differs in technology using a specific reagent wash of nucleotide triphosphates with synchronised optical detection and includes pyrosequencing, allele-specific (AS) PCR, mass spectrometry, and real-time qPCR with melt-curve analysis [19]. NGS is the new gold standard test in *BRAF* mutation detection given its superior detection and speed.

Pyrosequencing is referred to as sequencing by synthesis and relies on the release of pyrophosphate (PPi) by DNA polymerase. The test detects light emitted when nucleotides are added to the target DNA template by DNA polymerase releasing PPi via a chemiluminescence reaction. It is a more rapid and sensitive test in detecting *BRAF* V600E mutations in addition to other variants such as V600D, V600K, V600R, and K601E. It can provide the percentage of DNA that harbours the *BRAF* V600E mutations. However, this method is limited by the length of DNA template and is prone to error readings in homopolymer sequences (TTTTTTTT) [18].

AS-PCR enriches known mutations in samples to increase the sensitivity of detection and is particularly useful in tissue with low tumour content. Mass spectrometry-based sequencing relies on the analysis of matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF). This process is facilitated by the addition of mass-modified bases A, C, T, and G to the primed and amplified mutational hotspots. It is this flight time difference of the generated mass-modified complex that is measured by the mass spectrometer. Mass spectrometry-based

sequencing is an even more sensitive test compared to pyrosequencing, with a detection ratio of 1:10 and 1:8, respectively [18].

Melt-curve analysis involves detecting the melting temperature for WT *BRAF* at 61°C and the V600E MT melting at 53°C. PCR methods, on the contrary, can perform as well and has advantages in terms of reduced labour (1.25 vs 16 min), faster turnaround (4 min vs 10 h), and lower cost ($2.6 vs $10.4). The sensitivity and specificity of real-time qPCR is reported to be 100% [19]. **Table 1** details the comparisons among some of the available NGS techniques.

Method	Sensitivity	Accuracy	Time	Cost/1 mill bases (USD)	Advantages/ disadvantages
Chain termination (Sanger)	Low	99.9%	20 min–3 h	2400	Requires the time-consuming step of PCR of plasmid cloning; impractical for larger sequencing projects
Pyrosequencing (454)	Medium	99.9%	24 h	10	Homopolymer errors
Sequencing by synthesis (Illumina)	High	99.9%	1–11 days	0.05–0.15	Expensive equipment; requires high DNA concentrations
Sequencing by ligation	High	99.9%	1–2 weeks	0.13	Slower; issues sequencing palindromic sequences
Ion semi conductor	High	98%	2 h	1	Homopolymer errors
Single-molecule real-time sequencing	High	87%	30 min–4 h	0.13–0.60	Expensive equipment; moderate throughput

Table 1. Available NGS techniques in detecting BRAF V600E mutation [20, 21].

The IHC detection of *BRAF* V600E with a mutation-specific antibody (clone VE1) was first described in metastatic melanoma and papillary thyroid carcinoma and is currently commercially available [22]. The advantage of IHC lies in the minimal amount of tissue needed and the availability of this technique in most pathological laboratories. Most studies have reported high sensitivities and specificities (98.8–100%) compared to PCR-based methods or sequencing [23–25]. However, there is one study that has reported sensitivity and specificity of only 71% and 74%, respectively [26]. The choice of positive control tissue and the amplification protocol is regarded to be crucial in the successful detection of *BRAF* mutation by IHC [27].

Recently, examination of CTC in peripheral blood has been explored as a new non-invasive means for detecting *BRAF* mutation in CRC [28]. Blood collected from 44 patients was enriched for CTC using a size-based microsieve technology. By incorporating the high-resolution melt-curve analysis technique, the concordance rates between CTC and tumour mutations were

observed to be 90.9% (p=0.174) for *BRAF* mutation genotype status and 84.1% (p=0.000129) for KRAS mutation genotype status.

2.2. *BRAF* mutation and its frequency in CRC

A meta-analysis of 10 studies reported *BRAF* mutations in 4.8% to 20.8% of CRC [74]. **Table 2** further details the *BRAF* mutation rates and the corresponding detection methods in some notable metastatic CRC (mCRC) trials.

CRC trials	BRAF MT frequency	Method
PRIME [29, 30]	8%	Bidirectional Sanger sequencing
FIRE-3 [31]	10.5%	pyrosequencing
CRYSTAL [4]	6%	PCR clamping/melt-curve analysis
MAX [3]	10.6%	High-resolution melting point/PCR
PICCOLO [32]	14.8%	PCR/pyrosequencing
NORDIC-VII [33]	12%	Wobble enhanced ARMS*/real-time PCR
AGITG/NCIC CO.17 [59]	3.2% (overall) and 4.8% (KRAS WT)	PCR/sequencing
COIN [34]	8%	MALDI-TOF (Sequenom)/Sanger sequencing
TRIBE [35]	7.5%	Pyrosequencing

Table 2. BRAF mutation detection methods and reported frequencies in notable CRC trials.

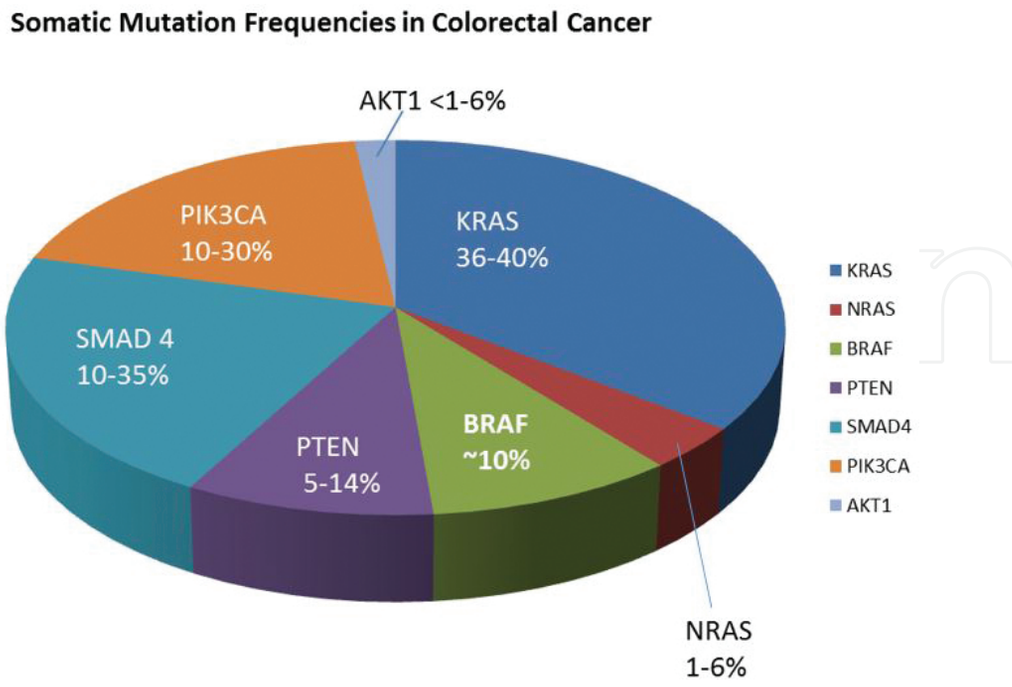

Figure 2. Somatic mutation frequencies in CRC.

Importantly, *BRAF* MT CRC is reported to be mutually exclusive to *KRAS* mutation [36]. *BRAF* mutation coexists with *PIK3CA* mutations in 13% and *PTEN* mutations in 22% of CRC [37]. **Figure 2** depicts the frequency of the different somatic mutations discovered in CRC patients.Chan, E. My Cancer Genome. Molecular Profiling of Colorectal Cancer [Internet]. January 26, 2016 [Updated:January 26, 2016]. Available from: https://www.mycancergenome.org/content/disease/colorectal-cancer/ [Accessed: January 26, 2016].

3. *BRAF* mutation and its clinical significance in CRC

3.1. CRC tumourigenesis pathways

The two main separate pathways observed in CRC development and progression are the chromosomal instability pathway (CIN), which accounts for 75% of the cases, and the microsatellite instability (MSI) pathway in 25% of the cases. Two processes are observed to contribute towards the MSI pathway: (1) germ-line mutations from Lynch syndrome and (2) sporadic MLH1 methylation from the serrated methylated pathway (**Figure 3**) [38] [100].

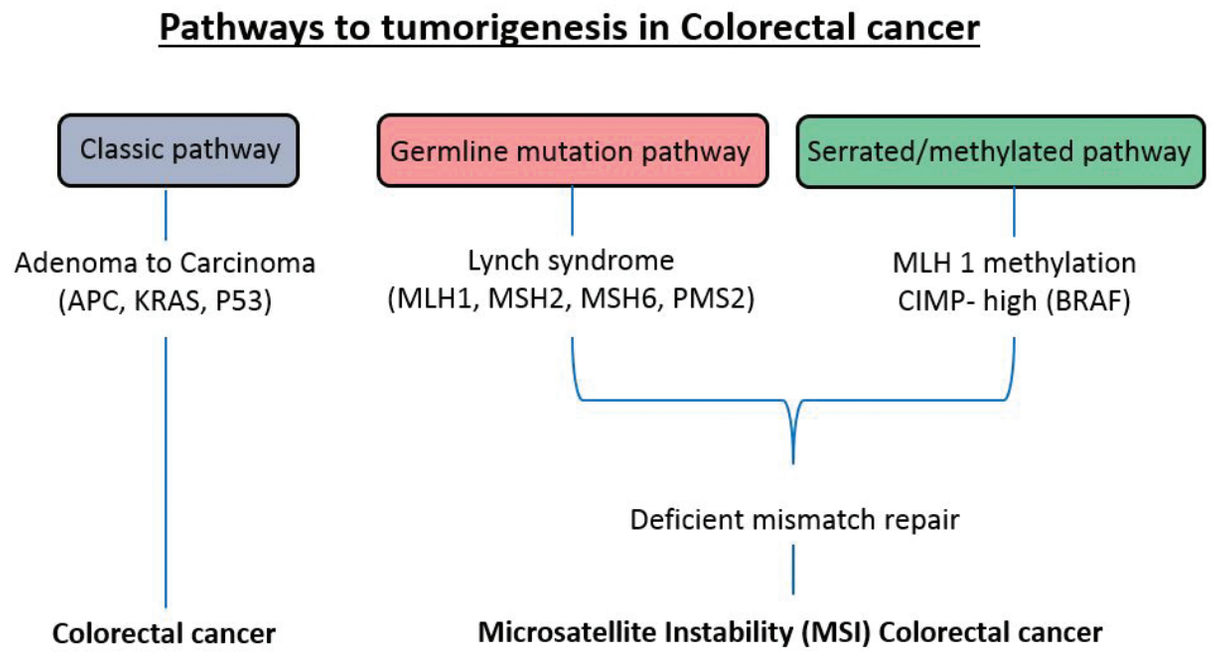

Figure 3. CRC tumourigenesis pathways.

The CIN pathway involves a defect in replication, mitosis, or DNA repair leading to genetic abnormalities, both structural and numeric, which are acquired sequentially. As a result, oncogenes are activated or tumour suppressor gene function is lost, which contributes towards malignant growth. This pathway is also often associated with aneuploidy by karyotyping. The genetic changes found in CRC arising via the CIN pathway include APC mutations (90%),

KRAS mutation (50%), TP53 mutations (70%), and allelic loss of 18q (80%) [39]. The CIN pathway has been traditionally associated with CRC arising in adenomatous polyps.

The MSI pathway is a result of defective mismatch repair (MMR) and occurs in a subset of CRC that arise from either adenomas or serrated polyps. It contributes towards tumour progression via the accumulation of tiny insertions and deletions in the repetitive sequences of microsatellites in coding genes, thereby retaining a near-diploid karyotype. This mechanism of tumourigenesis is readily recognized through a test for MSI, which categorises each tumour as MSI-high (MSI-H), MSI-low (MSI-L), or microsatellite stable (MSS), based on the proportion of microsatellites mutated. MSI-H cases usually imply an acquired or inherited defect in DNA repair.

In inherited cases of MSI-H CRC, germ-line mutation in one of the four genes that encode proteins responsible for MMR (MLH1, MSH2, PMS2, and MSH6) is responsible for a familial predisposition to cancer. This familial predisposition to CRC is known as Lynch syndrome [40], and the CRC that arise in this condition develop in adenomas.

In sporadic cases of MSI-H CRC, the serrated methylated pathway is increasingly implicated. Serrated polyps, not driven by CIN but by *BRAF* mutations, are observed to replace adenomas as precursor lesions in CRC. MSI-H CRC occur due to the epigenetic inactivation of *MLH1* by promoter methylation, which prevents MLH1 protein expression, resulting in defective MMR and producing MSI. This pathway is also closely associated with the widespread methylation of CpG islands, causing the transcriptional silencing of tumour suppressor genes, known as the CpG island methylator phenotype (CIMP) [38, 39].

3.2. *BRAF* testing to distinguish between sporadic versus germ-line MSI-H cases (Lynch syndrome)

Approximately 12% of MSI-H cases are sporadic in nature and *BRAF* mutation is implicated in nearly all (91%) of these cases [41, 42]. The methylation of *MLH1* is found only in 1.6% of germ-line Lynch syndrome cases [43], whereas it is typically found in sporadic tumour lacking MLH1 expression [44]. Hence, *BRAF* mutation testing is recommended in MSI-H CRC as a triage for Lynch syndrome. Only those lacking the *BRAF* mutation proceed with further workup for Lynch syndrome, as CRC harbouring the *BRAF* mutation are, with few exceptions, unlikely to have this condition.

MLH1 methylation testing is an alternative assay to distinguish sporadic from familial cases of CRC. However, given that methylation testing is more technically challenging than *BRAF* mutation testing, most would advocate *BRAF* testing as the more cost-effective assay to distinguish sporadic from familial MSI-H CRC [44].

3.3. Clinicopathological and molecular features of *BRAF* MT CRC

BRAF mutation has been reported in multiple studies to be associated with several clinicopathological parameters in CRC patients. *BRAF* V600E mutation is reported to increase from 10% in unselected patients to 37% in females ages >70 years [45]. *BRAF* mutations in the Western population tend to be more common in females and to have a more proximal location in the

colorectum [27, 46–52]. *BRAF* mutations are rarely found in the left-sided colon (4%) and rectal cancers (2%) compared to the right-sided colon (22%; p<0.0001) [53]. *BRAF* mutation also varies by pathology. Approximately 60% of *BRAF* MT tumours are poorly differentiated and only up to 36% of them are well to moderately differentiated. Mucinous cancers tend to have a higher rate of *BRAF* mutation (22–67%) compared to non-mucinous cancers (6–21%) [39, 54, 55].

The relationship between *BRAF* mutation and these clinicopathological features was confirmed in a meta-analysis reported in 2014 [36]. Twenty-five studies of 11,955 CRC patients were included in this analysis. The mutation rate was seen to vary with the highest reported at 21.8% [2], the lowest being 5.0% [1], and the overall rate being 10.8%. Nine of the 25 studies have shown that *BRAF* mutation was associated with advanced tumor-node-metastasis (TNM) stage at diagnosis [11.6% in stage III/IV CRC vs 8.0% in stage I/II CRC; odds ratio (OR)=1.59; 95% confidence interval (CI)=1.16–2.17]. Thirteen of these studies showed that *BRAF* MT CRC was more prevalent in poorly differentiated tumours than well to moderately differentiated tumours. Of 766 patients with poorly differentiated tumours, 25.6% were *BRAF* MT, whereas only 8% of 4257 patients with well to moderately differentiated tumours were *BRAF* MT (OR=3.89; 95% CI=2.94–5.17). Six studies have also shown that more *BRAF* MT were detected in the mucinous subgroup than in the non-mucinous subgroup (19.4% vs 8.1%; OR=2.99; 95% CI=2.20–4.07). Twenty studies have also significantly demonstrated that proximal cancers (21.6%) harbour more *BRAF* mutations than distal cancers (4.8%; OR=4.85; 95% CI=3.59–6.56) [36].

Another study [56] reported a significantly increased rate of peritoneal (46% vs 24%; p<0.001) and distant lymph node metastases (53% vs 38%; p=0.001) and a lower rate of lung metastases (35% vs 49%; p=0.049) in *BRAF* MT CRC compared to *BRAF* WT tumours that might help to explain their poor prognosis.

Clinicopathological features of BRAF V600E MT CRC patients	**Molecular features of BRAF V600E MT CRC**
1. Age >70 years	1. More prevalent in MSI-H>MSS CRC
2. Female patients	2. More CIMP
3. Proximal right-sided tumours	3. More *MLH-1* methylation
4. High-grade and poorly differentiated	4. Mutually exclusive to KRAS mutation
5. Mucinous>non-mucinous	
6. More peritoneal and lymph node metastases	
7. Less lung metastases	

Table 3. Clinicopathological and molecular characteristics of BRAF V600E MT CRC

Relationships between *BRAF* MT and some molecular characteristics were also reported [36]. *BRAF* MT were significantly more prevalent in MSI-H CRC (38.9%) than MSS CRC (9.3%; OR=8.18; 95% CI=5.08–13.17). As mentioned above, CIMP characterized by widespread

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aberrant DNA methylation at select CpG islands was implicated in a minority of CRC tumourigenecity cases. Two studies were analysed for CIMP status and demonstrated a positive relationship with *BRAF* MT CRC: 45.9% (CIMP) vs 9.1% (non-CIMP; (OR=16.44; 95% CI=6.72–40.21). The methylation of the *MLH1* promoter region is an underlying cause of sporadic non-Lynch cases of MSI-H CRC. Three studies reported a relationship between *BRAF* MT and *MLH1* methylation status; 62.5% of *MLH1* methylated CRC had *BRAF* mutations compared to 9.2% of non-methylated CRC (OR=13.84; 95% CI=1.75–109.24). *BRAF* MT and *KRAS* MT were found to be mutually exclusive in this meta-analysis.

Table 3 summarises the clinicopathological and molecular characteristics of *BRAF* MT CRC.

4. *BRAF* mutation and its prognostic and predictive significance

4.1. Prognostic role and nature of progression

Multiple studies have reported poorer median overall survival (OS) in the *BRAF* MT mCRC subgroup. Regardless of treatment modality, median survival is generally reported to be between 10 and 16 months shorter than the overall population. For instance, the COIN trial, which studied 1630 patients for the effect of cetuximab and doublet chemotherapy FOLFOX in mCRC patients, had reported a median OS of 8.8 months in *BRAF* MT patients versus 20.1 months in patients with (*BRAF* and *RAS*) WT [34]. The PRIME study had reported a median OS of 10.5 months in the *BRAF* MT/RAS WT subgroup, contrasting to a median OS of 25.8 months in RAS WT group and 15.5 months in the RAS MT group. In this study, both (BRAF and RAS) WT patients also had the longest median OS of 28.3 months [29]. The pooled analysis of CRYSTAL and OPUS had also reported lower median OS in the *BRAF* MT group compared to the *BRAF* WT group (9.9 vs 21.1 months in the chemotherapy arm and 14.1 vs 24.8 months in the chemotherapy in combination with cetuximab arms) [57]. In 2013, the PLoS ONE meta-analysis analysed 21 mCRC trials of 5229 patients treated with monoclonal antibodies [58]. Fourteen of these trials were retrospective; two trials were prospective and five trials were randomised-controlled trials (RCTs). *BRAF* mutation was detected in 7.4%. Patients with *BRAF* WT showed decreased risks of progression and death with an improved progression-free survival [PFS; hazard ratio (HR)=0.38; 95% CI=0.29–0.51] and an improved OS (HR=0.35; 95% CI=0.29–0.42) compared to *BRAF* MT. Compared to *BRAF* WT patients, the updated prognostic analyses from the TRIBE study in 2014, which compared standard doublet chemotherapy to triplet chemotherapy, also reported significantly shorter PFS and OS, in the *BRAF* MT group in unresectable mCRC patients, independent of the treatment received [35]. **Table 4** summarises the reported median OS in the *BRAF* MT CRC subgroup reported from various phase III trials. It is also noted here that the *BRAF* mutation rates decrease with lines of therapy, signifying the reducing likelihood of *BRAF* MT patients surviving long enough to receive further lines of treatment.

Study	No. of patients	Treatment line/arm	BRAF MT rate	BRAF MT median PFS (months)	BRAF MT median OS (months)	KRAS WT median PFS (months)	KRAS WT median OS (months)
CRYSTAL (2011) [4]	1198	First line: FOLFIRI vs cetuximab+ FOLFIRI	6%	5.6 vs 8.0 (HR=0.93; p=0.87)	10.3 vs 14.1 (HR=0.91; p=0.74)	8.8 vs 10.9 (HR=0.67; p=0.001)	21.6 vs 25.1 (HR=0.83; p=0.055)
PRIME (2013) [29, 30]	1183	First line: FOLFOX vs panitumumab+ FOLFOX	8%	5.4 vs 6.1 (HR=0.58; p=0.12)	9.2 vs 10.5 (HR=0.90; p=0.76)	RAS/BRAF WT 9.2 vs 10.8 (HR=0.68; p<0.01)	RAS/BRAF WT 20.9 vs 28.3 (HR=0.74; p=0.02)
FIRE-3 (2013) [31]	400	First line: Avastin +FOLFIRI vs cetuximab+FOLFIRI	10.5%	6 vs 4.9 (HR=0.87; p=0.65)	13.7 vs 12.3 (HR=0.87; p=0.65)	RAS WT 10.2 vs 10.4 (HR=0.93; p=0.54)	RAS WT 25.6 vs 33.1 (HR=0.70; p=0.011)
COIN (2011) [34]	1630	First line: FOLFOX/ XELOX vs cetuximab +FOLFOX/XELOX	8%	5.6 vs 9.0 (RAS/BRAF WT) p<0.0001	8.8 vs 14.4 (KRAS MT) p<0.001	8.6 vs 8.6 (HR=0.96; p=0.60)	17.9 vs 17.0 (HR=1.04; p=0.67)
NORDIC-VII (2012) [33]	566	First line: NORDIC FLOX+cetuximab vs FLOX alone vs intermittent FLOX +cetuximab	12%	5.1 vs 8.3 (BRAF WT) p<0.001	9.5 vs 22 (BRAF WT) p<0.001	8.7 vs 7.9 vs 7.5 (HR=1.07; p=0.66)	22.0 vs 20.1 vs 21.4 (HR=1.08–1.14; p=0.77–0.80)
CO.17 (2013) [59]	572	Chemorefractory: cetuximab vs BSC	3.2%	Median PFS not reported (HR=0.76; p=0.69)	1.77 vs 2.97 (HR=0.84; p=0.81)	Favours cetuximab (HR=0.4; p<0.001)	9.7 vs 5.0 (HR=0.52; p<0.0001)
MAX (2011) [3]	471	First line: capecitabine (C) vs capecitabine/ bevacizumab (CB) or capecitabine/ bevacizumab/ mitomycin (CBM)	10.6%	2.5 vs 5.5 (HR=0.86; p=0.71)	6.3 vs 9.2 (HR=0.67; p=0.34)	5.9 vs 8.8 (HR=0.66; p=0.006)	20 vs 19.8 (HR=0.86; p=0.38)
PICCOLO (2013) [32]	460	Second line: irinotecan vs irinotecan/ panitumumab (IrPan)	14.8%	Favours irinotecan (HR=1.40; p=0.018)	Favours irinotecan (HR=1.84; p=0.029)	Favours IrPan (~6M) (HR=0.78; p=0.015)	10.5 vs 10.4 (HR=1.01; p=0.91)
181 Peeters M, Oliner KS, Price TJ, Cervantes A, Sobrero AF, Ducreux M, et al.	1015	Second line: FOLFIRI vs panitumumab/ FOLFIRI	4.4%	RAS WT 1.8 vs 2.5 (HR=0.69; p=0.34)	RAS WT 5.7 vs 4.7 (HR=0.64; p=0.20)	RAS WT 5.5 vs 6.9 (HR=0.68; p=0.006)	RAS WT 15.4 vs 18.7 (HR=0.83; p=0.15)

Study	No. of patients	Treatment line/arm	BRAF MT rate	BRAF MT median PFS (months)	BRAF MT median OS (months)	KRAS WT median PFS (months)	KRAS WT median OS (months)
Updated analysis of KRAS/NRAS and BRAF mutations in study 20050181 of panitumumab (pmab) + FOLFIRI for 2nd-line treatment (tx) of metastatic colorectal cancer (mCRC). J Clin Oncol 2014;32(Suppl.). Abstract 3568.							
TRIBE (2015) [35]	508	First line: Avastin/ FOLFIRI vs Avastin/ FOLFOXIRI	7.5%	5.5 vs 7.5 (HR=0.56)	10.8 vs 19.1 (HR=0.55)	RAS WT 11.3 vs 13.3 (HR=0.77)	RAS WT 34.4 vs 41.7 (HR=0.84)

Table 4. Poorer survival in BRAF MT CRC and mutation frequencies in subsequent lines of treatment

The *BRAF* MT CRC patients of Eastern populations were also reported to share the same fate as those in Western populations. A retrospective study [60] reported a *BRAF* mutation rate of 4.2% in 212 Chinese CRC patients. This study, which did not specifically examine the lines of treatment administered, showed that *BRAF* MT was associated with advanced TNM ($p<0.001$), more distant metastases ($p=0.025$), and worse OS (3-year OS: 16.7% in the *BRAF* MT subgroup vs 73.2% in the *BRAF* WT subgroup; $p<0.001$). The *BRAF* mutation rate of 4.2% in the Chinese population was found similar to the rates (1–7%) reported for Taiwanese and Japanese populations [61–64].

BRAF MT is also associated with poor prognosis in other stages of CRC. A review in 2013 [65] on seven studies that included stages I to IV CRC patients has concluded that *BRAF* mutation served as an independent prognostic factor for reduced OS, disease-free survival (DFS), and cancer-specific survival, especially in MSS CRC. One of the studies that included 911 stage II to IV CRC patients demonstrated *BRAF* mutation to be associated with a poor 5-year OS (*BRAF* MT vs WT, 47.5% vs 60.7%; $p<0.01$) [66]. Another study [47] looked at 1307 patients with stage II to III CRC and reported reduced OS in *BRAF* MT group (HR=2.2; 95% CI=1.4–3.4; $p=0.0003$).

To further analyse the impact of MSI status in the *BRAF* MT CRC patients, Samowitz et al. [66] have shown that survival differs for stages II to IV CRC *BRAF* MT tumours with MSI compared to MSS tumours. Poor prognosis was only demonstrated in MSS tumours (5YS: *BRAF* MT vs WT, 16.7% vs 60.0%; log-rank $p<0.01$) from a multivariate analysis adjusted for age, stage, and

tumour sites. MSI tumours were reported to have good prognosis regardless of *BRAF* MT status, with 5YS 76.2% (with *BRAF* mutation) vs 75.0% (without *BRAF* mutation). Interestingly, a recent retrospective Japanese study also studied the role of *BRAF* MT in MSI tumours [67]. They examined KRAS, BRAF, and MSI status in 813 patients with curatively resected, stage I to III CRC. After adjusting for relevant variables, including MSI status, they reported that *BRAF* MT were poor prognostic factors for DFS (HR=2.20; 95% CI=1.19–4.06) and OS (HR=2.30; 95% CI=1.15–4.71) independent of MSI status. This small study, which excludes stage IV patients, suggests that MSI-H tumours without *BRAF* mutation may have the best prognosis compared to MSI-H tumours with *BRAF* mutation. MSS tumours with *BRAF* mutation would have the worst prognosis.

In accordance with their aggressive nature, *BRAF* MT cancers have also been reported to have poor PFS with sequential systemic treatments. A retrospective study on 1567 patients detected a *BRAF* mutation rate of 8%. These *BRAF* MT patients had received a median of two later lines of chemotherapy, with the median PFS for the first three lines of chemotherapy being 6.3, 2.5, and 2.6 months, respectively [68]. Another smaller study had reported even shorter median PFS (4.3 months) after first-line treatment in *BRAF* MT [69]. This observation highlights the importance of considering early intensified treatment given the propensity for these patients to not survive long enough for second- or third-line treatments.

Recently, other rare (<1%) subtypes of *BRAF* MT, which harbour mutations in codon 594 or 596, were reported to have markedly longer OS compared to *BRAF* V600E MT (median OS=62.0 vs 12.6 months; HR=0.36; 95% CI=0.20–0.64; p=0.002). These subtypes are noted to be MSS and also differ in other molecular and clinical characteristics, being more frequently rectal in origin, non-mucinous, and with no peritoneal spread [70].

4.2. Predictive role

Given that *RAS* MT are negative predictors of anti-epidermal growth factor receptor (EGFR) therapies, the predictive role of *BRAF* MT for anti-EGFR agents has been of interest given the relationship with RAS in the EGFR/RAS/RAF/MEK/ERK pathway. *BRAF* MT and its associated resistance to anti-EGFR agents have been suggested by several retrospective analyses [71–73].

To date, the predictive role of *BRAF* MT on anti-EGFR agents remains unclear, in light of differing conclusions from two separate meta-analyses [74, 75]. Pietrantonio et al. concluded that *BRAF* MT might be a negative predictor for anti-EGFR agents, supporting the meta-analysis by Yuan et al. [58]. This study included a pooled analysis of nine phase III trials and one phase II trial and shown that cetuximab- or panitumumab-based therapy did not increase the benefit of standard treatment versus best supportive care in RAS-WT/*BRAF*-MT CRC patients. Overall, the addition of cetuximab or panitumumab did not significantly improve the PFS (HR=0.88; p=0.33), OS (HR=0.91; p=0.63), and overall response rate [ORR; relative risk (RR)=1.31; p=0.25] in this subgroup population [74]. However, another recent meta-analysis reviewed seven RCTs for OS and eight RCTs for PFS and concluded on insufficient evidence to justify the exclusion of anti-EGFR agents in the *BRAF* MT population [75]. Nevertheless, these latest findings have supported the need for *BRAF* mutation assessment before the

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initiation of treatment to study and tailor the most effective strategies to the *BRAF* MT population.

5. Treatment strategies

5.1. Triplet chemotherapy effect

BRAF MT has not been known to be a predictor of benefit from chemotherapy or anti-vascular endothelial growth factor (VEGF) agents. The Italian TRIBE study [35] compared anti-VEGF therapy, bevacizumab added to intensified triplet chemotherapy, fluorouracil, oxaliplatin, and irinotecan (FOLFOXIRI), to standard first-line doublet chemotherapy with fluorouracil and irinotecan (FOLFIRI) plus bevacizumab in 508 unresectable mCRC patients. The study reported a higher response rate of 65% vs 53% with the triplet FOLFOXIRI and bevacizumab arm. Reassuringly, there was no increase in fatal or serious adverse events.

The updated analyses of the same study reported a BRAF mutation rate of 7.5%. In the *BRAF* MT group, there was a significant trend for better survival in the triplet arm compared to the doublet arm (19.1 vs 10.8 months; HR=0.55). Significantly, this is the only regimen to have resulted in a median OS of more than 15 months in the *BRAF* MT group compared to the more often reported median of 4.4 to 14 months in most studies [29, 57]. It was proposed that intensified triplet chemotherapy (FOLFOXIRI+bevaczicumab) is considered first line in the BRAF MT group, who usually have aggressive cancers with limited ability to undergo a more sequential approach to treat metastatic disease.

5.2. Maintenance treatment

A recent meta-analysis on five RCTs had failed to demonstrate a statistically significant OS benefit (HR=0.93; 95% CI=0.85–1.02; p=0.12; I^2=5%) with administering maintenance chemotherapy versus complete treatment interruption after first-line therapy in unselected CRC [76]. The chemotherapy free interval in the group not using maintenance treatment was 3.9 months (3.6–4.3 months). Nevertheless, the author had emphasized the importance of predictive markers to guide the selection of patients who would benefit from the maintenance strategy. Although not formally tested in the *BRAF* MT subgroup population, the maintenance strategy might prove more favourable than the intermittent strategy given its known aggressive nature. This is especially relevant given that the median reported PFS in BRAF MT as indicated previously ranged from 4.3 to 6.3 months after first-line treatment [68, 69].

In terms of the choice for maintenance treatment, there is no current recommended standard. However, practice trends could perhaps be extrapolated from the AIO KRK 0207 trial, which confirmed the prognostic impact of mutation status [77]. In all patients (irrespective of BRAF or RAS status), at a median follow-up of 27 months, the authors reported a time to failure of strategy of 3.6, 6.2, and 4.6 months among all patients receiving no treatment, fluoropyrimidine plus bevacizumab, or bevacizumab alone, respectively (p<0.001). However, in RAS/BRAF WT patients, bevacizumab monotherapy was as effective as combination treatment (fluoropyra-

midine/bevacizumab) for maintenance. In contrast, in the RAS or BRAF MT subgroup, the combination treatment was favoured, as single-agent bevacizumab was equivalent to no maintenance at all.

6. Investigated treatments targeting EGFR/RAF/MEK

6.1. BRAF/MEK inhibitors

As mentioned above, RAS proteins normally activate BRAF along with A-RAF and C-RAF [78]. BRAF mutations lead to the constitutive activation of BRAF kinase activity, resulting in phosphorylation and activation of the MEK kinases (MEK1 and MEK2). Once activated, MEK kinases phosphorylate and activate ERK kinases, which phosphorylate a multitude of cellular substrates involved in cell proliferation and survival (**Figure 1**).

RAF inhibitors, such as vemurafenib and dabrafenib, have produced response rates of 50 to 80% in melanomas that harbour the BRAF V600 mutations [79, 80]. This is disappointingly contrasting to the response rate of only 5%, and median PFS of 2.1 months achieved in *BRAF* MT CRC [81]. Previous observations have proposed that RAF inhibitor insensitivity in BRAF MT CRC was driven by the feedback reactivation of the RAS/RAF/MEK/ERK signalling cascade. In many BRAF MT CRC cell lines, EGFR-mediated activation of RAS and C-RAF was observed to be the culprit [82, 83]. Solit et al. had also demonstrated the critical dependency of *BRAF* MT colorectal cell lines and xenografts on MEK-ERK signalling, which renders them highly sensitive to pharmacological MEK inhibition. Pharmacological MEK inhibition completely abrogated tumour growth in BRAF MT xenografts, whereas *RAS* MT tumours were only partially inhibited [84].

Many RAF inhibitor combinations were hence evaluated in clinical trials in recent years and have shown promising results. A phase I to II clinical trial of combined RAF/MEK inhibition with dabrafenib (150 mg BD) and trametenib (2 mg OD) in 43 *BRAF* MT CRC resistant to anti-EGFR therapy produced partial responses in 12% and complete response in 2%. One patient achieved a durable complete response exceeding 36 months. Additionally, 56% achieved stable disease as the best confirmed response [85].

6.2. Dual and triplet targeting EGFR/BRAF/MEK inhibitors

The observations above have also led to a number of studies assessing the combined blockade at other sites in the EGFR pathway in addition to RAF/MEK inhibition. It was observed that the dual inhibition of anti-EGFR therapy in combination with RAF inhibition in resistant cell lines might still produce a lower degree of mitogen-activated protein kinase (MAPK) pathway inhibition in BRAF MT CRC compared to single-agent RAF inhibitors in *BRAF* MT melanoma patients. Dabrafenib and panitumumab doublet was trialled with a response rate of (partial and complete response) 2/20 (10%) and stabilised disease in 16/20 (80%) as the best overall response [86]. Another study examined the combination of vemurafenib (BRAF-inhibitor) and panitumumab in 15 patients. Two (13%) patients reported partial responses lasting 40 and 24

weeks, respectively. Eight (53%) patients stable disease lasting more than 6 months [87]. A phase II study studied dual inhibition with encorafenib (BRAF inhibitor) and cetuximab with 26 patients. Encorafenib and cetuximab doublet was reported to produce an overall RR (complete and partial) of 23.1% with a median PFS of 3.7 months. The most common treatment-related grade 3/4 adverse events associated with this doublet regimen were fatigue and hypophosphatemia (8% each) [88].

Encouragingly, the triplet combination of EGFR/RAF/MEK inhibition in *BRAF* MT CRC reported an improved response rate (26% complete and partial) in 35 patients compared to the doublet inhibition. The triplet regimen had also stabilised disease in 57%. The most common adverse events reported were diarrhoea (60% grade 1/2 and 9% grade 3) and dermatitis acneiform (47% grade 1/2 and 9% grade 3) [86].

6.3. Acquired resistance to EGFR/RAF/MEK targeted therapies

Although trials have demonstrated early efficacies of combination targeted therapies in these *BRAF* MT patients, attention was brought towards their eventual treatment resistance and disease progression. A group in Harvard recently compared pretreatment and postprogression *BRAF* MT CRC tumour biopsies by whole exome sequencing (WES) to examine the related changes that could explain treatment resistance in these cases [89]. They have identified four possible acquired molecular mechanisms that could lead to resistance to combination treatments with RAF/MEK and RAF/EGFR. These four mechanisms include (1) *KRAS* exon 2 mutation (G12D and G13D), (2) *KRAS* WT amplification [confirmed by fluorescence *in situ* hybridisation (FISH) to be ~25-fold overexpression], (3) *BRAF* MT allele amplification, and (4) *MEK1* mutation. These alterations converge on the MAPK pathway reactivation and promote resistance.

Interestingly, the group also discovered an ERK inhibitor that retained the ability to suppress MAPK signalling and overcome each of these mechanisms identified [89]. In conjunction with these findings, early-phase clinical trials are currently incorporating ERK inhibitors as potential future treatment strategies for *BRAF* MT CRC.

6.4. Other possible EGFR/RAF targeted combination treatments

6.4.1. Vemurafenib/irinotecan/cetuximab combination

The phase I vemurafenib/irinotecan/cetuximab triplet study reported a RR of 35% (partial response) in 18 mCRC patients with a median PFS of 7.7 months. The most common adverse effects were fatigue (94%), diarrhoea (89%), nausea (83%), and rash (78%). Following this, a U.S. cooperative group randomised phase II trial (NCT01787500) of irinotecan and cetuximab ±vemurafenib in BRAF-mutated mCRC (SWOG 1406) is now ongoing [90].

7. Alternative target signalling pathways

Although our increasing understanding of the complexity of the EGFR/RAF pathway has led to some advances in our understanding of possible mechanisms of resistance to BRAF inhibition, additional complex interactions with related pathways are likely to be involved, including the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, mammalian target of rapamycin (mTOR), and Wnt signalling.

7.1. PI3K/AKT and mTOR pathway

The PI3K/AKT pathway is an alternative resistance mechanism to BRAF inhibition in *BRAF* MT CRC. Approximately 40% of CRC have been shown to have alterations in one of eight PI3K pathway genes. These mutations are almost always mutually exclusive to each other [91]. In addition, *BRAF* mutation co-exists with *PIK3CA* mutations in 13% and *PTEN* mutations in 22% of CRC [37]. Compared to *BRAF* MT melanoma cell lines, *BRAF* MT CRC cell lines seemed to also display a higher rate of PI3K/AKT pathway activation. These cell lines were reported to be less sensitive to the BRAF inhibitor, vemurafenib [92].

Based on the above observations, the combination triplet inhibition treatment was studied with encorafenib (BRAF inhibitor), cetuximab, and PI3K inhibitor (alpelisib) in 28 patients and reported an overall RR of 32.1% with a median PFS of 4.3 months. The most common grade 3/4 adverse events reported were hyperglycemia (11%) and increased lipase (7%) [88].

Sustained PI3K/mTOR activity was demonstrated also by Corcoran et al. [82] in BRAF MT CRC cell lines upon BRAF inhibition. Pleasingly, a potent growth-inhibitory effect was recently observed in xenografts of BRAF MT CRC with the combined BRAF/PI3K/mTOR inhibition [93].

7.2. Wnt/β-catenin pathway

A study by Lemieux et al. demonstrated the Wnt/β-catenin pathway (**Figure 3**) as a potential novel target in MEK/ERK signalling involved in CRC tumourigenesis [94]. The Wnt/β-catenin pathway is activated via the binding of Wnt1 protein to the G-protein coupled receptor, Frizzled. After the activation by Wnt1, Dishevelled protein (Dsh) induces the dissociation of the destruction complex that usually degrades β-catenin. Without the destruction complex, β-catenin is accumulated in the cytoplasm and transported to the nucleus to act as a transcriptional coactivator of transcription factors as shown in **Figure 4**. The aforementioned destruction complex comprises Axin (A), adenomatous polyposis coli (APC), and glycogen synthase kinase 3 (GSK3β). In the absence of Wnt1 activation, the destruction complex phosphorylates the downstream ubiquinating process. Here, the β-transducin repeat containing protein (βTrCP) binds β-catenin, ubiquinating it and marks it for degradation by the proteasome. Although there is conflicting literature with regards to the role of MAPK signalling in activating Wnt/β-catenin pathway, this group found Wnt signalling induction in high-grade *BRAF* MT tumours. Their data also show that the oncogenic activation of KRAS/BRAF/MEK signalling stimulates the canonical Wnt/β-catenin pathway, which in turn promotes intestinal

tumour growth and invasion. This has in turn sparked trial designs to incorporate Wnt signalling as a treatment strategy.

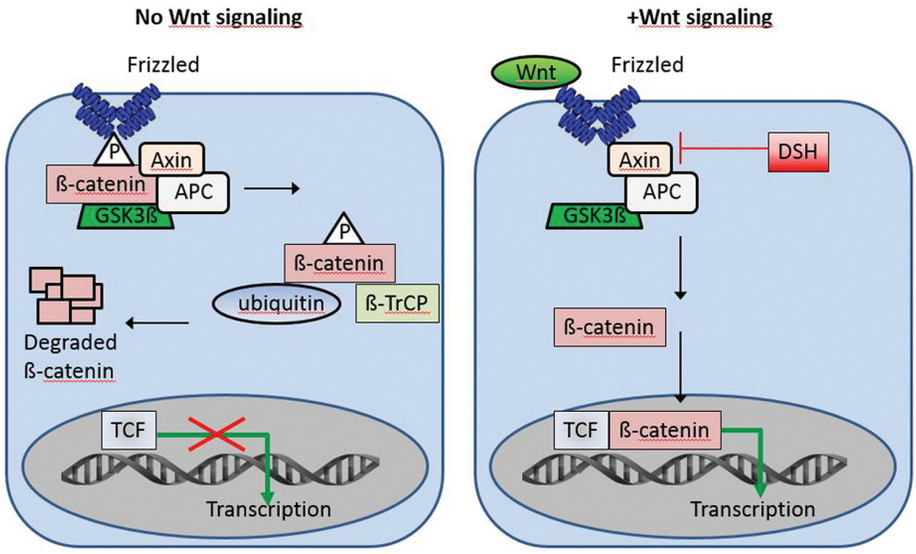

Figure 4. Wnt/β-catenin pathway.

8. Other possible therapeutic mechanisms

Recently, a number of other early studies have reported additional potential mechanisms of targeted treatment, which had shown promise in *BRAF* MT CRC xenografts or cell line studies.

8.1. Multi-targeted angiokinase inhibitor (dovitinib)

Dovitinib is a multi-target angiokinase inhibitor with activity against fibroblast growth factor receptors (FGFRs), platelet-derived growth factor receptors (PDGFRs), and VEGF receptors, which participate in tumour growth, survival, angiogenesis, and vascular development. Although not effective *in vitro*, *in vivo* studies have shown the inhibition of *BRAF* MT xenografts tumours with dovitinib. Lee et al. proposed that this observation is secondary to its angiogenesis-suppressing effect and could be a novel approach to improve the outcome of CRC patients in whom FGFR is overexpressed or amplified [95].

8.2. Proteasome inhibitor (carfilzomib)

A novel use of proteasome inhibitors (carfilzomib, bortezomib), known more for utility in haematological malignancy, has shown promising preclinical results in *BRAF* MT CRC [96]. Zecchin et al. have observed increased sensitivity of *BRAF* MT CRC to carfilzomib, whereas WT cells were significantly less affected ($p<0.05$). This response seemed to be independent of the phosphatase and tensin homologue (PTEN) or retinoblastoma protein (RB1) expression status in CRC. The mechanism of this activity was explained by the higher accumulation rate

of ubiquitinated proteins in MT cells with respect to WT. It was speculated that this is secondary to the non-oncogenic addiction of *BRAF* MT cells to the protein degradation function of proteasome to counterbalance the proteotoxic stress induced by the MT protein. Interestingly, carfilzomib was also found to have antagonistic effects with the RAF inhibitor, vemurafenib, and was proposed as a possible alternative treatment to BRAF/MEK inhibition.

8.3. microRNA (miR-145)

miR-145, a short RNA molecule of microRNA gene, which was observed to have tumour suppressor function, was found to be down-regulated in vemurafenib-resistant *BRAF* MT CRC cell lines [97]. Peng et al. reported that the overexpression of miR-145 increased the sensitivity of *BRAF* MT CRC cell lines both *in vitro* and *in vivo* and could be used as a potential therapeutic target.

8.4. *In situ* cancer vaccine (Allostim)

AlloStim is an innovative design based on immunotherapy principles. It is derived from the blood of normal blood donors and is intentionally mismatched to the recipient. $CD4^+$ T cells are initially separated from the blood and differentiated and expanded for 9 days in culture to make an intermediary called T-Stim. AlloStim is made by incubating T-Stim cells for 4 h with antibody-coated microbeads. The cells with the beads still attached are suspended in infusion media and loaded into syringes. The syringes are shipped refrigerated to the point-of-care. A phase I study was completed in May 2011 and a phase II/III study is due to recruit in 2016. It involves an *in situ* (in the body) cancer vaccine step that combines killing a single metastatic tumour (usually liver metastasis) lesion by the use of cryoablation to cause the release of tumour-specific markers to the immune system and then injecting bioengineered allogeneic immune cells (AlloStim) into the lesion as an adjuvant to modulate the immune response and educate the immune system to kill other tumour cells wherever they reside in the body [98].

8.5. Apoptosis regulator (BCL-2/BCL-XL) inhibitor (Navitoclax)

Apoptosis regulator (BCL-2/BCL-XL) inhibitor (Navitoclax) was explored as a novel approach in sensitising *BRAF* MT CRC to mTOR inhibition. The results showed that this combination strategy leads to efficient apoptosis in specifically *KRAS* and *BRAF* MT but not WT CRC cells [99]. These data showed promising results with the combination strategy of apoptosis regulator inhibitors with mTOR inhibitors in *BRAF* MT CRC.

9. Ongoing trials for BRAF MT CRC

Many phase I/II trials are currently ongoing for *BRAF* MT mCRC. Most of them focus on the RAS/RAF/MEK/ERK signalling pathway, trialling combination targeted treatments. **Table 5** lists these available trials.

Trial name/Reg	Phase	Trialed agents	Status
NCT01543698	I/II	RAF inhibitor (dabrafenib)+MEK inhibitor (trametenib)+CDK4/6 inhibitor (LEE011)	Recruiting
NCT 01719380	IB/II	RAF inhibitor (LGX818)+cetuximab+PI3K inhibitor (BYL-719) vs LGX818+ BYL-719	Recruiting
NCT01902173	I/II	Dabrafenib+trametenib: in stage IIIC+IV CRC	Recruiting
NCT02034110	II	Dabrafenib+trametenib: BRAF MT rare cancers	Recruiting
NCT00265824	III	Avastin±erlotinib: maintenance treatment in unresectable CRC	Closed; awaiting for results
NCT02175654 (PREVIUM)	II	Regorafenib: single-agent second-line post-FOLFOXIRI+Avastin	Recruiting
NCT01750918	I/II	Dabrafenib+trametenib+panitumumab	Recruiting
NCT01787500	I	Vemurafenib+cetuximab+irinotecan	Recruiting
S1406	II	Cetuximab+irinotecan±vemurafenib	Recruiting
NCT01596140	I	Vemurafenib+mTOR inhibitor (everolimus/temsirolimus)	Recruiting
NCT02041481	I	MEK inhibitor+FOLFOX: CRC failing standard treatment	Recruiting
NCT02380443	IIB	Allostim (*in situ* cancer vaccine): third-line treatment in KRAS/BRAF MT CRC	Pending
NCT02278133	IB/II	Wnt ligand inhibitor (WNT974), RAF inhibitor and cetuximab	Recruiting
NCT01351103	I	Wnt ligand inhibitor (LGK974)	Recruiting

Table 5. Ongoing trials in BRAF MT CRC

10. Conclusion

The *BRAF* V600E MT CRC typically presents with right-sided proximal high-grade mucinous tumours in older women and may arise from serrated polyps. Molecularly, they are associated with more *MLH1* methylation, MSI, and CIMP. This small subset of CRC, which generally affects approximately 10% of CRC patients, remains a challenging group with poor response to both anti-EGFR and standard doublet chemotherapy. This CRC subgroup is typically aggressive, has short median PFS between sequential lines of treatments, and emphasises the need to use effective treatments early. New evidence suggests that triplet chemotherapy with FOLFOXIRI could be considered in suitable patients with or without bevacizumab as first-line treatment. Many trials are currently studying the effective combinations of targeted treatments involving BRAF and MEK inhibitors in this subgroup and ways to overcome resistance.

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