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# *BRAF*-mutated colorectal adenocarcinomas: Pathological heterogeneity and clinical implications

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ABSTRACT

Advances in molecular biology have markedly increased our understanding of the heterogeneous molecular landscape of colorectal cancer (CRC). Up to 15% of CRCs harbor the *BRAF* p.V600E somatic mutation (*BRAF*mt), a well-established negative prognostic marker in patients with metastatic CRC (mCRC). The BEACON CRC trial set a new standard of care in patients with progressive *BRAF*mt cancers, consisting of the combination of encorafenib and cetuximab. On these bases, *BRAF* mutational testing is now recommended in patients with mCRC. However, efforts are needed to further stratify patients carrying this mutation.

Here, we discuss the heterogeneous pathologic and molecular landscape of *BRAF*mt CRCs, focusing on the promises and pitfalls of molecular diagnostics, on novel biomarkers to improve patients' stratification and on the current diagnostic scenario for CRC.

We believe that a better stratification based on histopathological features and novel molecular biomarkers should be performed to optimize patient management and therapeutic decision-making.

## 1. Background

Colorectal cancer (CRC) accounts for approximately 10% of all cancers and cancer-related deaths worldwide, with 1.9 million new cases and almost 0.9 million deaths in 2020 (Sung et al., 2021).

Advances in molecular biology have increased our understanding of the complex and heterogeneous molecular landscape of CRC, which is responsible for the significant variability in patients' prognosis and response to therapy (The Cancer Genome Atlas Network, 2012; Guinney et al., 2015).

About 8%–15% of CRCs harbor the p.V600E somatic mutation in the *BRAF* (v-raf murine sarcoma viral oncogene homolog B) gene (*BRAF*mt), which is almost always mutually exclusive with the *RAS* (rat sarcoma virus) gene mutation (Morkel et al., 2015). *BRAF* exerts its oncogenic activity by positively modulating the MAPK (mitogen-activated protein kinase) cascade, which plays a crucial role in tumor cell proliferation and survival (Davies et al., 2002). Several studies demonstrated that *BRAF*mt CRCs represent a distinct clinico-pathological subgroup with specific molecular features (Fanelli et al., 2020).

The presence of *BRAF* mutation is a well-established negative prognostic biomarker in metastatic CRC (mCRC), and patients bearing this alteration in most cases do not respond to therapeutic regimens based on multi-kinase inhibitors (Pietrantonio et al., 2015; Loupakis et al., 2009). The BEACON CRC trial set a new standard of care in patients with progressive $^{V600E}$*BRAF* cancers, consisting of the combination of the BRAF inhibitor encorafenib plus the anti-epidermal growth factor receptor (EGFR) monoclonal antibody cetuximab (Kopetz et al., 2019).

Because of its increasing clinical significance, the National Comprehensive Cancer Network (NCCN) (Benson et al., 2021) and European Society of Medical Oncology (ESMO) (Van Cutsem et al., 2016)

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guidelines recommend *BRAF* mutational testing in patients with mCRC.

However, the predictive role of *BRAF* mutation in mCRC is not fully elucidated yet and more efforts are needed to further stratify the *BRAF* mutant population.

In this review, we will provide insights into the heterogeneous pathologic and molecular landscape of *BRAF*mt CRCs, focusing on the promises and pitfalls of molecular diagnostics and on novel biomarkers to improve patients' stratification.

## 2. *BRAF* gene and $^{V600E}$*BRAF* mutation

*BRAF* is a proto-oncogene that encodes for a cytoplasmic serine/threonine kinase (STK), which activates the RAS/RAF/MEK/extracellular signal-regulated kinase (ERK) signaling cascade-known as the MAPK pathway. The MAPK pathway promotes cell proliferation, differentiation, migration, survival and angiogenesis and its dysregulation is a common event in tumorigenesis (Ascierto et al., 2012).

*BRAF* was reported to be mutated at several sites; however, the most common mutation in CRC (90%) and other cancer types as well is V600E, a single nucleotide change at residue 1799 (T1799A) leading to an amino acid substitution from valine to glutamic acid at codon 600 and resulting in a constitutive active-active kinase (Davies et al., 2002). $^{V600E}$*BRAF* mutation is common in many malignancies other than CRC, particularly melanoma (66%) (Thompson et al., 2009), papillary thyroid cancer (30%) (Kimura et al., 2003), serous ovarian cancer (30%) (Singer et al., 2003), cholangiocarcinoma (6%) (Tannapfel et al., 2003) and non-small cell lung cancer (4%) (Baik et al., 2017) (Fig. 1).

## 3. Clinico-pathological features of *BRAF*mt CRCs

*BRAF*mt CRCs are characterized by distinct clinico-pathological characteristics and specific molecular alterations defining a discrete subgroup (Fig. 2).

While the overall *BRAF* mutation frequency is estimated to be approximately 10%, its prevalence varies among different ethnic groups (Western countries have a higher frequency than Asian countries) (Chen et al., 2014). Among CRCs, the rate of *BRAF* mutations is significantly higher in the metastatic setting (stage IV) rather than in stage II-III (15–20% *vs* 10–5%) (Clarke and Kopetz, 2015).

According to several studies, *BRAF* mutation appears to be associated with advanced age (>70 years old), thus indicating that *BRAF* alteration is an acquired genetic event in the carcinogenetic cascade, sporadic CRC, female sex and proximal location (*BRAF* mutations are a rare finding in rectal cancers)(Chen et al., 2014; Clancy et al., 2013).

Additionally, a distinct pattern of metastatic spread characterizes *BRAF* mutant tumors, namely high rates of peritoneal metastases and distant lymph node metastases, together with low rates of lung metastases (Tran et al., 2011).

*BRAF*mt CRCs are associated with specific histopathological features, such as mucinous component and poorly differentiated histology (Chen et al., 2014; Clarke and Kopetz, 2015; Clancy et al., 2013; Loupakis et al., 2016; Guan et al., 2020; Jang et al., 2017). Other frequent findings include signet ring cell component, serrated architecture, lymphovascular invasion, tumor budding, growing infiltrative pattern and peritumoral lymphoid reactions (Jang et al., 2017).

## 4. *BRAF* mutation as a predictive factor for mCRC

Several treatment options are available for patients with *BRAF*mt mCRC. Systemic chemotherapy regimens, including FOLFOX, FOLFIRI, and capecitabine plus oxaliplatin, have been the standard of care for the past several decades (Benson et al., 2021; Van Cutsem et al., 2016).

Due to the inherent chemotherapy-refractory nature of *BRAF*mt mCRC, it is rational to explore novel approaches involving targeted therapeutic agents, alone and in combination with chemotherapy (Johnson and Kopetz, 2020).

Similar to patients with *BRAF* wild-type tumors, patients with *BRAF*mt cancers appear to benefit from anti-VEGF (vascular endothelial growth factor) therapy (Hurwitz et al., 2004). The TRIBE study evaluated FOLFOXIRI plus bevacizumab *versus* FOLFIRI plus bevacizumab as the first-line treatment for mCRC. However, no evidence of a clear benefit to triplet chemotherapy over doublet chemotherapy was found (Loupakis et al., 2014; Cremolini et al., 2020).

Although *BRAF* mutation is associated with wild-type *RAS*, several studies have identified *BRAF* mutation as a predictor of resistance to anti-EGFR therapy (Di Nicolantonio et al., 2008; Fakih, 2015). There is

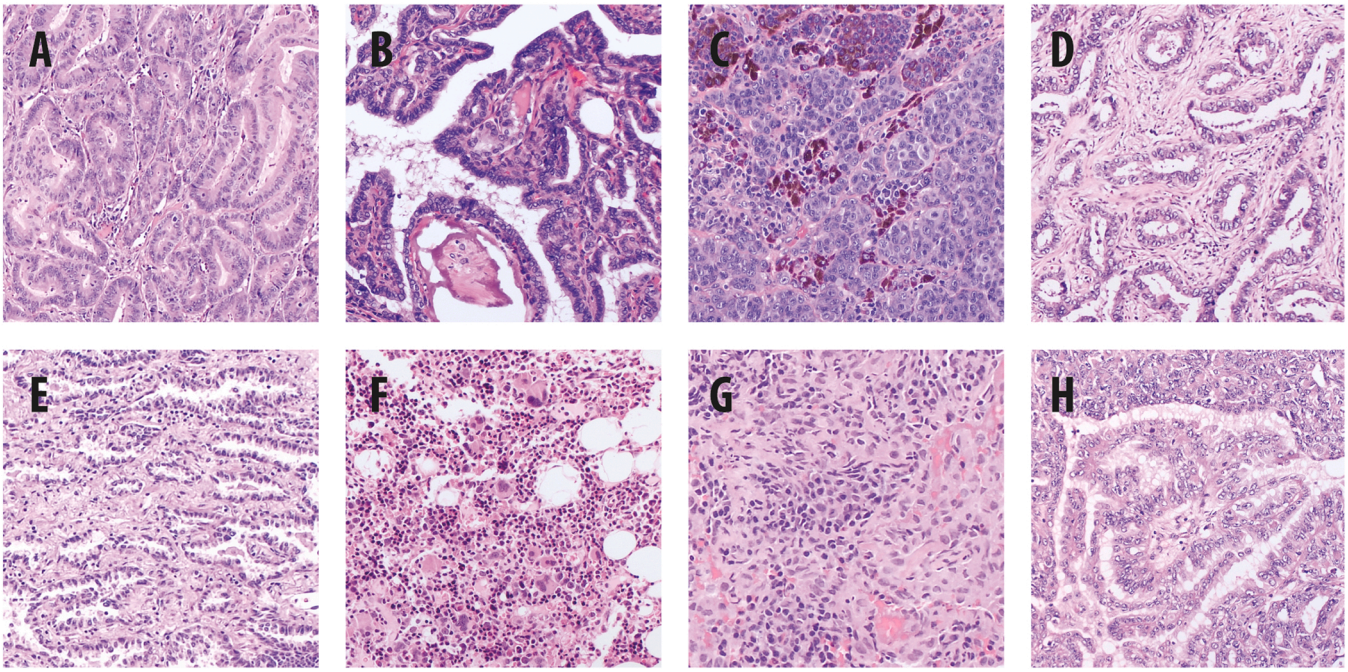


**Fig. 1.** Representative images of *BRAF* mutated neoplasms. A) Colorectal Cancer B) Thyroid Papillary Carcinoma C) Malignant melanoma D) Cholangiocarcinoma E) Lung adenocarcinoma F) Hairy cell leukemia G) Langerhans cell histiocytosis H) Low-grade serous ovarian carcinoma.

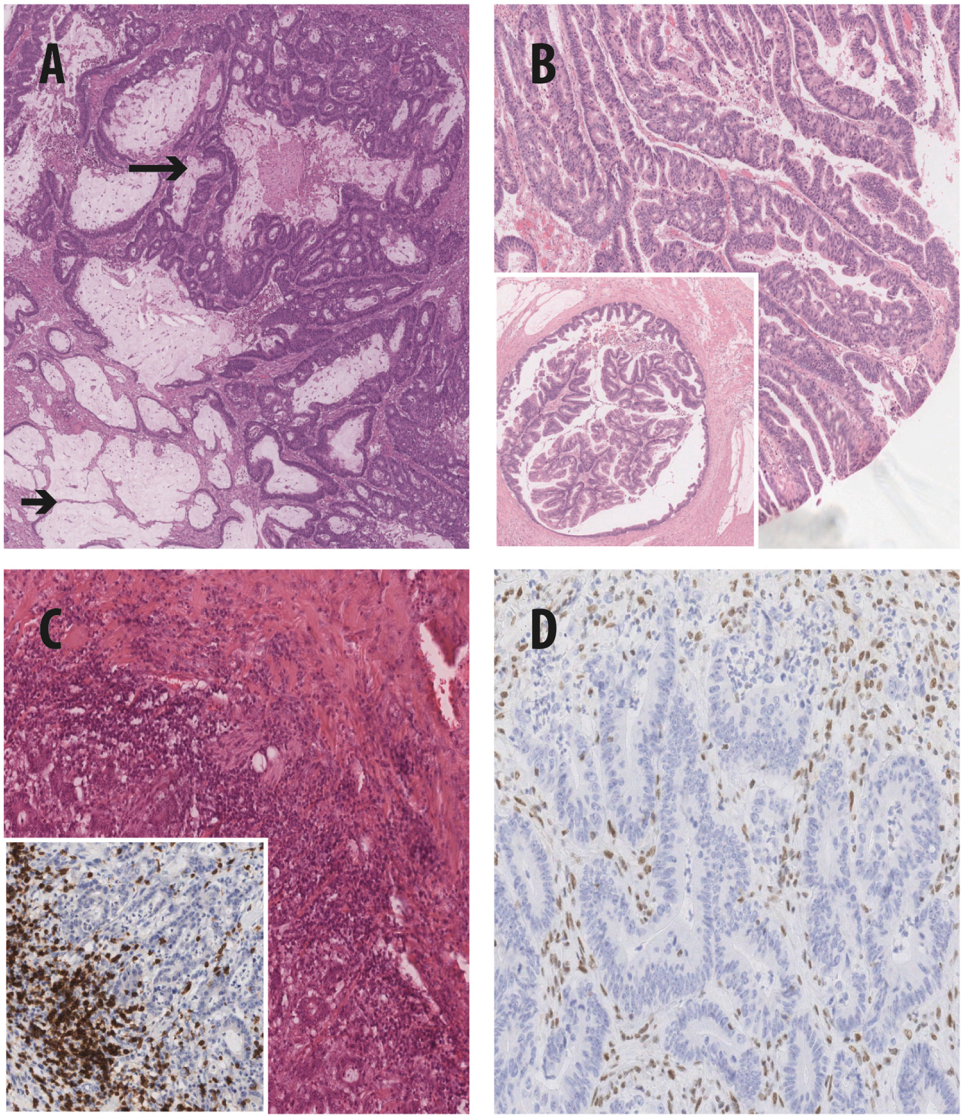


**Fig. 2.** The heterogenous pathological landscape of *BRAF* mutated colorectal adenocarcinoma. A) Mucinous histotype B) Serrated architecture C) Lymphocytes' infiltration D) loss of expression of MLH1 in a *BRAF*mt/MSI CRC.

currently limited and conflicting evidence regarding the use of anti-EGFR monoclonal antibodies in combination with cytotoxic agents in patients with *BRAF*mt cancers (Pietrantonio et al., 2015; Van Cutsem et al., 2011). For this reason, anti-EGFR therapy is indicated exclusively for patients who are *RAS/BRAF* wild-type (Benson et al., 2021; Van Cutsem et al., 2016).

In contrast to the successful results achieved for $^{V600E}$*BRAF*mt melanoma (Chapman et al., 2011), single-agent BRAF inhibition resulted in zero complete response and an overall response rate of 5% in *BRAF*mt mCRC (Kopetz et al., 2015). In CRC, BRAF blockade alone is responsible for feedback reactivation of EGFR and persistent MAPK signaling activation. On the contrary, the simultaneous BRAF and EGFR inhibition in preclinical models resulted in inhibition of tumor growth, by overcoming the MAPK feedback loop (Corcoran et al., 2012; Prahallad et al., 2012). Subsequent clinical trials showed promising activity of EGFR-inhibitors combined with anti-BRAF monoclonal antibodies (Yaeger et al., 2015; van Geel et al., 2017).

Preclinical and early clinical studies showed that the addition of a MEK inhibitor to *BRAF* inhibition exerts greater antitumor activity by strengthening inhibition of the MAPK pathway (Corcoran et al., 2018). Recently, the initial results of the BEACON CRC study, the first randomized phase III study and largest clinical trial for previously treated $^{V600E}$*BRAF* mCRCs, have been published. The BEACON CRC study evaluated the activity of a BRAF inhibitor encorafenib plus cetuximab, with or without the MEK inhibitor binimetinib versus irinotecan plus FOLFIRI and cetuximab. According to the updated results, encorafenib plus cetuximab with or without binimetinib significantly improved OS (overall survival) and PFS (progression-free survival) in comparison to the control group (Kopetz et al., 2019; Roviello et al., 2020). The outcomes of this landmark trial represent the first survival benefit over the current standard of care and led to the approval by the FDA and European Commission of the doublet encorafenib plus cetuximab in previously treated $^{V600E}$*BRAF* mCRC patients.

## 5. Molecular pathogenesis of *BRAF*mt CRC

CRC develops *via* a multistep carcinogenetic process, involving a gradual accumulation of genetic and epigenetic alterations that determine the intestinal mucosa's phenotypic transformation into precancerous lesion and invasive carcinoma (Vogelstein et al., 2013).

Two alternative carcinogenetic pathways have been described: i) the "conventional" pathway or "classic adenoma-carcinoma sequence" (tubular and tubulovillous adenomas → invasive CRC) and ii) the "serrated" pathway (traditional serrated and sessile serrated adenomas

→ invasive CRC) (Cappell, 2005; Higuchi and Jass, 2004).

At the molecular level, the "classic adenoma-carcinoma sequence" consists of an accumulation of genetic mutations associated with chromosomal instability (CIN) (Fearon and Vogelstein, 1990). CIN comprises aneuploidy, which is an imbalance in the chromosome number, and loss of heterozygosity (LOH) generated by chromosomal mis-segregation (Thompson et al., 2010). The *primum movens* of the CIN pathway is the inactivating mutation of the tumor suppressor gene *APC*, followed by mutations of *KRAS*, *SMAD4* and finally *TP53 (*Smith et al., 2002*)*.

The "serrated" pathway (15–30% of sporadic CRCs) is linked with two, often overlapping, molecular pathways: the CpG island methylator phenotype (CIMP), which can be either low level (CIMP-L) or high level (CIMP-H) and the microsatellite instability (MSI) (Weisenberger et al., 2006). The molecular trigger of the serrated pathway is MAPK pathway activation by either *BRAF* or *KRAS* mutation, followed by CpG island methylation that causes the silencing of critical tumor suppressor genes. Although important, MSI is not a defining molecular feature of the serrated neoplasia pathway (Rajagopalan et al., 2002; O'Brien et al., 2006).

In 2007, Jass proposed three molecular profiles for CRCs developing *via* the "serrated" pathway: i) *BRAF*mt/CIMP-H/MSI, ii) *BRAF*mt/CIMP-H/MSS (Microsatellite Stable) and iii) *KRAS*mt/CIMP-L/MSS (Jass, 2007).

In most cases, BRAFmt/CIMP-H tumors arise from the sessile serrated lesions (SSLs). The distinctive morphological feature of SSL is the distortion of the crypt profile, probably resulting from alterations of the proliferative zone, together with bland cytology and prominent serrations of the crypts (Galuppini et al., 2021).

*BRAF* mutation is present in 70–80% of SSLs and is thought to be the initiating event of the carcinogenetic process. *MLH1* methylation and MSI are late events and are limited to SSLs with dysplasia. Activation of the Wnt signaling pathway has also been associated with the progression of SSLs (Bettington et al., 2013; De Palma et al., 2019; Cappellesso et al., 2019).

Traditional serrated adenomas (TSAs) are rare (1–6% of serrated colorectal lesions). TSAs are characterized by slit-like serrations and tall cells that contain prominent eosinophilic cytoplasm and pencillate nuclei (Galuppini et al., 2021). TSAs can harbor either *KRAS* or *BRAF* mutations and are MSS. *KRAS*mt TSAs have CIMP-L phenotype and progress into *KRAS*mt/CIMP-L/MSS tumors *via* late Wnt signaling pathway activation and *TP53* mutation. *BRAF*mt TSAs and, more frequently CIMP-H. In addition, Wnt signaling pathway activation and *CDKN2A* silencing appear to be pivotal to malignant progression. Interestingly, residual presence of an SSL was found in approximately 30% of TSAs, suggesting that SSLs that do not undergo *MLH1* methylation could progress *via* a TSA to *BRAF*mt/MSS tumor (Bettington et al., 2015).

## 6. *BRAF*mt/MSI *versus BRAF*mt/MSS

MSI is a condition of genetic hypermutability resulting from a deficient DNA mismatch repair (dMMR) system. MSI is present in approximately 15% of CRCs and is associated with a better survival outcome (Vilar and Tabernero, 2013; Popat et al., 2005; Roth et al., 2012). A germline mutation in mismatch repair (MMR) genes is the cause of dMMR in Lynch syndrome (or Hereditary Non-Polyposis Colorectal Cancer, HNPCC), which accounts for 3% of CRCs (Sinicrope, 2018). In sporadic CRC, dMMR is caused by *MLH1* gene promoter hypermethylation. *BRAF* mutation is strongly linked with (~60%) the somatic inactivation of the DNA MMR genes and is virtually absent in Lynch syndrome (Weisenberger et al., 2006; Fassan et al., 2020). Hence, *BRAF* mutation analysis is a valid tool to implement in Lynch syndrome diagnosis (Fassan et al., 2020).

Several studies evaluated the prognostic role of *BRAF* mutation in relation to MSI status. Although some studies suggest that the adverse prognostic association of *BRAF* mutation is limited to MSS tumors (Samowitz et al., 2005; Roth et al., 2010; Fariña-Sarasqueta et al., 2010), others suggest that *BRAF* mutation remains prognostic regardless of MSI status (Lochhead et al., 2013; Sinicrope and Sargent, 2012). Tran and colleagues (Tran et al., 2011) reported poorer survival outcomes in MSI mCRC, indicating that, unlike early-stage disease, MSI is a negative prognostic factor in advanced disease and is likely driven by *BRAF* mutation. Of note, recent population-based data reported that *BRAF*mt/MSI mCRC patients have a worse survival outcome in comparison to *BRAF*mt/MSS ones (Chu et al., 2020).

The introduction of immune checkpoint inhibitors has revolutionized the therapeutic landscape of MSI mCRC (Le et al., 2020; Overman et al., 2017; Overman et al., 2018). A sporadic MSI phenotype is isolated in 20–30% of *BRAFmt* mCRCs, representing a subgroup that derives clinical benefit from treatment with programmed death-1 (PD-1) and cytotoxic T-lymphocyte- associated protein 4 (CTLA-4) inhibitors. Recent data from the KEYNOTE-177 (André et al., 2020) trial evaluating the anti-PD-1 antibody pembrolizumab *versus* standard-of-care reported durable response in treatment naïve *BRAF*mt/MSI mCRC patients. However, additional data should be collected in order to establish the best therapeutic sequential approach for this subgroup.

*BRAF*mt/MSS CRCs have not been as well-characterized as *BRAF*mt/MSI tumors. Although they both develop *via* the "serrated" pathway, *BRAF*mt/MSS CRCs represent a unique subgroup, with different clinicopathologic and molecular features.

*BRAF*mt/MSS tumors have a younger age of onset and equal gender distribution and present at a more advanced stage when compared to *BRAF*mt/MSI tumors (Bond and Whitehall, 2018). Histologically, *BRAF* mt/MSS CRCs are frequently mucinous and poorly differentiated. However, they display aggressive morphological features such as frequent tumor budding, lack of tumor-infiltrating lymphocytes (TILs), frequent angio-lymphatic and perineural invasion and increased lymph node metastases rate *BRAF*mt/MSI and *BRAF* wild-type cancers (Samowitz et al., 2005; Landau et al., 2014).

The more adverse clinico-pathologic features might be the product of specific molecular features. *BRAF*mt/MSS tumors originate from SSLs that do not methylate *MLH1*. Because the residual presence of an SSL has been found in approximately 30% of TSAs, It has been suggested that SSLs could progress *via* a TSA to *BRAF* mt/MSS cancer (Bettington et al., 2013). The *BRAF*mt/MSS subgroup is characterized by: i) a higher frequency of CIMP (60%), but lower than MSI (70–80%) (Bond et al., 2012; Bond et al., 2014), ii) a "focal" pattern of CIN (*i.e.,* high proportion of small, targeted copy number aberrations) *versus* "whole chromosome arm" pattern of *BRAF* wild-type cancers (Bond et al., 2014), iii) high rate of *TP53* mutations, which is frequently observed in the conventional pathway and is associated with advanced disease (Samowitz et al., 2002; Bond et al., 2012).

## 7. *BRAF*mt CRCs in the context of novel CRC molecular classifications

In 2012, The Cancer Genome Atlas Network proposed a novel genomic classification of CRCs, identifying two main groups by mutation rate: hypermutated and non-hypermutated cancers. Hypermutated CRCs (13%) are characterized by a high mutation rate, with the majority displaying MSI due to defective dMMR, CIMP and low rate of somatic copy number alterations (SCNAs). A further 2–3% are classified as ultramutated CRCs, with an exceptionally high mutation rate resulting from the DNA-replicating enzyme POLE mutation. Non-hypermutated cases (83%) have a low frequency of mutations, are MSS, but have a high rate of SCNAs. Approximately 46% of hypermutated CRCs harbor a *BRAF* mutation, and almost all *BRAF*mt CRCs are part of the hypermutated subgroup. However, a small part *BRAF*mt CRCs can be classified as non-hypermutated (The Cancer Genome Atlas Network, 2012).

By using multiple microarrays or RNA-sequencing datasets of primary CRC samples enriched with additional molecular data, the Colorectal Cancer Subtyping Consortium identified four gene expression

consensus molecular subtypes (CMS): CMS1 Immune (MSI, hypermutated; 14%), CMS2 Canonical (Wnt and Myc activation; 37%), CMS3 Metabolic (metabolic dysregulation, *KRAS* mutations; 13%) and CMS4 Mesenchymal (Stromal infiltration, TGF-β activation and angiogenesis; 23%), which were later found to be independent prognostic factors. The majority of *BRAF*mt CRCs (up to 70%) are classified as CMS1, while 7% and 17% are clustered in CMS3 and CMS4, respectively (Guinney et al., 2015). According to the results of a study by Smeby and colleagues (Smeby et al., 2018), the well-established poor prognostic impact of *BRAF* mutations in MSS tumors is strengthened among CMS1 tumors, probably due to strong mutation enrichment in this subtype, leading to a propensity for metastatic disease.

Being transcriptomic closely linked with tumor behavior and clinical phenotype, the uneven distribution of *BRAF*mt CRCs across the CMSs indicates that *BRAF*mt CRC is a molecularly heterogeneous subgroup. Further prognostic and predictive stratification is needed.

## 8. Gene expression-based molecular subtypes of *BRAF*mt CRC

Barras and colleagues (Barras et al., 2017) categorized *BRAF*mt CRCs into two subtypes based on gene expression profiling. BM1 subtype accounts for one-third of the cases, while BM2 for the remaining two-thirds. The BM1 subgroup is characterized by an upregulation of KRAS/mTOR/AKT/4EBP1 signaling pathway, an enhanced expression of genes associated with macrophage activity and epithelial-mesenchymal transition (EMT). In contrast, the BM2 subgroup displays an enrichment in cell cycle and cycle checkpoint associated genes. Interestingly, all CMS4 *BRAF* mutants are classified as BM1, whereas CMS1 *BRAF* mutants are distributed into both BM1 and BM2, indicating that the BM classification can capture a further level of heterogeneity within *BRAF*mt CMS1 tumors.

Although differences between the two subgroups in relapse-free and overall survival were not significant, it is currently under investigation whether this novel gene expression-based classification of *BRAF*mt CRCs could have therapeutic implications. In fact, it has been hypothesized that BM1 tumors could benefit from a combination of anti-BRAF, anti-MEK, and anti-AKT/mTOR/4EBP1 drugs, while drugs targeting cell-cycle components might be effective in BM2 patients (Barras et al., 2017).

According to the results of a recent Phase II clinical trial, the combination of dabrafenib, trametinib, and panitumumab resulted in longer OS and PFS of BM1 patients, who tend to have a worse outcome than BM2 patients. Therefore, BM subtype could represent a key biomarker to aid in the stratification of those patients who are more likely to benefit from the combination of BRAF, MEK and EGFR inhibitors (Middleton et al., 2020).

## 9. The heterogenous landscape of *BRAF* mutations: beyond V600E

Nearly all of our current knowledge of *BRAF*mt CRC clinical features has been derived from patients with the $^{V600E}$*BRAF* mutation. In recent years, the adoption of expanded mutational testing has allowed the identification of additional mutational hotspots within genes of interest. Overall, $^{non\text{-}V600E}$*BRAF* mutations occur in approximately 2% of mCRCs and 22% of *BRAF*mt mCRCs and cover 19 different codons, mainly mutations in codons 594,596 (Jones et al., 2017; Van Cutsem and Dekervel, 2017).

CRCs harboring $^{non\text{-}V600E}$*BRAF* mutations represent a molecular subtype with excellent prognosis and distinct clinico-pathologic features. First, patients with $^{non\text{-}V600E}$*BRAF* mutations present a less aggressive disease course and are likely to have significantly longer OS than patients harboring $^{V600E}$*BRAF* mutations (Cremolini et al., 2015; Calegari et al., 2021; Schirripa et al., 2019). Furthermore, $^{non\text{-}V600E}$*BRAF*mt CRCs arise more frequently in younger male patients and are often left-sided, non-mucinous, MSS, have no peritoneal spread and lower grade at presentation (Cremolini et al., 2015; Calegari et al., 2021; Schirripa et al., 2019).

Based on studies on preclinical models, *BRAF* mutations have been categorized in three different classes: activating RAS-independent *BRAF* mutations signaling as monomers (class 1; V600E mutation) or as dimers (class 2; mutations in codons 464, 469, 597 or 601) and RAS-dependent *BRAF* mutations with impaired kinase activity or kinase-dead (class 3; mutations in codons 287,459,466,467,469,581,594,595 or 596) (Zheng et al., 2015; Yao et al., 2017).

Class 3 mutants bind more tightly than wild-type *BRAF* to RAS-GTP, and they activate ERK signaling by heterodimerizing with CRAF. Hence, class 3 *BRAF* mutations can coexist with RAS/EGFR activating mutations, while these genetic alterations are mutually exclusive in other cases (Yao et al., 2017; Yao et al., 2015).

To date, there are no specific guidelines for the management of patients with $^{non\text{-}V600E}$*BRAF* mCRC. However, several clinical trials are exploring tailored treatment options.

Yeager and colleagues (Yaeger et al., 2019) investigated whether patients with class 2 and class 3 $^{non\text{-}V600}$*BRAF*mt CRC might benefit from EGFR inhibitors. According to the results, patients with class 2 mutation have limited or no benefit, while class 3 mutation carriers achieved 50% response rate with some durable responses when treated with anti-EGFR monoclonal antibodies (Fontana and Valeri, 2019). This is an intriguing finding; however, there is some contradictory evidence in the literature. In a small cohort of patients harboring $^{non\text{-}V600}$*BRAF* mutations of both class 2 and class 3, Johnson and colleagues (Johnson et al., 2019) found no complete or partial responses to anti-EGFR therapy in any of the cases.

The lack of efficacy of EGFR inhibitors indicates that new targeted approaches are needed. Novel inhibitors of MEK or ERK alone or in combination with next-generation RAF inhibitors that target dimerization are currently under evaluation (Johnson and Kopetz, 2020). In this context, the BIG BANG study assesses the efficacy of a combinatorial approach of simultaneous inhibitions of MEK, BRAF and EGFR, which exhibited potent antitumor activity in the preclinical model (Kotani et al., 2020).

## 10. Novel biomarkers for risk stratification

Despite its recognized prognostic value (Seligmann et al., 2017), there is great heterogeneity in survival outcome among $^{V600E}$*BRAF* mutated mCRCs. Indeed, some patients with *BRAF*mt mCRC achieve durable responses, while other patients rapidly develop resistance. A better stratification based on histopathological features and novel molecular biomarkers should be performed to optimize patient management and therapeutic decision-making.

In a study by Loupakis and colleagues (Loupakis et al., 2019), a large cohort of *BRAF*mt mCRC patients was analyzed to identify novel prognostic markers. The major findings were that: i) CDX2 loss, ii) high CK7 expression, iii) fewer TILs, iv) CMS2–3 or CMS4 (compared to CMS1) were associated with worse survival outcomes (both OS and PFS).

In most cases, CRC is CK7 negative and CK20/CDX2 positive (Werling et al., 2003). However, some evidence in the literature suggests that *BRAF*mt mCRC may have a different cytokeratins' profile, with lower CDX2 and higher CK7 expression (Landau et al., 2014; Zlobec et al., 2011; Kim et al., 2013). Furthermore, it has been hypothesized that $^{V600E}$*BRAF* mutation cooperates with CDX2 silencing to promote serrated carcinogenesis (Sakamoto et al., 2017) and that CK7 acts as a promoter of EMT (Harbaum et al., 2011).

The finding that a high number of TILs and CMS1, which are both tightly linked with MSI, are associated with a better survival outcome pinpoints the role of the tumor-immune system interaction in affecting prognosis in *BRAF*mt mCRCs (Guinney et al., 2015; Deschoolmeester et al., 2010).

Neuroendocrine differentiation has been associated with a worse prognosis and has been identified as mechanisms of therapy resistance

in several cancers, mainly in lung adenocarcinomas and prostate cancers (Antonescu et al., 2005; Davies et al., 2018). Fassan and colleagues (Fassan et al., 2021) reported that, among a large cohort of *BRAF*mt mCRCs, 22% of tumors showed positivity for synaptophysin at immunohistochemistry (IHC) and that synaptophysin expression was linked with a worse survival outcome, thus identifying a new subgroup with worse prognosis.

In a recent study, Angerilli and colleagues (Angerilli et al., 2021) found that approximately 20% of *BRAF*mt mCRCs displayed intratumor morphologic heterogeneity *(i.e.* the coexistence of two different histologic variants within the primary tumor). Of note, intratumor heterogeneity is a rather frequent finding in MSI tumors because the mutator phenotype of MSI tumors may produce in several molecular clones with possible distinct morphological features. According to the results of this study, intratumor phenotypic heterogeneity was linked with gene-expression intratumor heterogeneity and with enrichment of CMS4 among the samples analyzed. CMS4 being associated with worse survival outcomes, it might be speculated that morphologic heterogeneity might predispose patients to inferior clinical outcomes.

## 11. The diagnostic scenario of *BRAF*mt mCRC

1. Molecular testing

   The NCCN panel recommends *BRAF* mutational analysis of tumor tissue (either primary tumor or metastasis) at diagnosis of stage IV disease (Benson et al., 2021). Testing for the *BRAF* mutation is carried out on formalin-fixed paraffin-embedded (FFPE) tissues and is usually done by Sanger Sequencing, Mass Spectrometry, quantitative real-time PCR (qRT-PCR) or next-generation sequencing (NGS) (Fig. 3).

   When choosing an assay for routine diagnostics, several factors are needed to be addressed, such as pre-analytical factors (tissue availability, DNA quality, percentage of tumor cells), sensitivity and specificity of the various tests and additional factors such as workload, hands-on time, turnaround time and assay and equipment cost (Angerilli et al., 2021).

   NGS allows the sequencing of thousands of genes in a single run. It has greater sensitivity in comparison to the other methods (approximately to 0.001–5% of mutant alleles in a background of wild-type alleles) and can detect the broad spectrum of *BRAF* mutations. However, the implementation of NGS in routine diagnostics has many challenges, such as high costs and turnaround time, along with the need of bioinformatic expertise. qRT-PCR and Mass Spectrometry have a high sensitivity (1–5% of mutant alleles), faster turnaround time and lower cost. Nonetheless, only hotspot *BRAF* mutations can be detected. On the contrary, Sanger sequencing allows the identification of all *BRAF* mutations but has a limit of detection of 10–20% of mutant alleles and is a time-consuming technology because it sequences a single DNA fragment at a time (Ihle et al., 2014; Surrey et al., 2019; McCourt et al., 2013; Gao et al., 2016; Malapelle et al., 2015).

2. Immunohistochemistry

   Mutation-specific $^{V600E}$*BRAF* IHC is an accepted screening tool for melanoma (Pearlstein et al., 2014) (Fig. 3). As previously stated, *BRAF* mutation is usually detected through various PCR-based molecular tests in CRC. However, these methods can be expensive, multi-step and time-consuming.

   Studies evaluating the concordance between $^{V600E}$*BRAF* IHC and molecular testing and the diagnostic accuracy of $^{V600E}$*BRAF* in colorectal adenocarcinomas have provided mixed results. Though most have reported high sensitivity and specificity with a good interobserver agreement and concordance between surgical resection specimens and biopsies as well (Affolter et al., 2013; Sinicrope et al., 2013; Rössle et al., 2013; Day et al., 2015; Vakiani et al., 2015; Galuppini et al., 2017), others have found a lower sensitivity and specificity using the same VE1 antibody (Adackapara et al., 2013). Furthermore, $^{V600E}$*BRAF* IHC assay demonstrated high rates of intratumoral homogeneity of VE1 immunostaining, showing a uniform staining pattern in most cases (Bledsoe et al., 2014).

   $^{V600E}$*BRAF* immunostaining could represent a viable alternative to molecular profiling for mCRC and may help identify Lynch syndrome patients. IHC has the advantages of being faster, less expensive compared to molecular testing, and can be implemented in community-based laboratories when there is a lack of molecular

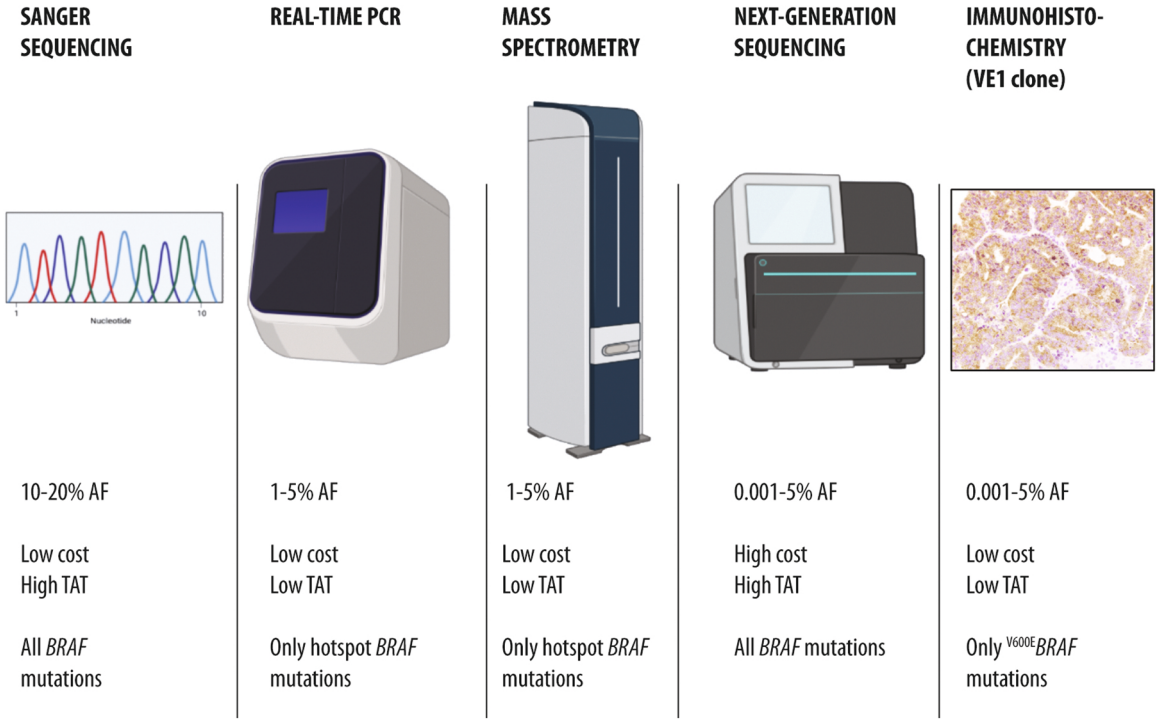


**Fig. 3.** Principal molecular diagnostics used in the clinical practice in the assessment of *BRAF* status.

pathology infrastructures and expertise. In addition, IHC could provide an alternative method for mutation detection in cases where the samples are inadequate for molecular testing (*i.e.* biopsies with few tumor cells). However, being the VE1 antibody highly specific for the $^{V600E}$*BRAF* mutation, molecular profiling is preferred over IHC also for the possibility of detecting $^{non\text{-}V600E}$BRAF mutations, which accounts for 22% of all *BRAF* mutations (Galuppini et al., 2017).

3. Liquid biopsy

Liquid biopsy is a promising tool in the diagnostic scenario of mCRC. Liquid biopsy can be defined as detecting tumor-derived biomarkers isolated from biological fluids (*i.e.* blood, urine, saliva) of cancer patients. It is rapid and minimally invasive and allows to recapitulate tumor spatial and temporal heterogeneity (Normanno et al., 2018).

In mCRC patients, liquid biopsy could represent an alternative to tissue sampling to assess predictive and prognostic biomarkers, such as *RAS* genes mutations. A landmark study by Thierry and colleagues (Thierry et al., 2014) circulating free DNA (cfDNA) analysis showed 100% concordance with CRC tissue analysis for $^{V600E}$*BRAF* mutation.

However, the results obtained from liquid biopsies must be interpreted with caution. The main limitations include: i) limited diagnostic sensitivity in low-tumor-burden patients; ii) the inability to distinguish free non-tumor cell DNA from tumor cell DNA; iii) the lack of information regarding the histological type; iv) impossibility to perform IHC (Angerilli et al., 2021). An additional point to consider is that *BRAF* mutations are common in several cancer types, so a silent *BRAF*mt tumor (i.e. papillary thyroid carcinoma) could cause a false positivity in a CRC patient. For the aforementioned reasons, it is unlikely that liquid biopsy will substitute tissue analysis in the future, but will instead provide additional clinically relevant information.

Various studies demonstrated that high levels of total cfDNA concentration and mutant cfDNA concentration were linked with a worse prognosis in mCRC (Thierry et al., 2014; Fan et al., 2017). Furthermore, the analysis cfDNA may find application in identifying minimal residual disease and in predicting recurrence in surgically resected patients (Tie et al., 2016; Tie et al., 2019; Tarazona et al., 2019). However, one of the most flourishing fields of application of liquid biopsy testing is the possibility of monitoring the response to therapy and tracking CRC evolution over time. Longitudinal profiling of cfDNA has been used to model resistance to anti-EGFR therapy and identity resistant clonal and subclonal populations harboring various genetic alterations, including *BRAF* mutations (Siravegna et al., 2015; Misale et al., 2014; Parseghian et al., 2019; Zhao et al., 2017).

## 12. Conclusion

Approximately 8–15% of CRCs harbor a *BRAF* mutation. Although *BRAF* mutation identifies a discrete subgroup of CRC with specific clinico-pathologic features and a detrimental prognosis, its predictive role is still blurred. Several studies have portrayed the heterogenous molecular landscape of *BRAF*mt CRCs on various levels to identify novel biomarkers for risk stratification and the optimization of therapeutic strategies (Table 1). However, more efforts are needed to provide the knowledge for rational use of targeted and combined therapies. It is fundamental to implement accurate and efficient molecular testing in routine diagnostics and advance in diagnostic technologies for translational and clinical research to achieve this goal.

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Not required.

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**Table 1**
Predictive and prognostic biomarkers for stratification of *BRAF*mt CRCs.

| Prognostic factor | Prognostic value | References |
|---|---|---|
| MSI | No prognostic value of BRAF mutation in MSI CRCs | Samowitz et al. (2005)<br>Roth et al. (2010)<br>Fariña-Sarasqueta et al. (2010) |
| MSI | Poorer survival outcomes in both MSI and MSS CRCs | Lochhead et al. (2013)<br>Sinicrope and Sargent (2012) |
| CDX2 loss<br>High CK7 expression<br>Fewer TILs<br>CMS2–3/CMS4 | Worse OS and PFS | Loupakis et al. (2019) |
| Synaptophysin expression | Worse OS and PFS | Fassan et al. (2021) |
| **Predictive factor** | **Predictive value** | **References** |
| MSI | Response to Immune Check-Point Inhibitors (improved OS and PFS) | André et al. (2020) |
| BM classification (BM1, BM2) | Response to BRAF, MEK and EGFR inhibitors of BM1 subgroup (improved OS and PFS) | Middleton et al. (2020) |
| $^{non\text{-}V600E}$*BRAF* (class 2, class 3) | Limited or no benefit to anti-EGFR therapy in class 2<br>Response to anti-EGFR therapy in class 3 | Yaeger et al. (2019) |
| $^{non\text{-}V600E}$*BRAF* (class 2, class 3) | Resistance to anti-EGFR therapy in class 2 and 3 | Johnson et al. (2019) |

Abbreviations: Colorectal cancer (CRC); microsatellite instability (MSI); Tumor-Infiltrating Lymphocytes.

## CRediT authorship contribution statement

Study concept and design: Matteo Fassan e Massimo Milione. Acquisition and analysis of data: Valentina Angerilli, Giovanna Sabella, Giovanni Centonze. Interpretation of data: All. Drafting of manuscript: Valentina Angerilli, Giovanna Sabella, Giovanni Centonze. Critical revision of the manuscript for important intellectual content: Sara Lonardi, Francesca Bergamo, Filippo Pietrantonio. Manuscript editing: All. Approval to submit: All. Obtained funding: Massimo Milione. Study supervision: Matteo Fassan, Massimo Milione.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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This work is dedicated to the memory of Laura Salvaterra, a courageous woman who battled against cancer. This is an invitation to fight cancer every day in her name, even after she has left us.

*Consent to participate*

Not required.

*Consent for publication*

Not required.

## References


Adackapara, C.A., Sholl, L.M., Barletta, J.A., Hornick, J.L., 2013. Immunohistochemistry using the BRAF V600E mutation-specific monoclonal antibody VE1 is not a useful surrogate for genotyping in colorectal adenocarcinoma. Histopathology 63, 187–193.

Affolter, K., Samowitz, W., Tripp, S., Bronner, M.P., 2013. BRAF V600E mutation detection by immunohistochemistry in colorectal carcinoma. Genes Chromosomes Cancer 52, 748–752.

André, T., et al., 2020. Pembrolizumab in microsatellite-instability-high advanced colorectal cancer. N. Engl. J. Med. 383, 2207–2218.

Angerilli, V., et al., 2021. Intratumor morphologic and transcriptomic heterogeneity in (V600E)BRAF-mutated metastatic colorectal adenocarcinomas. ESMO Open 6, 100211.

Angerilli, V., et al., 2021. The role of the pathologist in the next-generation era of tumor molecular characterization. Diagnostics 11.

The Cancer Genome Atlas Network, 2012. Comprehensive molecular characterization of human colon and rectal cancer. Nature 487, 330–337.

Antonescu, C.R., et al., 2005. Acquired resistance to imatinib in gastrointestinal stromal tumor occurs through secondary gene mutation. Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res. 11, 4182–4190.

Ascierto, P.A., et al., 2012. The role of BRAF V600 mutation in melanoma. J. Trans. Med. vol. 10, 85.

Baik, C.S., Myall, N.J., Wakelee, H.A., 2017. Targeting BRAF-mutant non-small cell lung cancer: from molecular profiling to rationally designed therapy. Oncologist 22, 786–796.

Barras, D., et al., 2017. BRAF V600E mutant colorectal cancer subtypes based on gene expression. Clin. Cancer Res. 23, 104–115.

Benson, A.B., et al., 2021. Colon cancer, version 2.2021, NCCN Clinical Practice Guidelines in Oncology. J. Natl. Compr. Canc. Netw 19, 329–359.

Bettington, M., et al., 2013. The serrated pathway to colorectal carcinoma: current concepts and challenges. Histopathology 62, 367–386.

Bettington, M.L., et al., 2015. A clinicopathological and molecular analysis of 200 traditional serrated adenomas. Mod. Pathol. Off. J. United States Can. Acad. Pathol. Inc 28, 414–427.

Bledsoe, J.R., Kamionek, M., Mino-Kenudson, M., 2014. BRAF V600E immunohistochemistry is reliable in primary and metastatic colorectal carcinoma regardless of treatment status and shows high intratumoral homogeneity. Am. J. Surg. Pathol. 38, 1418–1428.

Bond, C.E., Whitehall, V.L.J., 2018. How the BRAF V600E mutation defines a distinct subgroup of colorectal cancer: molecular and clinical implications. Gastroenterol. Res. Pract. 2018, 9250757.

Bond, C.E., et al., 2012. Chromosomal instability in BRAF mutant, microsatellite stable colorectal cancers. PLoS One 7, e47483.

Bond, C.E., et al., 2014. Microsatellite stable colorectal cancers stratified by the BRAF V600E mutation show distinct patterns of chromosomal instability. PLoS One 9, e91739.

Bond, C.E., et al., 2012. p53 mutation is common in microsatellite stable, BRAF mutant colorectal cancers. Int. J. Cancer 130, 1567–1576.

Calegari, M.A., et al., 2021. Clinical, pathological and prognostic features of rare BRAF Mutations in Metastatic Colorectal Cancer (mCRC): a bi-institutional retrospective analysis (REBUS Study). Cancers 13.

Cappell, M.S., 2005. From colonic polyps to colon cancer: pathophysiology, clinical presentation, and diagnosis. Clin. Lab. Med. 25, 135–177.

Cappellesso, R., et al., 2019. Molecular characterization of 'sessile serrated' adenoma to carcinoma transition in six early colorectal cancers. Pathol. Res. Pract. 215, 957–962.

Chapman, P.B., et al., 2011. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. N. Engl. J. Med. 364, 2507–2516.

Chen, D., et al., 2014. BRAFV600E mutation and its association with clinicopathological features of colorectal cancer: a systematic review and meta-analysis. PLoS One 9, e90607.

Chu, J.E., et al., 2020. Population-based screening for BRAF (V600E) in metastatic colorectal cancer reveals increased prevalence and poor prognosis. Clin. cancer Res. Off J. Am. Assoc. Cancer Res. 26, 4599–4605.

Clancy, C., Burke, J.P., Kalady, M.F., Coffey, J.C., 2013. BRAF mutation is associated with distinct clinicopathological characteristics in colorectal cancer: a systematic review and meta-analysis. Color. Dis. Off. J. Assoc. Coloproctology Gt. Britain Irel. 15, e711–e718.

Clarke, C.N., Kopetz, E.S., 2015. BRAF mutant colorectal cancer as a distinct subset of colorectal cancer: clinical characteristics, clinical behavior, and response to targeted therapies. J. Gastrointest. Oncol. 6, 660–667.

Corcoran, R.B., et al., 2012. EGFR-mediated re-activation of MAPK signaling contributes to insensitivity of BRAF mutant colorectal cancers to RAF inhibition with vemurafenib. Cancer Discov. 2, 227–235.

Corcoran, R.B., et al., 2018. Combined BRAF, EGFR, and MEK inhibition in patients with BRAF(V600E)-mutant colorectal cancer. Cancer Discov. 8, 428–443.

Cremolini, C., et al., 2020. Upfront FOLFOXIRI plus bevacizumab and reintroduction after progression versus mFOLFOX6 plus bevacizumab followed by FOLFIRI plus bevacizumab in the treatment of patients with metastatic colorectal cancer (TRIBE2): a multicentre, open-label, phase 3, ran. Lancet Oncol. 21, 497–507.

Cremolini, C., et al., 2015. BRAF codons 594 and 596 mutations identify a new molecular subtype of metastatic colorectal cancer at favorable prognosis. Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. 26, 2092–2097.

Van Cutsem, E., et al., 2016. ESMO consensus guidelines for the management of patients with metastatic colorectal cancer. Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. 27, 1386–1422.

Van Cutsem, E., et al., 2011. Cetuximab plus irinotecan, fluorouracil, and leucovorin as first-line treatment for metastatic colorectal cancer: updated analysis of overall survival according to tumor KRAS and BRAF mutation status. J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 29, 2011–2019.

Van Cutsem, E., Dekervel, J., 2017. Not All BRAF-mutant metastatic colorectal cancers are identical: distinct clinical consequences of (non-V600) BRAF mutations. J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. vol. 35, 2598–2599.

Davies, H., et al., 2002. Mutations of the BRAF gene in human cancer. Nature 417, 949–954.

Davies, A.H., Beltran, H., Zoubeidi, A., 2018. Cellular plasticity and the neuroendocrine phenotype in prostate cancer. Nat. Rev. Urol. 15, 271–286.

Day, F., et al., 2015. A mutant BRAF V600E-specific immunohistochemical assay: correlation with molecular mutation status and clinical outcome in colorectal cancer. Target. Oncol. 10, 99–109.

Deschoolmeester, V., et al., 2010. Tumor infiltrating lymphocytes: an intriguing player in the survival of colorectal cancer patients. BMC Immunol. 11, 19.

Fakih, M.G., 2015. Metastatic colorectal cancer: current state and future directions. J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 33, 1809–1824.

Fanelli, G.N., et al., 2020. The heterogeneous clinical and pathological landscapes of metastatic Braf-mutated colorectal cancer. Cancer Cell Int. 20, 1–12.

Fan, G., et al., 2017. Prognostic value of circulating tumor DNA in patients with colon cancer: systematic review. PLoS One 12, e0171991.

Fariña-Sarasqueta, A., et al., 2010. The BRAF V600E mutation is an independent prognostic factor for survival in stage II and stage III colon cancer patients. Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. 21, 2396–2402.

Fassan, M., et al., 2020. Current prognostic and predictive biomarkers for gastrointestinal tumors in clinical practice. Pathologica 112, 248–259.

Fassan, M., et al., 2021. Synaptophysin expression in (V600EBRAF-)mutated advanced colorectal cancers identifies a new subgroup of tumours with worse prognosis. Eur. J. Cancer 146, 145–154.

Fearon, E.R., Vogelstein, B., 1990. A genetic model for colorectal tumorigenesis. Cell 61, 759–767.

Fontana, E., Valeri, N., 2019. Class(y) dissection of BRAF heterogeneity: beyond non-V600. Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res. 25, 6896–6898.

Galuppini, F., et al., 2021. The histomorphological and molecular landscape of colorectal adenomas and serrated lesions. Pathologica 113, 218–229.

Galuppini, F., et al., 2017. BRAF p.V600E-specific immunohistochemical assessment in colorectal cancer endoscopy biopsies is consistent with the mutational profiling. Histopathology vol. 71, 1008–1011.

Gao, J., et al., 2016. Comparison of next-generation sequencing, quantitative PCR, and Sanger Sequencing for Mutation Profiling of EGFR, KRAS, PIK3CA and BRAF in clinical lung tumors. Clin. Lab. 62, 689–696.

van Geel, R.M.J.M., et al., 2017. A phase Ib dose-escalation study of Encorafenib and Cetuximab with or without Alpelisib in metastatic BRAF-mutant colorectal cancer. Cancer Discov. 7, 610–619.

Guan, W.-L., et al., 2020. Clinicopathologic features and prognosis of BRAF mutated colorectal cancer patients. Front. Oncol. 10, 563407.

Guinney, J., et al., 2015. The consensus molecular subtypes of colorectal cancer. Nat. Med. 21, 1350–1356.

Harbaum, L., et al., 2011. Keratin 7 expression in colorectal cancer–freak of nature or significant finding? Histopathology 59, 225–234.

Higuchi, T., Jass, J.R., 2004. My approach to serrated polyps of the colorectum. J. Clin. Pathol. 57, 682–686.

Hurwitz, H., et al., 2004. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N. Engl. J. Med. 350, 2335–2342.

Ihle, M.A., et al., 2014. Comparison of high resolution melting analysis, pyrosequencing, next generation sequencing and immunohistochemistry to conventional Sanger sequencing for the detection of p.V600E and non-p.V600E BRAF mutations. BMC Cancer 14, 13.

Jang, M.H., et al., 2017. BRAF-mutated colorectal cancer exhibits distinct clinicopathological features from wild-type BRAF-expressing cancer independent of the microsatellite instability status. J. Korean Med. Sci. 32, 38–46.

Jass, J.R., 2007. Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. Histopathology 50, 113–130.

Johnson, B., Kopetz, S., 2020. Applying precision to the management of BRAF-mutant metastatic colorectal cancer. Target. Oncol. 15, 567–577.

Johnson, B., et al., 2019. Atypical, non-V600 BRAF mutations as a potential mechanism of resistance to EGFR inhibition in metastatic colorectal cancer. JCO Precis. Oncol. 1–10. https://doi.org/10.1200/po.19.00102.

Jones, J.C., et al., 2017. (Non-V600) BRAF mutations define a clinically distinct molecular subtype of metastatic colorectal cancer. J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 35, 2624–2630.

Kimura, E.T., et al., 2003. High prevalence of BRAF mutations in thyroid cancer: genetic evidence for constitutive activation of the RET/PTC-RAS-BRAF signaling pathway in papillary thyroid carcinoma. Cancer Res. 63, 1454–1457.

Kim, J.H., Rhee, Y.-Y., Bae, J.M., Cho, N.-Y., Kang, G.H., 2013. Loss of CDX2/CK20 expression is associated with poorly differentiated carcinoma, the CpG island

methylator phenotype, and adverse prognosis in microsatellite-unstable colorectal cancer. Am. J. Surg. Pathol. 37, 1532–1541.
Kopetz, S., et al., 2019. Encorafenib, binimetinib, and cetuximab in BRAF V600E–mutated colorectal cancer. N. Engl. J. Med. 381, 1632–1643.
Kopetz, S., et al., 2015. Phase II pilot study of vemurafenib in patients with metastatic BRAF-mutated colorectal cancer. J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 33, 4032–4038.
Kotani, D., et al., 2020. BIG BANG study (EPOC1703): multicentre, proof-of-concept, phase II study evaluating the efficacy and safety of combination therapy with binimetinib, encorafenib and cetuximab in patients with BRAF non-V600E mutated metastatic colorectal cancer. ESMO Open 5, e000624.
Landau, M.S., Kuan, S.-F., Chiosea, S., Pai, R.K., 2014. BRAF-mutated microsatellite stable colorectal carcinoma: an aggressive adenocarcinoma with reduced CDX2 and increased cytokeratin 7 immunohistochemical expression. Hum. Pathol. 45, 1704–1712.
Le, D.T., et al., 2020. Phase II open-label study of pembrolizumab in treatment-refractory, microsatellite instability-high/mismatch repair-deficient metastatic colorectal cancer: KEYNOTE-164. J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 38, 11–19.
Lochhead, P., et al., 2013. Microsatellite instability and BRAF mutation testing in colorectal cancer prognostication. J. Natl. Cancer Inst. 105, 1151–1156.
Loupakis, F., et al., 2009. KRAS codon 61, 146 and BRAF mutations predict resistance to cetuximab plus irinotecan in KRAS codon 12 and 13 wild-type metastatic colorectal cancer. Br. J. Cancer 101, 715–721.
Loupakis, F., et al., 2016. Clinico-pathological nomogram for predicting BRAF mutational status of metastatic colorectal cancer. Br. J. Cancer 114, 30–36.
Loupakis, F., et al., 2014. Initial therapy with FOLFOXIRI and bevacizumab for metastatic colorectal cancer. N. Engl. J. Med. 371, 1609–1618.
Loupakis, F., et al., 2019. CK7 and consensus molecular subtypes as major prognosticators in (V600E)BRAF mutated metastatic colorectal cancer. Br. J. Cancer 121, 593–599.
Malapelle, U., et al., 2015. Ion Torrent next-generation sequencing for routine identification of clinically relevant mutations in colorectal cancer patients. J. Clin. Pathol. 68, 64–68.
McCourt, C.M., et al., 2013. Validation of next generation sequencing technologies in comparison to current diagnostic gold standards for BRAF, EGFR and KRAS mutational analysis. PLoS One 8, e69604.
Middleton, G., et al., 2020. BRAF-mutant transcriptional subtypes predict outcome of combined BRAF, MEK, and EGFR blockade with Dabrafenib, Trametinib, and Panitumumab in patients with colorectal cancer. Clin. Cancer Res. 26, 2466–2476.
Misale, S., et al., 2014. Blockade of EGFR and MEK intercepts heterogeneous mechanisms of acquired resistance to anti-EGFR therapies in colorectal cancer. Sci. Transl. Med. 6, 224ra26.
Morkel, M., Riemer, P., Bläker, H., Sers, C., 2015. Similar but different: distinct roles for KRAS and BRAF oncogenes in colorectal cancer development and therapy resistance. Oncotarget 6, 20785–20800.
Di Nicolantonio, F., et al., 2008. Wild-type BRAF is required for response to panitumumab or cetuximab in metastatic colorectal cancer. J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 26, 5705–5712.
Normanno, N., Cervantes, A., Ciardiello, F., De Luca, A., Pinto, C., 2018. The liquid biopsy in the management of colorectal cancer patients: current applications and future scenarios. Cancer Treat. Rev. 70, 1–8.
Overman, M.J., et al., 2017. Nivolumab in patients with metastatic DNA mismatch repair-deficient or microsatellite instability-high colorectal cancer (CheckMate 142): an open-label, multicentre, phase 2 study. Lancet. Oncol. 18, 1182–1191.
Overman, M.J., et al., 2018. Durable clinical benefit with Nivolumab plus Ipilimumab in DNA mismatch repair-deficient/microsatellite instability-high metastatic colorectal cancer. J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 36, 773–779.
O'Brien, M.J., et al., 2006. Comparison of microsatellite instability, CpG island methylation phenotype, BRAF and KRAS status in serrated polyps and traditional adenomas indicates separate pathways to distinct colorectal carcinoma end points. Am. J. Surg. Pathol. 30, 1491–1501.
De Palma, F.D.E., et al., 2019. The molecular hallmarks of the serrated pathway in colorectal cancer. Cancers 11.
Parseghian, C.M., et al., 2019. Anti-EGFR-resistant clones decay exponentially after progression: implications for anti-EGFR re-challenge. Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. 30, 243–249.
Pearlstein, M.V., et al., 2014. Validation of the VE1 immunostain for the BRAF V600E mutation in melanoma. J. Cutan. Pathol. 41, 724–732.
Pietrantonio, F., et al., 2015. Predictive role of BRAF mutations in patients with advanced colorectal cancer receiving cetuximab and panitumumab: a meta-analysis. Eur. J. Cancer 51, 587–594.
Popat, S., Hubner, R., Houlston, R.S., 2005. Systematic review of microsatellite instability and colorectal cancer prognosis. J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 23, 609–618.
Prahallad, A., et al., 2012. Unresponsiveness of colon cancer to BRAF(V600E) inhibition through feedback activation of EGFR. Nature 483, 100–103.
Rajagopalan, H., et al., 2002. Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status. Nature 418, 934.
Rössle, M., et al., 2013. Ultra-deep sequencing confirms immunohistochemistry as a highly sensitive and specific method for detecting BRAF V600E mutations in colorectal carcinoma. Virchows Arch. 463, 623–631.
Roth, A.D., et al., 2012. Integrated analysis of molecular and clinical prognostic factors in stage II/III colon cancer. J. Natl. Cancer Inst. 104, 1635–1646.
Roth, A.D., et al., 2010. Prognostic role of KRAS and BRAF in stage II and III resected colon cancer: results of the translational study on the PETACC-3, EORTC 40993, SAKK 60-00 trial. J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 28, 466–474.
Roviello, G., et al., 2020. Encorafenib, Binimetinib, and Cetuximab in BRAF V600E-mutated colorectal cancer. Transl. Oncol. 13, 100795.
Sakamoto, N., et al., 2017. BRAF(V600E) cooperates with CDX2 inactivation to promote serrated colorectal tumorigenesis. Elife 6.
Samowitz, W.S., et al., 2005. Poor survival associated with the BRAF V600E mutation in microsatellite-stable colon cancers. Cancer Res. 65, 6063–6069.
Samowitz, W.S., et al., 2002. Prognostic significance of p53 mutations in colon cancer at the population level. Int. J. Cancer 99, 597–602.
Schirripa, M., et al., 2019. Class 1, 2, and 3 BRAF-mutated metastatic colorectal cancer: a detailed clinical, pathologic, and molecular characterization. Clin. cancer Res. Off. J. Am. Assoc. Cancer Res. 25, 3954–3961.
Seligmann, J.F., et al., 2017. Investigating the poor outcomes of BRAF-mutant advanced colorectal cancer: analysis from 2530 patients in randomised clinical trials. Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. 28, 562–568.
Singer, G., et al., 2003. Mutations in BRAF and KRAS characterize the development of low-grade ovarian serous carcinoma. J. Natl. Cancer Inst. 95, 484–486.
Sinicrope, F.A., 2018. Lynch Syndrome-associated colorectal cancer. N. Engl. J. Med. 379, 764–773.
Sinicrope, F.A., Sargent, D.J., 2012. Molecular pathways: microsatellite instability in colorectal cancer: prognostic, predictive, and therapeutic implications. Clin. cancer Res. Off. J. Am. Assoc. Cancer Res. 18, 1506–1512.
Sinicrope, F.A., et al., 2013. Mutation-specific antibody detects mutant BRAFV600E protein expression in human colon carcinomas. Cancer 119, 2765–2770.
Siravegna, G., et al., 2015. Clonal evolution and resistance to EGFR blockade in the blood of colorectal cancer patients. Nat. Med. 21, 795–801.
Smeby, J., et al., 2018. CMS-dependent prognostic impact of KRAS and BRAFV600E mutations in primary colorectal cancer. Ann. Oncol. 29, 1227–1234.
Smith, G., et al., 2002. Mutations in APC, Kirsten-ras, and p53–alternative genetic pathways to colorectal cancer. Proc. Natl. Acad. Sci. USA 99, 9433–9438.
Sung, H., et al., 2021. Global Cancer Statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA. Cancer J. Clin. 71, 209–249.
Surrey, L.F., et al., 2019. Next-Generation Sequencing (NGS) methods show superior or equivalent performance to non-ngs methods on BRAF, EGFR, and KRAS proficiency testing samples. Arch. Pathol. Lab. Med. 143, 980–984.
Tannapfel, A., et al., 2003. Mutations of the BRAF gene in cholangiocarcinoma but not in hepatocellular carcinoma. Gut 52, 706–712.
Tarazona, N., et al., 2019. Targeted next-generation sequencing of circulating-tumor DNA for tracking minimal residual disease in localized colon cancer. Ann. Oncol. 30, 1804–1812.
Thierry, A.R., et al., 2014. Clinical validation of the detection of KRAS and BRAF mutations from circulating tumor DNA. Nat. Med. 20, 430–435.
Thompson, S.L., Bakhoum, S.F., Compton, D.A., 2010. Mechanisms of chromosomal instability. Curr. Biol. 20, R285–R295.
Thompson, J.F., Scolyer, R.A., Kefford, R.F., 2009. Cutaneous melanoma in the era of molecular profiling. Lancet 374, 362–365.
Tie, J., et al., 2016. Circulating tumor DNA analysis detects minimal residual disease and predicts recurrence in patients with stage II colon cancer, 346ra92 Sci. Transl. Med. 8, 346ra92.
Tie, J., et al., 2019. Circulating tumor DNA analyses as markers of recurrence risk and benefit of adjuvant therapy for stage III colon cancer. JAMA Oncol. 5, 1710–1717.
Tran, B., et al., 2011. Impact of BRAF mutation and microsatellite instability on the pattern of metastatic spread and prognosis in metastatic colorectal cancer. Cancer 117, 4623–4632.
Vakiani, E., et al., 2015. Immunohistochemical detection of the BRAF V600E mutant protein in colorectal neoplasms. Appl. Immunohistochem. Mol. Morphol. AIMM 23, 438–443.
Vilar, E., Tabernero, J., 2013. Molecular dissection of microsatellite instable colorectal cancer. Cancer Discov. 3, 502–511.
Vogelstein, B., et al., 2013. Cancer genome landscapes. Science 340, 1546–1558.
Weisenberger, D.J., et al., 2006. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. Nat. Genet. 38, 787–793.
Werling, R.W., Yaziji, H., Bacchi, C.E., Gown, A.M., 2003. CDX2, a highly sensitive and specific marker of adenocarcinomas of intestinal origin: an immunohistochemical survey of 476 primary and metastatic carcinomas. Am. J. Surg. Pathol. 27, 303–310.
Yaeger, R., et al., 2015. Pilot trial of combined BRAF and EGFR inhibition in BRAF-mutant metastatic colorectal cancer patients. Clin. cancer Res. Off. J. Am. Assoc. Cancer Res. 21, 1313–1320.
Yaeger, R., et al., 2019. Response to anti-EGFR therapy in patients with BRAF non-V600-mutant metastatic colorectal cancer. Clin. cancer Res. Off. J. Am. Assoc. Cancer Res. 25, 7089–7097.
Yao, Z., et al., 2017. Tumours with class 3 BRAF mutants are sensitive to the inhibition of activated RAS. Nature 548, 234–238.
Yao, Z., et al., 2015. BRAF mutants evade ERK-dependent feedback by different mechanisms that determine their sensitivity to pharmacologic inhibition. Cancer Cell 28, 370–383.
Zhao, B., et al., 2017. Mechanisms of resistance to anti-EGFR therapy in colorectal cancer. Oncotarget 8, 3980–4000.
Zheng, G., et al., 2015. Clinical detection and categorization of uncommon and concomitant mutations involving BRAF. BMC Cancer 15, 779.
Zlobec, I., Bihl, M., Foerster, A., Rufle, A., Lugli, A., 2011. Comprehensive analysis of CpG island methylator phenotype (CIMP)-high, -low, and -negative colorectal

cancers based on protein marker expression and molecular features. J. Pathol. 225, 336–343.

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