University of Insubria
Department of Medicine and Surgery
PhD in Experimental and Translational Medicine
XXXIII cycle
Coordinator: Prof. Daniela Negrini

BRAF MUTATION IN ADVANCED COLORECTAL CANCER

Tutor: Prof. Daniela Furlan

PhD candidate:
Dr. Nunzio Digiacomo

Academic year: 2019-2020

SUMMARY

Metastatic colorectal cancer (mCRC) is one of the leading causes of mortality and morbidity in the world [1]. It is a heterogeneous disease characterized by different pathways of carcinogenesis and can be classified into different subtypes with specific molecular and morphological alterations. In this contest, *BRAF* mutations are found in about 10% of mCRC and define a particular subtype (*BRAF*-mCRC) characterized by a dismal prognosis and resistance to standard therapies with a median survival of less than 12 months [2]. Furthermore, *BRAF*-mCRCs are significantly associated with female gender, right site, advanced stage, mucinous histology, serrated pathway during carcinogenesis and CpG island methylator (CIMP) phenotype, defective mismatch repair (dMMR) and presence of microsatellite instability (MSI) [3].

Although they are clinically considered an unique entity with a specific histopathological profile, a significant biological heterogeneity has been described.

The vast majority of *BRAF* mutations (~96%) occur at the V600 amino acid residue where a DNA point mutation (transversion of a thymidine to adenosine in coding nucleotide 1799) results in a Valin to Glutamate (p.V600E) amino acid substitution, causing a constitutive activation of BRAF kinase activity regardless of upstream signaling [4]. Non-*BRAF* V600E mutations are much less common and have been reported to be associated with different clinical impact and biological features [5].

BRAF p.V600E is found in ~60% of CRC with microsatellite instability (MSI) and in only 5% to 10% of microsatellite stable (MSS) CRC [6, 7]. Both *BRAF* mutation and MMR status should be determined in all CRCs to distinguish sporadic tumors from Lynch CRCs. Indeed, because *BRAF* p.V600E mutation has been demonstrated to cause *MLH1* epigenetic silencing, this mutation and/or *MLH1* methylation analysis have an established clinical utility to recognize sporadic CRC, excluding Lynch tumors [8].

MSI in CRC has been associated with a proximal location, poor histologic differentiation, mucinous or signet ring cell differentiation, and is common in stages II (20%) and III (12%) but rare in stage IV CRCs (4%) [9]. Moreover, MSI is strongly associated with the presence of tumor-infiltrating lymphocytes (TILs) because DNA mismatch repair (MMR) defects lead to a high rate of immunogenic neoantigens and stimulates the activation of host immune response which is generally correlated with the survival benefit of presence of MSI in early stages disease. Instead, the prognostic role of MSI remains less clear in metastatic setting [10, 11, 12].

At present, the prognostic and predictive implications of TILs and inhibitory programmed cell death 1 (PD-1/programmed cell death ligand 1 (PD-L1) proteins in mCRCs are poorly understood,

especially in the *BRAF*-mCRC subtype in which the clinical relevance of a pronounced host immune reaction remains elusive, including both MSI and MSS tumors [13]. Moreover, considering the dramatic prognosis conferred by *BRAF* mutation, the groundbreaking results of the immune checkpoint inhibitors against the PD1/PD-L1 axis in MMR-deficient tumors could provide a remarkable therapeutic promise for patients *BRAF*-mCRC patients with MSI. [14]

In contrast with MSI/*BRAF*-CRC, *BRAF*-CRC with proficient MMR (MSS/*BRAF*-CRC) represents a less defined subset which shares clinical and molecular features with both serrated pathway and conventional pathway [15,16,17]. Indeed, although the *BRAF* mutation occurs early in tumorigenesis and is correlated with the serrated pathway, the MSS/*BRAF*-CRCs have been found to have an high rate of molecular alterations, such as *TP53* mutations and chromosomal instability (CIN) which are typically correlated with classic pathway and found in *BRAF* wild type (*BRAF* wt) tumors [15,17]. Furthermore, in a few reports, prevalent or partial neuroendocrine differentiation, including patterns of high-grade neuroendocrine carcinoma or mixed adenoneuroendocrine carcinoma (MANEC), has been found more commonly in MSS/*BRAF*-CRC than in MSI/*BRAF* tumors [18,19,20].

To our knowledge, no extensive clinical pathological studies focused on *BRAF*-mCRC have been reported to clarify whether the MSI status and associated biological and/or morphological characteristics will allow the identification of clinically different tumor subgroups.

Although the standard of care for *BRAF*-CRC patients remains fluoropyrimidine-based cytotoxic regimen, the aggressive biology of this subgroup has stressed the importance of studying chemotherapy intensification strategies in order to develop new therapeutic agents and improve outcomes in these patients [21]. Despite the lack of a randomized phase III trial dedicated to *BRAF*-mCRC, chemotherapy intensification combining a quadruple association of 5-fluorouracil, oxaliplatin, irinotecan (FOLFOXIRI) associated with antiangiogenic agent, bevacizumab, seems like a valid option [22, 23].

BRAF mutations, resulting in constitutive activation of MAPK pathway, have garnered a great deal of attention over past decade since the inhibition of this pathway by *BRAF*/MEK inhibitors has revolutionized the treatment of melanoma, becoming a standard of care option and its efficacy has been replicated in selected tumors such as NSCLC, thyroid cancer, hairy cell leukemia [24,25,26]. However, these results contrast with the low activity of *BRAF*/MEK-targeted therapy in patients with *BRAF*-mCRC, suggesting that responses may be histology-dependent and that the

molecular landscape of CRC is more complex [2]. Early data suggested that epidermal growth factor receptor (*EGFR*)-mediated reactivation of the MAPK pathway may be a mechanism underlying resistance to therapy [2]. This hypothesis has now been confirmed in a randomized phase III study, which demonstrated a survival benefit combining *BRAF* inhibitors with both anti EGFR monoclonal antibodies and MEK inhibitors (BEACON trial) [27].

Interestingly, *BRAF* mutation was found as driver mutation in multiple tumor types, including colorectal rhabdoid carcinomas (CRbCs), which represents a very rare and aggressive subtype of CRC with poor prognosis [28]. Although the hallmark diagnostic is based on the presence of the rhabdoid cell, this entity remains often underdiagnosed because misrecognized and confused with poorly differentiated histological subtypes of CRC which may share some histomorphological aspects with CRbCs but with very distinct genetic and/or molecular features from each other [29].

The events involved in CRbCs pathogenesis are poorly elucidated and to our knowledge only 39 cases have been reported in literature so far. *SMARCB1* (INI-1) inactivation, *BRAF* V600E mutation, epigenetic alterations involved in the serrated pathway and centrosomal inactivation due to *CROCC* mutation are the main molecular mechanisms commonly observed in the development of these tumors [28, 30, 31, 32].

The complex heterogeneity among sporadic CRC subtypes implies that there is no standardized therapeutic approach for these patients. The development of new biomarkers and their clinical significance will improve the overall survival and disease management of these tumors, especially for aggressive and lethal entities such as *BRAF*-mCRC or CRbCs without current valid treatments.

In the present study we performed an extensive literature search concerning the role of *BRAF* mutation in CRC with particular regard to the histological characteristics, to the molecular mechanisms underlying the pathogenesis of the mutated *BRAF* subtype and its biological and molecular heterogeneity. Furthermore, we provided a review of the major studies aimed at defining the clinical significance of the *BRAF* mutation in colorectal cancers in terms of prognosis, predictive markers of response to different treatments and newly developed innovative therapeutic approaches or currently under study.

Secondly, we analyzed a selected cohort of *BRAF*V600E-mCRC in order (1) to compare the clinico-pathological profile of MSI *versus* MSS group, highlighting the most significant differences associated with MMR status; (2) to analyze the extent of neuroendocrine differentiation and the

types of neuroendocrine neoplasms between these two tumor groups; and (3) to evaluate the density of intratumor CD8+ T lymphocytes and the PD-L1 immunohistochemical expression in tumor cells and their prognostic role.

Finally, since the *BRAF* mutation has been reported to be a common feature of CRbCs, we herein report seven new cases of this rare entity, examining in detail their clinicopathologic and molecular features. For comparison, we included four poorly differentiated medullary carcinomas (PDMCs) with focal aspects mimicking rhabdoid features. Immunohistochemical, genetic, and epigenetic analyses were performed to clarify the molecular alterations associated with this phenotype, with special emphasis on *BRAF* V600E mutation. In addition, we reviewed the literature on the cases of this entity reported so far.

INTRODUCTION

1. BRAF gene and MAP Pathway

The *BRAF* gene (homolog B of the murine sarcoma viral oncogene v-raf) is located on chromosome 7 and encodes a protein that belongs to the Raf family of serine-threonine protein kinases [33]. It is involved in the MAP kinase (MAPK) cascade (mitogen activated protein) that regulates cell growth and differentiation (Figure 1). MAPK signaling starts when epidermal growth factor (EGF) binds to its receptor (EGFR) in the cell membrane, activating it by phosphorylation in the cytoplasmic domain. Phosphorylated EGFR activates RAS which subsequently activates and phosphorylates *BRAF* which, upon stimulation, forms an active dimer. This in turn phosphorylates and activates MEK and then ERK. Activated ERK subsequently translocates to the nucleus where it can stimulate transcription factors involved in the promotion of cellular proliferation and survival. A negative feedback mechanism exists whereby phosphorylated ERK inhibits the upstream components. Two other Raf kinase family isoforms exist: ARAF and CRAF (or RAF-1) that have lower kinase activity, affinity and efficiency for MEK binding, confirming that BRAF is the strongest RAF isoform in driving this signaling cascade [2].

BRAF is reported to be mutated at different codons, however the most frequent mutation is V600E (up to 90% of all *BRAF* mutations) where a point mutation at the V600 aminoacidic residue (transversion of a thymidine to adenosine at coding nucleotide 1799 in exon 15) results in substitution of valine to glutamate [2]. This confers a constitutive activation of the MAPK pathway with an activity approximately 500-fold greater than the wild type form [24].

BRAF and its isoforms ARAF and CRAF not only activate MAPK pathway, but also affect other key cellular processes such as cell migration (through RHO small GTPases), apoptosis (through the regulation of BCL-2), and survival (through the HIPPO pathway). Thus, it is not a surprise that *BRAF* gene is found constitutively activated by mutation in around 15% of all human known cancer types [2, 34].

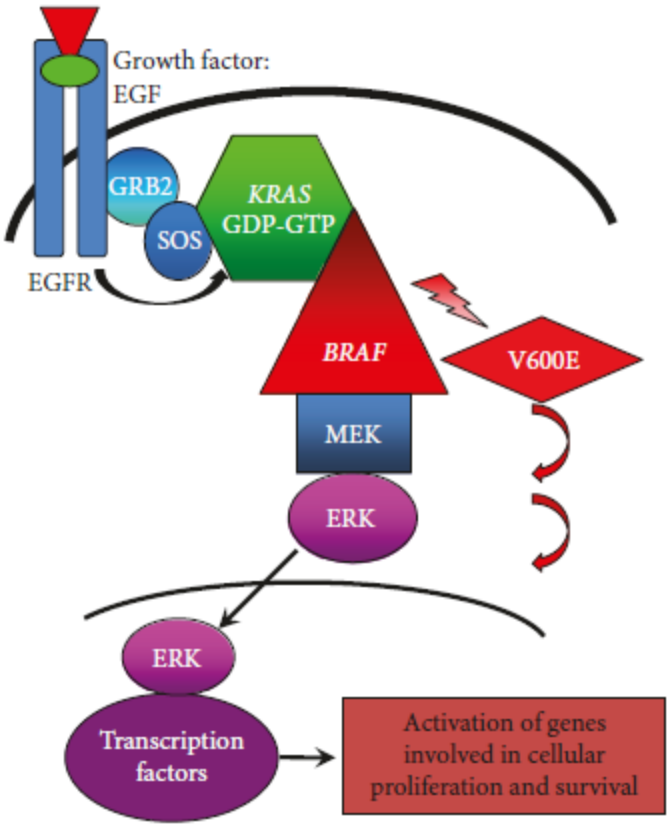


Figure 1. Mitogen activated protein kinase pathway. A signaling epidermal growth factor binds to the receptor (EGFR) on the cell surface causing its phosphorylation and activation. The active signal is passed to scaffolding proteins (GRB2 and SOS) which in turn promotes the removal of GDP from membrane bound KRAS. KRAS then binds GTP allowing its activation and undergoes conformational change to bind and phosphorylate BRAF. The signaling cascade continues through MEK and ERK. Activated ERK translocates to the nucleus where it recruits transcription factors involved in cellular survival and growth. The V600e BRAF mutationj allows for constitutive activation of BRAF and continuation of downstream signaling regardless of upstream regulation.

C.E. Bond. How the BRAF V600E mutation defines a distinct subgroup of colorectal cancer: molecular and clinical implications. Review Article; 2018.

2. Frequency of *BRAF* mutation in CRC.

The frequency of *BRAF* mutations varies widely in human cancers, from nearly half of all melanomas to at lower frequencies, ranging from 0 to 18%, in other tumours [24].

In mCRC, the *BRAF* mutation (nearly always V600E) occurs in approximately 10% of cases with recent estimates ranging from 5% to 21% [33].

In a report examining 2530 patients with mCRC included in three randomized trials (COIN, FOCUS, and PICCOLO), the prevalence of *BRAF* mutations was 9.1% [33, 35]. In a population-based study that could better reflect the true incidence, 12% of the patients had *BRAF*V600E mutant tumors [33, 36]. In another population based report the percentage of *BRAF*-mutant tumors reaches 20% [33, 37]. *BRAF* and *RAS* mutations are generally mutually exclusive. When both mutations are found in the same tumor, they can be traced to different clones, each with a single mutation. These cases are rarely observed: only eight cases on 2530 patients (0.3%) from the three randomized COIN, PICCOLO and FOCUS trials and 0.01% of cases in another series [33, 38]. There are more *BRAF* mutations in right-sided CRC than in left-sided tumors. The SPECTAcolor trial revealed that the percentage of *BRAF*-mutant tumors was 10.5% in the total population of 370 patients, and was 22.6% in patients with right-sided colon cancer *versus* only 5.1% in patients left-sided colon cancer [33, 38]. In a large pooled biomarker analysis evaluating the role of biological markers in defining the prognosis of stage II and III colon cancer beyond TNM classification, a stepwise decrease in the prevalence of *KRAS* or *BRAF* V600E mutations was observed when moving from right-sided to left-sided colon cancer. *BRAF* mutations (and *KRAS*) were approximately twice as likely to be found in the caecum than in the sigmoid colon [33, 39].

3. Clinicopathological profile of the *BRAF*-CRC subtype

BRAF mutations define a particular subtype of CRC associated with morphological, clinical and therapeutic features that differ substantially from patients not carrying this genetic alteration.

BRAF V600E-mCRCs are strongly associated with female sex, older age, mucinous histology, poor differentiation, right-sided onset, T4 stage, microsatellite instability, serrated adenomas pathway and DNA hypermethylation (CIMP phenotype) [2]. *BRAF*-CRC patients often have a poor performance status (PS) and multiple metastatic sites at diagnosis with higher rate of nodal and peritoneal metastases and a lower rate of lung involvement [40, 41]

Furthermore, there is an association between heavy smoking (current or former smokers) and the presence of the *BRAF* mutation that can be also related to the CIMP phenotype. Although the

exact mechanism remains unknown, preclinical studies have shown that tobacco exposure can stimulate the DNA methyltransferase activity that is associated with CIMP [42].

Compared to *BRAF* wt patients, *BRAF*-CRCs are characterized by a worse prognosis and resistance to standard therapies, with a median OS (mOS) less than 12 months [43]

The patterns of *BRAF*-CRC have been shown to be so specific that a nomogram predictive for *BRAF* V600E status was developed and published by Loupakis et al [43]. In this study, a predictive score was assigned to each of the following variables: the primary site of the tumor, the patient's sex, and the mucinous characteristics of the cancer. The sum of the scores was converted to the probability of *BRAF* mutation occurrence and was 81% in female patients with mucinous-type right-sided colon cancer. The probability to carry a *BRAF*-mutated tumour ranged from 4% to 81% with a predictive accuracy > 80% and with high sensitivity and specificity (81.2% and 72.1%, respectively). In particular, a *RAS*-wt mCRC, not mucinous and originated from a left-sided primary occurring in male patients have an extremely poor likelihood to be *BRAF* mutant (4%). In the era of molecular characterization, the present nomogram should not be considered a tool to replace the mutational analysis of CRC, but it could allow physicians to better estimate patients' prognosis where *BRAF* testing is not available or reimbursed because of regulatory restrictions. Furthermore, the poor prognosis of *BRAF*-CRC patients makes their earliest identification essential to enable enrolment in clinical trials. This nomogram may also potentially guide for prospective stratification of future randomized trials thus avoiding costly and time-consuming upfront testing procedures. In addition, the identification of subgroups where *BRAF* mutation is very likely to occur would theoretically help to decrease the attrition bias of retrospective studies when tumour blocks are no longer available or difficult to retrieve [43].

4. Heterogeneity of the *BRAF* CRC subtype

Although *BRAF*-CRCs are typically considered an unique clinical entity, a significant biological heterogeneity has been described in literature.

4.1 Different molecular patterns: BM1 and BM2.

CRCs with *BRAF* V600E mutation segregate into two different gene expression subtypes with distinct molecular features and different potential therapeutic targets [2, 44] Barras et al. have recently described in a series of 218 *BRAF*-CRCs patients, two distinct groups based on their

expression profiles regardless of gender, primary tumour location, mismatch repair (MMR) status and PI3KCA status [44].

The BM1 subtype, representing one-third of patients, is associated with strong activation of KRAS/mTOR/AKT/4EBP1 signalling, macrophage infiltration, epithelial–mesenchymal transition (EMT) and poor survival. Overall, BM1 shows a strong immune profile characterized by activation of pathways such as IL2/STAT3, TNF alfa signalling via NF-KB, IL6/JAK/STAT3 and high score apoptosis signatures (for example protein BH3, the only pro-apoptotic BIM protein). Moreover, BM1 presents a higher inflammatory response correlated with the different expression of protein like SYK that transmits signals in B and T cells and STAT5, whose expression correlates with immune activation [2, 44].

The BM2 subtype, representing the remaining two-thirds of *BRAF*-mCRC patients, displays deregulation of the cell cycle with high levels of cyclin-dependent kinase (CDK)1 and low cyclin D1. This group is associated with an improved prognosis and MSI. Moreover, the mTORC1 signature (complex activation can lead to 4EBP1 and S6K pathway activation) is mostly detected in BM2, which confirms a major involvement in the metabolic process [2, 44].

4.2 Frequency and spectrum of *BRAF* mutations

Little is known about non-V600E *BRAF* mutations and their biological and clinical impact. These mutations identify a rare and unexplored molecular subtype of mCRC with clinical and pathological features very different from *BRAF* V600E CRC [21].

A study of almost 10,000 mCRCs sequenced with MSK-IMPACT showed that non-V600E *BRAF* mutations occurred in 2.2% of cases, the vast majority being found in MSS cancers. Interestingly, these non-V600 mutant cancers are more frequently in younger male patients, are low-grade left-sided tumors. Additionally, non-V600 mutant cancers were associated with more favourable overall survival rates compared to both *BRAF* V600 mutant and wild-type cancers [5, 45].

Recently, mutations in 594 or 596 codons have been found in <1% of mCRC. Cremolini et al. showed that patients with CRCs mutated in these two codons (n = 10) exhibited better overall survival (OS) than patients with *BRAF* V600E CRC (n = 77) (mOS 62.0 vs 12.6 months; HR= 0.36; 95% CI, p= 0.002) and even better than those of *BRAF* wild- type tumors. Moreover, compared to V600E, the 594 or 596 mutations were more frequently detected in the rectum, showing a non-mucinous histology, no peritoneal dissemination and were not associated with MSI [46].

Similarly, Jones et al. [47] confirmed these results on a larger cohort of 1014 patients with mCRC where non-V600E variants were observed in 29 of 137 (21.2%) with mainly BRAF codon 594 mutations. These data have been confirmed by more recent studies [5, 48].

Three classes of *BRAF* mutations have been described according to their functional effect and are grouped in activating RAS-independent signaling as monomers (class 1–V600E) or as dimers (class 2–codons 597/601) and RAS-dependent with impaired kinase activity (class 3–codons 594/596) [45, 50].

From a therapeutical point of view, *BRAF* inhibitors, such as vemurafenib and dabrafenib, effectively inhibit only mutant monomers but not dimers, because their binding to one site in the dimer significantly reduces their affinity for the second site [50]. So, tumors with non-*BRAF*V600E mutations are insensitive to these drugs as well as the increased expression of *BRAF* V600E dimers causes acquired resistance [50]. Next-generation *BRAF* inhibitor compounds are aimed at overcoming the problems associated with dimerization and in preclinical models have been shown to bind to class 1 monomers, dimers in class 2 mutants, as well as all RAF isoforms. In contrast to classes 1 and 2, class 3 BRAF mutants concurrently express high levels of phosphorylated EGFR which made them susceptible to EGFR antibody treatment [45].

The class 1 *BRAF* V600E mutation and also class 2 mutations allow for constitutive activation of the pathway, therefore a concurrent KRAS mutation is redundant. Indeed they are mutually exclusive in a single cancer. Interestingly, the V600E mutation conferred more elevated levels of phosphorylated MEK compared to KRAS mutations, which suggests the potency of this mutation in upregulation of the MAPK pathway. In contrast, class 3 *BRAF* mutations are dependent on activated KRAS and can coexist and even synergize with KRAS mutation [45].

As regards the clinical behaviour and outcome several trials demonstrated that class 3 was associated with better OS than classes 1 and 2 [2].

Schirripa et al. demonstrated a correlation between this classification and the clinical behavior of distinct subtypes. Indeed, class 3 subtypes were more frequently left-sided, node-negative, with no peritoneal metastasis compared to class 1, whereas class 2 was similar to class 1. About overall survival, the mOS was 21, 23.4, 44.5 and 42.2 months for *BRAF* patients with class mutations 1, 2, 3 and *BRAF*-WT ($p < 0.0001$), respectively [2, 50].

These data confirmed the findings reported by Jones et al: they showed important differences in terms of prognosis between *BRAF* V600 and non-V600 CRCs (> 50% class 3), with a substantially longer mOS of 60.7 months in *BRAF* non-V600E-mutated patients compared to 11.4 months in

BRAF V600E-mutated, but also compared to the 43.0 months of *BRAF*-WT population, emphasizing the less aggressive behavior of the *BRAF* non-V600E-mutated CRCs [2, 5].

In conclusion, although relatively rare, the presence of non-V600E mutations in colorectal cancer has important implications for clinical management with the choice of therapy and indicates that more gene-wide mutation screening regimes are warranted.

4.3 MSI/*BRAF*-CRC and MSS/*BRAF*: two different sites of the same coin?

BRAF p.V600E mutation has been found in ~ 60% of CRC cases with microsatellite instability (MSI) and in 5% - 10% of microsatellite stable (MSS) CRC [6, 7].

MSI/*BRAF*-CRC represents the most well-characterized subgroup associated with specific clinicopathological and molecular features [45]. The sporadic MSI tumor phenotype is mainly caused by biallelic hypermethylation of the *MLH1* promoter, resulting in silencing of the gene's expression. Because the *BRAF* p.V600E mutation has been demonstrated to cause the epigenetic silencing of the MLH1 promoter, this mutation and/or MLH1 methylation analysis have an established clinical utility to recognize sporadic CRC, excluding Lynch tumors [8, 51]. From a clinico-pathological point of view, MSI in CRC has been associated with proximal location, poor differentiation, mucinous or signet ring cell differentiation and high degree of tumor-infiltrating lymphocites (TILs) as immune response to the high rate of truncated proteins formed as a result of multiple frameshift mutations induced by inactivated MMR [3, 45].

MSI is commonly observed among stage II (20%) and III (12%) whereas is rare in stage IV CRC (4%) [9]. While in early stages MSI is positive prognostic factor, in metastatic setting the prognostic role of MSI remains unclear. At present, numerous evidences suggest that the prognostic benefit conferred by MSI, linked to an activated immune profile, is lost in the late-stages [10, 11].

Moreover, it has been hypothesized that detrimental prognosis seen in late-stage MSI cancers is driven by the presence of the *BRAF* mutation [45]. In this subgroup, there is a positive correlation among *BRAF* mutation, MSI and the presence of CpG island methylator (CIMP) phenotype [42].

The remaining *BRAF*-CRC exhibiting no MLH1 methylation are microsatellite stable (MSS) [45]. Compared to MSI/*BRAF* tumors, MSS/*BRAF* subset represents a distinct entity that shares clinical and molecular features with both MSI/*BRAF* CRCs of the serrated pathway and *BRAF* wild-type CRCs of the conventional pathway [15, 16, 17, 45].

Like MSI/*BRAF* tumors, MSS/*BRAF*-CRCs frequently occur in the proximal colon and are often mucinous and poorly differentiated. However, they do show more adverse morphological features

associated with aggressive phenotype such as frequent tumour budding, lack of TILs, frequent lymphatic, perineural, and venous invasion and increased lymph node metastases, compared to *BRAF* mutant/MSI and *BRAF* wildtype cancers. Finally, MSS/*BRAF*-CRCs are associated with worse outcome than the other two subsets of CRCs [15, 16, 17, 45]

In CRC, immunohistochemical markers such as CDX2, CK20, and CK7 are frequently used to ascertain the colorectal origin of metastases. As MSS/*BRAF*-CRCs frequently metastasize, the expression of these markers is important for an accurate diagnosis of the primary tumour site. Reduced levels of CDX2 staining, which were correlated with epigenetic silencing in MSI and CIMP tumors [45, 52, 53] were also found in MSS/*BRAF* CRCs. CK20 expression is commonly found in both MSS/*BRAF* and in *BRAF*-wt tumors, but is lost in MSI/*BRAF* cancers [52, 53]. CK7 is minimally present in CRC but more frequently upregulated in MSS/*BRAF* group compared to the other cancer subtypes. Interestingly, CK7 has been found in tissue areas of tumour budding and is a morphological feature commonly associated with tumor aggressiveness [45].

MSS/*BRAF*-CRCs have multiple genetic aberrations that are representative of typical changes associated with both serrated and conventional pathways [45]. As all BRAF CRC, also this subset is characterized gene hypermethylation (CIMP), although this epigenetic feature is more frequently observed in MSI/BRAF-CRCs than in MSS/*BRAF*-CRCs (70–80% *versus* 60% of cases, respectively) [15, 17, 45]. *TP53* mutation, typically associated with advanced stage, has been correlated with conventional pathway cancers and found uncommon in MSI cancers. MSS/*BRAF*-CRCs of the serrated pathway have been found to have a comparably high rate of *TP53* mutation as observed in *BRAF* wt CRCs. This finding provides evidence of a molecular overlap between MSS/*BRAF* and *BRAF* wt CRCs. [45, 54]. Moreover, MSS/*BRAF*-CRCs have been found to have a comparably high rate of chromosomal instability (CIN) as observed in *BRAF* wt CRCs [16, 45].

4.4 *BRAF* CRCs subtype in the Consensus Molecular Subtype (CMS) classification

From a comprehensive framework using aggregated gene expression data from multiple datasets, Guinney et al. studied almost 2000 colorectal cancers with known *BRAF* mutational status (about 10% of all cancers analysed) and identified four consensus molecular subtypes (CMSs) [55].

The majority of the *BRAF* mutant cancers (about 70% of cases) grouped into CMS1, also known as “MSI immune” subset, which was enriched with cancers positive for MSI, gene hypermethylation and activated immune pathways [55]. From a clinical point of view, CMS1 CRCs are more common

in females and in the right colon location, and they are characterized by higher histopathological grade [55, 56].

In the metastatic setting, considering the strong immunogenicity of these tumors, the use of checkpoint inhibitors is a novel therapeutic approach in MSI CRC. Indeed nivolumab (anti PD1), pembrolizumab (anti PD1) and the combination of nivolumab and ipilimumab (anti CTLA4) have been approved for this subset of patients. A retrospective analysis of the Alliance for Clinical Trials in Oncology suggests that patients with CMS1 tumors may have more benefit from bevacizumab (anti-VEGF) treatment, when compared to anti-EGFR targeted agents, such as cetuximab [56, 57].

The next highest proportion of *BRAF* mutant cancers (about 17% of BRAF mutant CRCs) fell into CMS4 subset, as known as "mesenchymal". These tumors are mainly MSS cancers, show upregulation of genes involved in epithelial-to-mesenchymal transition (EMT) and are associated with worse survival rates [45, 55]. In terms of survival, CMS4 metastatic CRCs have the worst 5-year OS and PFS, when compared to the remaining subtypes. A recent analysis of the FIRE3 trial showed that, in contrast with CMS1, patients with CMS4 CRC possibly benefit more from adding cetuximab, instead of bevacizumab to first line treatment with FOLFIRI (5-fluorouracil + irinotecan) [56, 58]. Few cases of *BRAF* CRCs were in CMS3 group and no case was observed in CMS2 subset.

In Table 1 the main genetic and epigenetic features observed in the four different CMSs are reported.

Table 1. Consensus Molecular Subtype Classification.

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

Legend: *CIMP* CpG island methylator phenotype, *-H high*, *-L* low, *MSI* microsatellite instability, *MSS* microsatellite stability, *CIN* chromosomal instability.

Colorectal Cancer Subtype – Target therapy of Colorectal cancer subtype; cap.1 Colorectal Cancer Subtype- Current Portrait; P.Jordan. Springer.

5. Molecular mechanisms

5.1 The central role of *BRAF* mutation and CIMP phenotype in the serrated pathway.

The "serrated neoplastic pathway" describes the progression of serrated polyps to colorectal cancer, representing an alternative multistep mechanism of carcinogenesis in addition to the conventional adenoma-carcinoma model [42].

Approximately, 15 to 30% of all sporadic CRCs arise from neoplastic serrated polyps, including sessile serrated adenomas (SSA) and traditional serrated adenomas (TSA), histologically characterized by a "serrated" (or saw-toothed) appearance of the epithelial glandular crypts within the precursor polyps [42]. In CRCs arising from serrated lesions, *BRAF* mutation is an early event as indicated by its presence in early neoplastic serrated lesions. Indeed it has been found in the vast majority of SSA and TSA but almost never (0.4 – 5%) in conventional adenoma [42, 59].

At present two main molecular mechanisms have been described in the serrated pathway, namely the "sessile" and the "traditional" sequences. The sessile serrated pathway begins in the proximal colon with a *BRAF* activating mutation that alters the MAPK-ERK pathway inducing apoptosis arrest, cellular proliferation and up-regulation of *p16* and *IGFBP7* genes. p16 (*CDKN2A* gene) and *IGFBP7* are important tumor suppressor genes. *CDKN2A* encodes for the p16 protein that negatively regulates the p16/cyclin-dependent kinase/retinoblastoma gene pathway involved in the cell cycle control, while IGFBP7 is a direct target and mediator of p53-dependent growth suppression [42, 59]. Up-regulation of *p16* and *IGFBP7* genes cause the transformation of the normal mucosa into hyperplastic polyps (HP) followed by cell senescence. HP lesions can stay dormant for a long time due to senescence which inhibits the tumorigenic progress, until an aberrant methylation of the *p16* and *IGFBP7* promoters subverts this protective mechanism and leads to evolution towards SSA [42, 60 ,61]. In the vast majority of cases, the next step of progression toward serrated adenocarcinoma from SSA may be related to *MLH1* aberrant methylation causing MSI. More rarely, it may occur epigenetic silencing of other targets, rather than *MLH1*, such as MGMT, leading to serrated adenocarcinoma without MMR deficiency [42, 62, 63]. Compared to SSA and sessile sequences, the traditional serrated (TSA) pathway is less characterized and more controversial. TSA lesions exhibit gene hypermethylation at variable levels. Sometimes they can be characterized by *BRAF* or KRAS mutations, but rarely show *MLH1* hypermethylation [42, 62]. A recent study of 200 TSAs found that all lesions retained MLH1 expression, leading to MSS phenotype. In addition, this study found residual presence of an SSA in approximately 30% of TSAs which suggests SSAs without *MLH1* methylation could progress via a

TSA to MSS/*BRAF* CRCs [45, 64]. Cancers arising through the traditional serrated pathway are predominantly localized in the distal colon and rectum [42]. TSAs can progress toward dysplasia via two different molecular pathways [42, 65]. Usually, TSA lesions which harbor *KRAS* mutations evolve toward conventional adenomatous dysplasia, while TSAs, which display *BRAF* mutations progress toward serrated dysplasia. Moreover, both dysplastic lesions can advance toward high grade dysplasia due to aberrant gene methylation. Finally, progression toward invasive carcinoma is driven by *TP53* alterations [42, 65]. Thus, the serrated carcinomas that originate from traditional serrated adenomas generally are MSS or MSI-L and those that originate from a sessile serrated adenoma are MSI-H [42].

5.2 CIMP phenotype and clinical implications

The *BRAF* mutation is strongly associated with the CIMP phenotype. DNA methylation is one of the epigenetic mechanisms that regulate gene expression. It consists of an enzymatic process that adds a methyl group (CH3) to the 5-position of cytosine by DNA methyltransferases (DNMT) to produce 5-methylcytosine. When a CpG site is methylated within the promoter region of a gene its transcription is inhibited, whereas methylation that happens in CpG sites outside of promoter site, called gene body methylation, may cause transcriptional activation [66, 67]. It is known that a hallmark of the human cancer genome is both the hypermethylation of specific genes involved in the control of cell growth that are transcriptionally silenced, and global DNA hypomethylation. CIMP represents a distinct phenotype in CRC, and can be graded as low (CIMP-L), high (CIMP-H) or negative (CIMP-0), depending on the degree of simultaneous hypermethylation of different promoter regions of tumor suppressor genes [68, 69]. Each single group is characterized by specific clinico- pathological and molecular features (Table 2) [42].

Table 2. Characteristics of CpG islad methylator phenotype in CRC

CIMP Phenotype	CIMP-0	CIMP-L	CIMP-H
Location	Distal colon	Proximal colon	Proximal colon
Gender	no gender bias	Male	Female
Pathway	Conventional adenoma	Serrated or Conventional	Serrated adenoma
Gene mutations	*TP53* mutations	*KRAS, TP53* mutations	*BRAF* mutations
Epigenetic alterations	no *MLH1* methylation	no *MLH1* methylation	*MLH1* hypermethylation
MSI rate	MSS	MSS	MSI
CIN association	positive	positive	negative
Prognosis	Variable	High	Poor

De Palma. The Molecular Hallmarks of the Serrated Pathway in Colorectal Cancer. Cancers; 2019.

CIMP-H, which is preferentially localized in the proximal colon, occurs more frequently in females and in older age, and shows the poorest prognosis compared to CIMP-L. At the molecular level, CIMP-H is often associated with BRAF mutation, MSI and inactivation of WNT/β-catenin pathway, Rarely these tumors exhibit *TP53* mutations. In comparison, CIMP-L is associated with KRAS mutations while CIMP-0 is characterized by frequent *TP53* mutations [42, 70, 71].

CIMP classification has been revised many times during the last two decades and many gen panels have been proposed to recognize CIMP-H, CIMP-L and CIMP-0 groups. Currently, there are two main gene panels frequently used to define CIMP. The first panel was described by Toyota and includes these five genes: *p16, hMLH1, MINT1, MINT2, and MINT31*. The second panel has been described by Weisenberger and comprises these five genes: *CACNA1G, IGF2, NEUROG1, RUNX3, and SOCS1* [42, 72, 73].

FANG et al have identified a specific pathway that mediates CIMP in *BRAF*-CRCs: *BRAF* V600E mutation stimulates ERK activity and subsequent phosphorylation of MAFG, which recruits a corepressor complex, including BACH1, CHD8 and DNMT3B, that binds to MLH1 promoter causing hypermethylation and silencing of the gene transcription. This mechanism explains why *MLH1* and other tumor suppressor genes are repressed in CRCs *BRAF*V600E mutant, but not in normal cells. This study has provided for the first time that *BRAF* V600E mutation has a direct causal role to determine gene hypermethylation [74].

From a clinical point of view, CIMP tumors are heterogeneous. CIMP-L CRCs are associated with male gender, while CIMP-H tumors show a female preponderance and tend to occur at a later age and are associated with cigarette smoking. The prognostic value of CIMP remains controversial especially for metastatic CRCs, perhaps because of their high molecular heterogeneity. In fact, although CIMP is mostly reported as a negative prognostic factor in CRC patients, several studies demonstrated that its prognostic value can be influenced by *BRAF*/KRAS and MSI status. Among them, CIMP-H, MSS, *BRAF* mutated tumors show the worst prognosis of all CRCs. The role of CIMP in tumor response to therapy is also controversial. Nevertheless, CIMP-H stage III CRC patients can benefit from adjuvant irinotecan plus 5-fluorouracil chemotherapy [42, 75].

6. Clinical impact of *BRAF* mutation in CRC

6.1 Prognostic role

Several clinical studies have been conducted aiming at defining the role of *BRAF* mutations as a potential prognostic biomarker for both localized and metastatic CRC patients. Current available data derive mainly from patients presenting *BRAF* V600E mutations, being the most common variant [2].

In metastatic setting all the published series recognised that *BRAF* V600E mutation is a strong negative prognostic factor, associated with a poor life expectancy of less 12 months [2, 76.]

In the pooled analysis of the phase III clinical trials CAIRO, CAIRO-2, COIN, and FOCUS, by Venderbosh et al, *BRAF* V600E mutation had a negative impact on both PFS and OS (mPFS 6.2 vs 7.7 months, HR = 1.34, 95% CI, P<0.001; mOS = 11.4 vs. 17.2 months, HR = 1.91, 95% CI, P<0.001). These patients showed a rapidly progressive multisite disease with a lower chance to receive a second or subsequent line of treatment, compared with *BRAF*-wild-type CRC [41, 77].

In the prognostic analysis of the MRC FOCUS trial, by Richman et al, *BRAF* mutation alone had a relevant impact on OS (HR 1.82; 95% CI; p < 0.0001) but there was no evidence of its effect on PFS (HR 1.14; 95%; p = 0.37) [76].

Innocenti et al. reported the aggressive behaviour of *BRAF* V600E tumors with a shorter OS than patients with *BRAF* wt (OS= 13.5 months vs. 30.6 months, respectively; HR, 2.01 [95% CI, *p* < .001) [78].

The pooled analyses of five randomized trials (FIRE-1, FIRE-3, AIOKRK0207, AIOKRK0604, RO91) by Modest et al, analyzing a total of 1239 patients, showed that *BRAF* mutation was associated with inferior PFS (multivariate HR: 2.19, P < 0.001) and OS (multivariate HR: 2.99, P < 0.001) compared with patients with non-mutated tumors [79].

Based on analyses from three large phase III randomized trials (COIN, FOCUS, PICCOLO), Seligmann et al explored the poor outcome of 231 patients (of 2530 total) with *BRAF* mCRC mutant. The study showed that the poor survival of *BRAF*-mutated patients is driven by accelerated decline following progression and a lower probability of receiving further lines of therapy. *BRAF* mutation confers a markedly worse prognosis regardless of associated clinicopathological features.

Moreover, compared to *BRAF* wt, the *BRAF* V00E mutated population exhibited higher rates of peritoneal metastasis (22 *versus* 14%, p = 0.003) [80].

These findings were in line with results from study of Tran et al which, analysing 57 patients *BRAF* mutated CRC, showed a distinct pattern of metastatic spread compared to *BRAF* wt CRC, namely higher rates of peritoneal metastases (46% v 24%, p=0.001), distant lymph node metastases (53% v 38%, p=0.008) and lower rates of lung metastases (35% v 49%, p=0.049) and poorer survival (10.4 months vs 34.7 months, $P < .001$). Moreover, in survival analysis, in contrast to early-stage disease, MSI was associated with shorter survival in metastatic CRC, possibly related to its association with the *BRAF* mutation [81].

In the AIO KRK0207 trial, *BRAF* mutation was reported as the strongest unfavourable prognostic factor (HR 3.16; 95% CI 2.17–4.60; p < 0.0001) compared to RAS status and primary tumor location [82].

While the detrimental prognostic impact of the *BRAF*V600E mutation in metastatic CRC is well established, conflicting results have been reported in early-stage CRC [21].

Several *post hoc* analyses of phase 3 trials did not show any significant prognostic impact on disease-free survival (DFS) [83, 84, 85]. Moreover, in the updated 10-years survival and outcomes of the MOSAIC trial (Multicenter International Study of Oxaliplatin/Fluorouracil/Leucovorin in the Adjuvant Treatment of Colon Cancer) the *BRAF* mutation was not prognostic for OS (p = 0.965) [86].

However, in the pooled analysis of the NSABP C-07 and C-08 trials, *BRAF*V600E mutation was associated with worse OS. Interestingly, the authors reported HRs of 1.02 (95% CI, P = 0.86), 1.46 (95% CI, P = 0.0002), and 2.31 (95% CI, P= 0.0001) for DFS, OS, and survival after relapse respectively, suggesting that *BRAF*V600E mutation negatively impacts the prognosis of patients with early stage CRC through a major decrease of survival after relapse but without a clear effect on DFS [83, 86].

Similarly, Farina-Sarasqueta et al. showed that *BRAF* V600E mutation is an independent negative prognostic factor for OS in stage II–III CRCs (HR 0.45, 95% CI) but it does not seem to influence DFS These data suggest that patients with *BRAF* V600E-mutated CRC have a similar probability of relapse compared to *BRAF*-wt but a significantly shorter post-relapse survival [87].

These apparent discrepancies on the prognostic relevance of *BRAF* mutation in CRC early stage, may be explained by the interrelation between the *BRAF* V600E mutation and MMR deficiency as shown by retrospective analyses of several studies [21].

Taking into account the MMR status, in the *post hoc* analysis of the PETACC-3, EORTC 40993, SAKK 60-00 trials, by Roth, *BRAF*V600E mutation was significantly associated with worse OS in patients bearing MSS (HR 1.78, 95%CI, reference *BRAF* wild type) [88].

Taieb et al, in the PETACC-8 phase III trial, found that the *BRAF*V600E mutation influenced neither DFS nor OS in the overall population of stage III colon cancers, whereas a significant pejorative impact was observed specifically in the MSS subgroup on both DFS (HR = 1.74; 95% CI; p = 0.01) and OS (HR = 1.84; p = 0.046). Moreover, in the MSI-H subpopulation, the presence of *BRAF* V600E mutation was associated with longer DFS as compared to *BRAF*-WT patients (DFS: HR 0.23, 95% CI, p = 0.04) suggesting that MSI-H is a protective factor against *BRAF* mutation in early-stage CRC [89].

From intergroup trial CALGB 89803, by Ogino et al, 75 *BRAF*-mutated patients in stage III CRC experienced significantly worse overall survival (OS; log-rank P = 0.015; multivariate HR = 1.66; 95% CI) compared with 431 *BRAF* wild-type patients. Combining *BRAF* and MSI status, *BRAF*-mutated MSS tumor was an unfavourable subtype compared with *BRAF* wt/MSI tumors, while *BRAF*-mutated MSI tumor and *BRAF* wild-type MSS tumor were considerate intermediate subtypes [90].

Howewer, other studies reported no impact of *BRAF* mutation on MSI-H early-stage CRCs. Kalady et al study analysed 56 CRC patients with *BRAF* mutation of 475 totals (12%). The survival data were analysed for 322 patients with stage I to III disease: CRCs with *BRAF* mutation had decreased OS than those without mutation (p = 0.018), regardless of MSI (HR 1.79, CI 1.05-3.05, p = 0.03) [91].

In the study by Sinicrope et al, patients in stage III CRC with *BRAF* V600e mutations were associated with worse DFS (HR, 1.37; 95% CI, 1.08-1.70; P <0.009), irrespective of MMR status [92].

Other studies including patients with both early and advanced CRC (I – IV stages) have demonstrated the negative impact of *BRAF* V600E mutation on clinical outcome.

Samowitz et al showed that *BRAF* V600E mutation in MSS CRC was associated with a significantly poorer survival in patients with II-IV stages disease but has no effect on the excellent prognosis of MSI tumors (reduced OS, HR= 3.06, 95%CI) [93].

Eklof et al, demonstrated that CRCs with *BRAF* mutation in stage I-IV showed reduced cancer specific survival (HR= 2.00, 95% CI) compared to *BRAF* wt patients. In this study archival CRC tissue from two different cohorts from Northern Sweden, NSHDS and CRUMS, were analysed for *KRAS, BRAF, PIK3CA* mutation status and loss of PTEN expression. As it is known that all four genes are

involved in EGFR signalling pathway, Sartore-Bianchi suggested a Quadruple index to indicate as positive any tumour with at least one mutation in *KRAS, BRAF, PIK3CA* and/or loss of PTEN protein expression.

The authors found a shorter cancer-specific survival in patients with Quadruple index positive tumours in the NSHDS cohort, while this result as not statistically significant in the CRUMS cohort. When analysing each gene separately, only BRAF mutations had a significant prognostic value in the NSHDS cohort, especially in combination with MSS or CIMP-low. By contrast, only KRAS mutations indicated a significantly poorer prognosis in the CRUMS cohort, especially together with MSS or CIMP-negative tumours. Finally, PIK3CA and PTEN aberrations did not add any significant prognostic information [94].

Although these results did not support the use of the full Quadruple index they emphasized the prognostic value of *KRAS* and *BRAF* mutation status, indicating that the establishment of molecular subgroups of CRC, based on KRAS and *BRAF* mutation status, can supply important information, not only in prediction of the EGFR-treatment response but also in prediction of patient prognosis [94].

Within the Nurses' Health Study and Health Professionals Follow-up Study, Lochhed *et al* investigated survival in 1253 CRC patients, I-IV stages, of which 182 *BRAF* mutated. Compared with MSS/*BRAF*-wt group, the MSS/*BRAF*-mutant, MSI-H/*BRAF*-mutant, and MSI-high/*BRAF*-wt subtypes showed colorectal cancer-specific mortality hazard ratios of 1.60 (95%CI; P = .009), 0.48 (95% CI; P =0 .02), and 0.25 (95% CI; P < .001), respectively [95].

Pai et al analysed 243 patients with CRC of which 20 *BRAF* mutated. When adjusting for tumor stage, survival analysis demonstrated that patients with *BRAF*-mutated CRC had a significantly poor OS and DFS (HR 6.63, 95% CI; and 6.08, 95% CI, respectively) compared with patients with KRAS/*BRAF*-wt CRCs. No significant difference in OS or DFS was identified between patients with KRAS-mutated and KRAS/*BRAF* wt. These results demonstrated that *BRAF*-mutated proximal colon adenocarcinomas with proficient DNA mismatch repair have a dismal prognosis with an aggressive clinical course, often display mucinous differentiation, focal signet ring histology, and other adverse histologic features such as lymphatic and perineural invasion and high tumor budding [96].

Finally, in the meta-analyses of Safaee that included 26 CRC studies, *BRAF* mutation was found to increase the risk of mortality (HR= 2.25,95% CI) [97].

Table 3 summarizes the main clinical trials showing *BRAF* mutation as negative prognostic marker, (overall survival, disease free survival or cancer specific survival) especially in MSS tumors.

Table 3. *BRAF* mutation as prognostic factor in CRC.

BRAF mutated	Stage	impact on OS	Reference
3063 (250)	IV	reduced OS HR: 1.91 and PFS HR: 1.34 (reference BRAF wt)	Venderbosh et al
711 (56)	IV	reduced OS HR: 1.82 (reference BRAF wt)	Richman et al
843 (100)	IV	reduced OS HR: 2.01 (reference BRAF wt)	Innocenti et al
74 (664)	IV	reduced OS HR: 2.99 and PFS HR: 2.19 (reference BRAF wt)	Modest et al
231 (2530)	IV	worse OS in both 1st-line studies FOCUS HR = 1.55 ;	Seligmann
		COIN HR = 1.77; in PICCOLO: PFS HR = 1.06. (reference BRAF wt)	
57 (524)	IV	reduced OS (10.4 months vs 34.7 months, p < 0.001	Tran et al
	IV	reduced OS HR 3.16 (reference BRAF wt)	Becker et al AIO KRK0207
18 (243)	IV	reduced PFS HR: 2.39 (reference BRAF wt)	Peeters et al
490 (77)	II - III	no effect on DFS (HR= 1.0) and OS (HR= 1.2) (reference BRAF wt)	French et al
822 (10%)	II - III	no effect on DFS, HR= 1.07, 95% CI:0.66–1.73 (reference BRAF wt)	Mouradov et al
297 (59)	II-III	reduced OS HR: 0.45 and cancer-specific survival HR: 0.47	Farina-Sarasqueta et al
		(reference BRAF mut)	
1307 (103)	II-III	reduced OS HR: 1.78 (reference BRAF wt)	Roth et al
506 (75)	III	reduced OS HR: 1.66 (reference BRAF wt)	Ogino et al
475 (56)	I - III	reduced OS HR: 1.79 (reference BRAF wt)	Kalady et al
911 (87)	II - IV	MSS, age tumor site, CIMP adjusted, reduced OS, HR= 3.06	Samowitz et al
475 (56)	I - III	reduced OS HR: 1.79 (reference BRAF wt)	Kalady et al
196 (35)	I - IV	reduced cancer-specific survival, HR: 2.00 (reference BRAF wt)	Eklof et al
1253 (182)	I - IV	higher cancer-specific mortality in MSS HR: 1.60 (reference BRAF wt)	Lochhead et al
181 (20)	I - IV	MSS, stage adjusted,reduced OS HR: 6.63 and DSF HR: 6.08 (ref.BRAF wt)	Pai et al

A.Thiel and A.Ristimaki. Toward a molecular classification of colorectal cancer: the role of BRAF. Frontiers in Oncology; 2013.

6.1.1 Impact of BRAF mutation in post-metastasectomy settings.

The impact of the *BRAF* mutation remains negative also in post-metastasectomy settings.

The first study of surgery for patients with liver metastases secondary to *BRAF*-mutant tumors suggested that the risk of liver metastases is higher in these patients and that OS is poorer after liver resection of their metastases [33, 98].

Schirripa et al. confirmed these data analysing BRAF and KRAS mutation status in 309 CRCs of patients undergoing liver resection, demonstrating that cases with *BRAF*-mutant tumors (*n* = 12) had a shorter recurrence-free survival (5.7 months) compared with *RAS*-mutant patients (11.0 months; *n* = 160) and with *RAS*-wild-type cases (14.4 months; *n* = 137) [33, 99].

In the pooled analyses of clinical studies by GONO, Cremolini et al. reported that after response to FOLFOXIRI + bevacizumab, resected patients shared the same prognosis, with a median disease-free survival of 11–12 months irrespective of *BRAF* status. However, this retrospective study included only seven patients with *BRAF*-mutant tumors [100].

In the largest series published by the Mayo Clinic of 21 patients who underwent resection of liver metastases from *BRAF*-mutated CRC, the median PFS and median OS were longer than in the non metastasectomy cohort (13.6 and 29.1 months vs. 6.2 and 22.7, respectively), with one patient who remained relapse-free for more than two years. In multivariate analysis, metastasectomy remained significant for improved survival outcomes (HR 0.52; 95% CI; p = 0.02) [2, 101].

Recent cohorts have given slightly discordant results. In a French cohort, 66 patients underwent resection for *BRAF*-mutant liver metastases of mCRC. A case-matched comparison was made with 183 patients who underwent resection for *BRAF*-wild-type liver metastases of mCRC during the same period. The 1- and 3-year DFS rates were respectively 46% and 19% in *BRAF*-mutant and 55% and 28% in *BRAF*-wt patients (p = 0.430). However, the 1- and 3-year OS rates after surgery were 93% and 54% in *BRAF*-mutant and 96% and 83% in *BRAF*-wt patients (p = 0.004). The median survival after disease progression was shorter in patients with *BRAF*-mutant tumors [33, 102].

In a large US cohort including 1497 patients who had complete resection, 35 (2%) patients had *BRAF*-mutant tumors (71% had BRAF V600E mutation). Compared with patients with *BRAF*-wild-type tumors, patients with *BRAF*-mutant tumors were older and appeared to have more advanced disease in the liver (more major hepatectomies, for instance) but less extrahepatic disease. Median OS was 81 months for patients with *BRAF*-wild-type tumors and 40 months for patients with *BRAF*-mutant tumors ($p < 0.001$). Median recurrence-free survival was 22 and 10 months for patients with *BRAF*-wildtype and *BRAF*-mutant tumors, respectively ($p < 0.001$). However, long-term survival was possible; it was associated with node-negative primary tumors, CEA $\leqslant$ 200 µg/l, and a clinical risk score <4 [33, 103]. A multivariate analysis of a smaller cohort of 849 patients, including 43 (5%) patients with *BRAF*-mutant tumors, revealed that the presence of a *BRAF*-V600E mutation but not a non-*BRAF*V600E mutation was associated with significantly poorer prognosis OS [33, 104].

In conclusion, these data show that results of liver surgery are poorer in patients with *BRAF*-V600E tumors, although this is still the only hope of cure for these patients. Considering the aggressiveness associated to *BRAF* mutation, it can be stated that surgery must be done as soon as possible in the treatment of these patients [33].

6.2 Predictive role

6.2.1 Efficacy of response to chemotherapy and to anti-angiogenic agents

Current standard first-line chemotherapy for metastatic CRC patients involves the combination of a fluoropyrimidine and either oxaliplatin or irinotecan [105].

Regarding standard chemotherapy treatments, there is no association between the presence of *BRAF* mutations and the response to chemotherapy with oxaliplatin compared to irinotecan, as demonstrated by Richman in the MRC FOCUS trial (The Medical Research Council Fluorouracil, Oxaliplatin and Irinotecan: Use and Sequencing). This study compared treatment sequences with first-line fluorouracil (FU), FU plus irinotecan versus FU plus oxaliplatin in mCRC, demonstrating that mutation in *KRAS* or *BRAF* was an unfavourable prognostic factor for OS (HR= 1.40; 95% CI; p <0.0001) and had minimal impact on PFS (HR, 1.16; 95% CI; p = 0.05) but the mutation status had no impact on the choice of using irinotecan versus oxaliplatin on PFS or OS. Therefore, the *KRAS* and *BRAF* mutation was associated with poor prognosis but is not a predictive biomarker for either drug [76].

Similar results were found by trial of Morris et al, in which standard chemotherapy has been evaluated in a retrospective cohort of 127 *BRAF*-mt mCRC patients, and has shown very poor outcomes in terms of PFS for the first three lines of chemotherapy (median PFS of 6.3, 2.5 and 2.6 months, respectively). The choice of systemic therapy used (oxaliplatin-based or irinotecan-based regimen) did not significantly affect PFS in first-line treatment (6.4 versus 5.4 months, P = 0.99) [106].

About also adjuvant setting, from a chemotherapeutic point of view, there are not differences between regimens applied for *BRAF* mutated and wild type tumors. In fact, the MOSAIC trial, previously reported, showed a slight increase of OS with the FOLFOX4 (standard regimen containing oxaliplatin) compared with LV5FU2 (weakened regimen without oxaliplatin) in the *BRAF* V600E population. This result confirms that, in daily clinical practice, determination of the *BRAF*V600E mutation in stage II–III CRC does not impact the therapeutic decision; thus the

standard of care for stage III CRC remains 6 months of fluoropyrimidine-based chemotherapy combined with oxaliplatin, regardless of the *BRAF* mutational status [86].

Since approximately 60% of patients with metastatic CRC *BRAF* mutated will not receive subsequent chemotherapy and their prognosis remains very poor, they should really be treated more aggressively [107].

Intensified first-line strategies of chemotherapy, including triple combination of oxaliplatin, irinotecan and 5-fluorouracil (FOLFOXIRI), combination of doublet with the EGFR inhibitors (cetuximab, panitumumab) or triplet with anti VEGFs (bevacizumab, aflibercept) have been evaluated in several studies [2, 21, 105].

Based on results from two phase 2 trials [108, 109], showing manageable toxicity and high antitumor activity of this regimen, FOLFOXIRI has been compared to FOLFIRI in a phase 3 randomized trial as first-line treatment for mCRC regardless of their mutational status.

The Gruppo Oncologico Nord Ovest, by Falcone et al., reported improved outcomes with FOLFOXIRI in terms of response rates, PFS, and OS (median OS = 22.6 versus 16.7 months; HR = 0.70, 95% CI 0.50–0.96, P = 0.032) [110].

As regards association strategies with anti-VEGF agents, the FOLFOXIRI regimen has been evaluated in combination with bevacizumab in phase 2 trials, showing promising antitumor activity. In 2010, an exploratory post hoc analysis reported by Masi et al. found that the subgroup of patients with *BRAF*V600E tumors (n = 10) exhibited tremendous outcomes (median PFS = 12.8 months and median OS = 23.8 months). Since data on OS and PFS were very similar between patients with *BRAF* mutation and those with *BRAF* wt, the author suggests that this aggressive treatment could also lead to the loss of the negative prognostic impact of *BRAF* mutation [111].

In order to prospectively validate this therapeutic strategy, Loupakis et al. designed a phase 2 trial conceived to explore FOLFOXIRI plus bevacizumab specifically in *BRAF*-mCRC patients (n=15) showing interesting results (median OS 24.1 months, median PFS 9.2 months). In the pooled population of patients with *BRAF*V600E-mutated tumors from both phase 2 trials (n = 25), with a median follow-up of 40.4 months, the progression-free rate at 6 months was 84%, median PFS and OS were 11.8 and 24.1 months, respectively [112].

The TRIBE phase 3 trial randomized patients with metastatic CRC between FOLFIRI plus bevacizumab and FOLFOXIRI plus bevacizumab, with a preliminary analysis of the *BRAF*V600E mutation in the study protocol. In the overall population, FOLFOXIRI plus bevacizumab was statistically superior to FOLFIRI plus bevacizumab on PFS and OS. Interestingly, similar proportions

of patients (76%) in both groups received second-line chemotherapy. The *BRAF*V600E CRC subgroup (28 patients were identified of which 16 for FOLFOXIRI plus bevacizumab and 12 for FOLFIRI plus bevacizumab) experimented better PFS (7.5 versus 5.5 months, HR = 0.57, 95% CI) and OS (19.0 versus 10.7 months, HR = 0.54, 95% CI) with the quadruple combination. However, these results did not reach statistical significance, probably due to a lack of power considering the small sample size [113, 114].

These data led to the recommendation of FOLFOXIRI + bevacizumab as valid option for chemotherapy-naive patients with *BRAF*-mCRC, by the most recent guidelines [115].

However, it is important to emphasise that this treatment considered "standard of care" is based on the observation of a limited number of patients in three studies and even if the level of evidence remains weak, this strategy is well accepted because it offers an aggressive upfront treatment, including all major chemotherapeutic agents for mCRC and a targeted therapy, with a manageable toxicity profile, to treat patients with a particularly aggressive disease who are rarely able to receive a second-line treatment [105].

Recently, the TRIBE-2 study did not confirmed the advantage of FOLFOXIRI plus bevacizumab versus the doublet regimens plus bevacizumab in the BRAFV600E mutant mCRC patients [172]. This has been recently confirmed by a meta-analysis from the same group, demonstrating no benefit from FOLFOXIRI plus bevacizumab if compared to standard doublet cytotoxic combinations [173]. These data relight the debate on current clinical guidelines recommendation, making FOLFOXIRI plus bevacizumab no longer the treatment of choice in first line for BRAFV600E mutant mCRC patients [173, 178].

Although the rationale for the use of anti-VEGF is based on the fact that the MAPK signalling cascade can increase VEGF expression and *BRAF* mutation might also modulate tumour response to anti-angiogenic treatments, the impact of antiangiogenic drugs in *BRAF*-mt patients has not yet been clinically demonstrated [105].

In fact, the results of the previously reported studies demonstrate that the addition of oxaliplatin to FOLFIRI (FOLFIRINOX) plus bevacizumab seem to be beneficial over FOLFIRI plus bevacizumab. However, the added value of the bevacizumab has not been shown [105, 114].

Even if no randomised data evaluating the influence of adding bevacizumab to standard chemotherapy (i.e., FOLFIRI or FOLFOX) are available for patients with *BRAF*-mCRC, in *post-hoc analyses* of the AVF2107g and AGITG MAX trials the addition of bevacizumab to first-line IFL [bolus

irinotecan, fluorouracil and leucovorin (folinic acid)] or capecitabine has shown a numerical improvement in survival outcomes in patients with *BRAF*-mt mCRC [105, 116, 117].

Analogously, in the VELOUR trial the impact of the addition of aflibercept (a fusion protein that binds circulating VEGF-A, VEGF-B and placental growth factor) to FOLFIRI in second line treatment (progressed after oxaliplatin-based first-line chemotherapy) of mCRC was tested. In the survival analysis stratified by prognostic biomarkers, the *BRAF*-mutated subgroup had a relevant improvement in OS compared to the *BRAF*-wt population, even if data did not reach statistical significance (HR 0.49; 95%CI; p = 0.08) [118]. Similar results have been reported with another anti-angiogenic agent that targets VEGFR2, ramucirumab, in the RAISE trial using FOLFIRI in second-line treatment [119].

It is possible to conclude that although these post-hoc analyses of randomised trials suggest that anti-angiogenic agents might be of interest in *BRAF*-mCRC patients, prospective trials comparing an aggressive chemotherapy alone or in combination with an anti-angiogenic therapy are still awaited.

6.2.2 Efficacy of response to anti EGFR agents

Regarding to response to anti-EGFR agents, the predictive role of *BRAF*V600E mutation is still debated and controversial data have been published [2].

In first line treatment, the pooled analysis of CRYSTAL and OPUS randomised studies evaluating the addition of cetuximab to FOLFIRI or FOLFOX chemotherapy in KRAS-wt mCRC patients, has shown an improvement of ORR, PFS and OS in the subgroup of *BRAF*-mt mCRC patients, suggesting that the *BRAF* mutation does not confer resistance to anti-EGFR agents in this setting but represents only a marker of poor prognosis [120, 121].

Similarly, the addition of panitumumab to FOLFOX first-line chemotherapy was associated with a numerical improvement of efficacy outcomes in the KRAS-wt/*BRAF* mutated subgroup [122].

Recently, Geissler et al. presented results from the VOLFI trial (AIO-KRK0109), in which FOLFOXIRI was compared to FOLFOXIRI + anti-EGFR (panitumumab) in first-line setting. The combination with panitumumab resulted in a significantly higher ORR compared to FOLFOXIRI alone (ORR 85.7% vs. 60.6% p = 0.0096) while there was no difference in PFS between both arms. The best results were obtained in symptomatic patients and *BRAF*-mutated mCRC; an outstanding ORR of 71% in *BRAF*-mutated CRC vs 22% in *BRAF*-WT was observed, even though statistical significance was not reached, probably due to the small number of patients (n=16) [123].

Although anti-EGFR agents do not confer any benefit to pre-treated *BRAF*-mt mCRC patients, these results suggest that they might be of value in the first-line treatment of such patients, especially if the goal of the treatment is tumour shrinkage.

In second-line treatment, two studies have been evaluated the addition of anti-EGFR to FOLFIRI reporting the same results, with no clinical benefit to *BRAF*-mt mCRC patients. The PICCOLO trial even reported a deleterious effect, in terms of OS (HR, 1.84; 95% CI, p= 0.029) when adding panitumumab to irinotecan in patients with *BRAF* mutated tumours [124, 125].

Two meta-analyses have been performed on the results from phase 2 and 3 clinical trials using cetuximab or panitumumab alone or combined with chemotherapy in first-, second- or beyond-second-line treatment. In the first meta-analyses by Pietrantonio et al, the addition of anti-EGFR agents to standard treatment (chemotherapy or best supportive care) in the RAS wild-type/*BRAF*V600E subgroup did not significantly improve PFS (HR = 0.88; 95%CI; P = 0.33) and OS (HR = 0.91; 95% CI; P = 0.63), supporting the need for determination of the *BRAF* status before initiation of anti-EGFR agents [126]. Conversely, Rowland et al. performed another meta-analysis showing that there may be insufficient evidence to justify the exclusion of anti-EGFR therapies for patients with RAS wild-type/*BRAF*V600E-mutated mCRC. In this meta-analysis, HRs for PFS and OS which benefit with anti-EGFR therapies were 0.86 and 0.97 for RAS wt/*BRAF*V600E tumors and 0.81 and 0.62 for RAS wt/*BRAF* wt ones. Tests of interaction of PFS and OS HRs between the two populations were not statistically significant (P = 0.43 and P = 0.07, respectively), highlighting the fact that the observed differences of survival benefit with anti-EGFR agents according to *BRAF* mutational status may be due to chance alone. Thus, authors concluded that the *BRAF* mutation could not actually be considered as a negative predictive biomarker for anti- EGFR monoclonal antibodies in mCRC and that further data are required to clarify this observation [127].

However, both meta-analyses have many limitations and overall cannot definitively guide the clinical practice. First, not all available studies were included in these two meta-analyses; second, several lines of treatment with different populations were included; third, negative trials for anti-EGFR agents with irrelevant backbone chemotherapeutic regimens (such as capecitabine plus oxaliplatin) were comprised. Moreover, the control arms mixed various chemotherapy regimens or even best supportive care. Finally, both panitumumab and cetuximab trials were considered although they might give different results in *BRAF* patients. All these points are likely to present significant confounding factors when evaluating *BRAF*- mCRC patients [105].

Finally, the FIRE-3 trial compared FOLFIRI plus bevacizumab with FOLFIRI plus cetuximab in the first-line treatment of RAS wt mCRC patients. For the 48 (n = 14%) *BRAF* patients identified in this trial, the ORR was higher in the cetuximab arm than in the bevacizumab arm (52% versus 40%), while no statistical differences were observed for PFS (HR, 0.84, p = 0.56) and OS (HR, 0.79, p = 0.45) suggesting that EGFR and VEGF inhibitors have equivalent therapeutic efficacy in *BRAF*-mCRC patients, except for response rate that favours anti-EGFRs [128].

7. Targeting *BRAF* and resistance mechanisms to *BRAF* inhibitors

7.1 Why are single *BRAF* inhibitors not effective in *BRAF-CRC*?

The monotherapy with *BRAF* inhibitors (i*BRAF*), such as vemurafenib, dabrafenib, encorafenib, demonstrated an impressive antitumor activity in advanced melanoma with objective response rates around 50% and their clinical efficacy has been replicated in other selected cancers such as NSCLC, thyroid cancer, hairy cell leukemia [2].

Compared to the remarkable success gained in melanoma, i*BRAF* alone show insufficient clinical activity in mCRC with fewer than 10% of responders and PFS of 2.1 – 4.3 months [129, 130, 131].

Kopetz et al. led a phase II study evaluating vemurafenib in patients with previously treated *BRAF*-mCRC. Among the 21 patients enrolled, only one reached a partial response, while seven reported to have stable disease, with mOS and mPFS of 7.7 months and 2.1 months, respectively [129]. In a phase 1 trial, no objective response was observed in 18 patients treated with encorafenib, another highly selective *BRAF*V600E inhibitor [130].

Pre-clinical studies investigated the mechanism that explains the lower response to *BRAF* inhibitors of *BRAF*-CRC subtype, demonstrating that molecular landscape of the colon cancer is more complex and heterogeneous as compared to melanoma [2].

In CRC *BRAF* mutant, MAPK activity is driven by mutated *BRAF* and the RTK-mediated activation of RAS is restricted by ERK negative feedback signals. The treatment with *BRAF* inhibitors cause an initial decrease of MAPK signalling leading to a loss of expression of ERK negative feedback and an increase of RTK-mediated RAS activation with the recruitment of other RAF kinases, which produce RAF dimers (such as CRAF) restoring MAPK pathways signalling. Significantly, RTK signalling is present at a higher level in CRC than in melanoma, and one of them (EGFR) is mainly responsible for MAPK reactivation in *BRAF*-mutated mCRC. Thus, although i*BRAF* induces transient

impairment of MAPK pathway signalling, a rapid activation occurs through phosphorylation and activation of ERK by the EGFR-mediated activation of RAS and CRAF [2].

This phenomenon, in which i*BRAF* leads to reactivation of MAPK activity, is known as "paradoxical activation of the MAPK pathway" and represents the main primary resistance mechanism that explains the persistence of tumor proliferation despite i*BRAF* and consequently their lower efficacy in mCRC [2].

The MAPK pathway reactivation can also occur through several mechanisms such as KRAS mutation or amplification, *BRAF* amplification, or MEK mutation, which underlie the development of acquired resistance to RAF inhibitor combinations. Based on these mechanisms, in order to maintain the MAPK pathway impairment, combinations of targeted therapy, such as anti-EGFR, *BRAF* and MEK and/or chemotherapy have been explored by many clinical trials with the aim of overcoming resistances [2].

7.2 Strategies to overcome the resistance mechanisms to *BRAF* inhibitors and rationale for new treatment approaches

As reported above, inhibition of *BRAF* alone has limited activity in mCRC. For this purpose, in a phase II "basket" trial, the combination of vemurafenib and cetuximab was evaluated in 27 patients with *BRAF* V600E-mCRC. One patient had a partial response and 69% had stable disease with mOS and mPFS of 7.1 and 3.7 months, respectively [2, 131].

In another trial, the combination of vemurafenib and panitumumab was investigated in 15 pre-treated patients with *BRAF* V600E-mCRC. Two patients had a partial response and six had stable disease [2, 132].

Similarly, the combinations such as encorafenib and cetuximab, dabrafenib and panitumumab, have been evaluated in other trials with response rates ranging from 4% to 23% [2, 133, 134, 135].
Preclinical studies have suggested that combined inhibition of *BRAF* and MEK is more effective than *BRAF* inhibitors combined with anti- EGFR agents. This finding was validated clinically in subsequent phase 1 and phase 2 trials that combined *BRAF* inhibitors with both anti-EGFR monoclonal antibodies and MEK inhibitors [2].
Given the revolutionary impact of the combination of *BRAF* and MEK inhibitors in the clinical management of melanoma, Corcoran et al. demonstrated that targeting the MAPK pathway may

be an effective therapeutic strategy for *BRAF*V600E-CRC. Among 43 patients treated with dabrafenib and trametinib, 56% of cases showed stable disease and an ORR of 12% [2, 136].

Triplet drug combinations have also been evaluated to provide the most effective inhibition of the MAPK pathway.

In a trial by Corcoran et al., three cohorts were treated with dabrafenib plus panitumumab, dabrafenib plus trametinib plus panitumumab, and trametinib plus panitumumab, respectively. The authors showed an ORR for triplet therapy of 21%, better than doublet therapy, but with an increase of adverse events, in terms of diarrhea and skin toxicity (rash and dermatitis acneiform); mPFS was 4.1 months, while mOS reported was 9.1 months [2, 137].

The BEACON trial by Loupakis et al was designed to assess a combination of anti *BRAF*, MEK and EGFR (encorafenib plus binimetinib plus cetuximab, respectively). In this phase 3 trial, 665 patients with pre-treated *BRAF*V600E-mCRC were randomized to receive encorafenib, binimetinib, and cetuximab (triplet-therapy group) vs encorafenib and cetuximab (doublet-therapy group) vs a control group of investigator's choice of either cetuximab and irinotecan or cetuximab and FOLFIRI. The median OS was 9.0 months in the triplet-therapy group vs 8.4 months in the (HR for death vs. control, 0.60; 95% CI; P<0.001) and 5.4 months in the control group (HR for death, 0.52; 95%; P<0.001). The response rate was 26% in the triplet-therapy group, 20% doublet-therapy group and 2% in the control group (P<0.001). Median PFS was 4.3 months and 4.2 months for triplet and doublet arms, respectively. The authors concluded that a combination of encorafenib, cetuximab, and binimetinib resulted in significantly longer overall survival and a higher response rate than standard therapy in patients with mCRC with the *BRAF* V600E mutation [27]. Interestingly, this trial is the largest cohort ever studied and the first phase III trial to demonstrate a survival and response advantage in the setting of pre-treated *BRAF* mutated CRC.

An ongoing study (ANCHOR-CRC) is investigating the effects of the same triplet therapy as a first-line treatment for patients with *BRAF*-mutated CRC [2, 138].

Other combinations were tested, including chemotherapy. Based on preclinical data showing a great antitumor activity by using doublet or triplet therapy, a phase 1 trial, combining vemurafenib, cetuximab and irinotecan was developed. A total of 18 *BRAF*-mutated CRC patients were included, with an ORR of 35% and mPFS of 7.7 months. The following randomized phase II trial (SWOG 1406) combining irinotecan and cetuximab with or without vemurafenib included 106 patients. mPFS was 4.3 months in vemurafenib arm vs. 2.0 months of the control arm; in addition, response and disease control rates were higher in the vemurafenib arm [2].

Another phase 3 randomised trial designed to investigate FOLFOXIRI plus cetuximab or FOLFOXIRI plus bevacizumab as firstline treatment in *BRAF*-mt mCRC patients is currently underway, with a main objective of ORR (FIRE-4.5/AIO KRK-0116) [139].
Other pathways have been involve in resistance mechanisms, such as PI3K/AKT pathway, the Wnt pathway and the overexpression of RAC1B [2].

It has been shown that *BRAF*-mCRC cell lines have higher activation of several proteins of PI3K/AKT pathway compared to melanoma cell lines, and that mCRC cell lines with mutations in this pathway or loss of PTEN are more resistant to growth inhibition by *BRAF* inhibitors as compared to cell lines without these alterations. Thus, the combination of *BRAF* inhibition and PI3K inhibition seems attractive. A phase 1b trial has evaluated the therapeutic effect of encorafenib with cetuximab (doublet) ± alpelisib (an α-specific PI3K inhibitor) (triplet) in 28 refractory *BRAF*-mCRC patients. Best ORR and PFS were, respectively, 23.1% and 3.7 months (95% CI, 2.8–10.6) in the dual arm versus 32.1% and 4.3 months (95% CI, 4.1–5.4) in patients treated with the triplet, which seemed relatively well tolerated [140]. Tabernero et al. investigated the same triplet in a phase 2 trial, in which 102 patients with *BRAF*V600E-mCRC were randomized to receive encorafenib plus cetuximab (n = 50) or encorafenib plus cetuximab and alpelisib (n = 52). Results were encouraging with an overall response rate of 22 and 27% for the doublet and the triplet regimen, respectively. Median PFS was 4.2 and 5.4 months (HR = 0.69; 95%CI; P = 0.064). Major grade 3 and 4 adverse events were anemia (17 versus 6%), hyperglycemia (13 versus 2%), and increased lipase (8 versus 18%) for the triplet and doublet arms, respectively [134].

Other potential targets include the Wnt/beta-catenin pathway. The Wnt/β-catenin pathway, which is frequently dysregulated in non-MSI CRC, was also recently implicated in the mechanisms of *BRAF* V600E CRC resistance. Treatment with i*BRAF* was found to upregulate this pathway in preclinical models of *BRAF* V600E-mCRC, including cell line and patient-derived xenografts. Stimulation of the Wnt signalling occurred through activation of focal adhesion kinase (FAK) upon inhibitor treatment. Notably, FAK activation did not require EGFR or ERK1/2 activation, indicating that the observed hyperactivation of Wnt signalling was a MAPK pathway reactivation-independent event. Importantly, combined inhibition of *BRAF*/Wnt pathway or *BRAF*/FAK exerted strong synergistic antitumor effects, both in cell lines and mouse xenograft models. The results of the trial NCT02278133, which enrols patients with both *BRAF*-V600E and Wnt pathway mutations

and treated with the Wnt inhibitor WNT974 in combination with encorafenib and cetuximab, are expected [2, 140].

Finally, regarding the tumor microenvironment, persistent inflammatory cues were shown to promote the overexpression of tumor-related RAC1B GTPase. RAC1B overexpression facilities malignant progression by promoting evasion from *BRAF* V600E-induced senescence. Importantly, it was shown that one non-steroid anti-inflammatory drug, ibuprofen, specifically downregulated inflammation related RAC1B overexpression and led to reduced tumour growth in mouse xenografts of *BRAF* V600E CRC cell lines. This could represent another therapeutic opportunity for combination therapy in patients with the *BRAF* CRC subtype [141].

Emerging data confirm that tumor microenvironment can impact disease prognosis. It has been demonstrated that chronic inflammation with a high expression of COX2-PGE2 plays a fundamental role in the genesis of CRC as PGE2 suppress the anti-tumor effect deployed by the immune system [2].

BRAF mutation determines an upregulation of the RAF-MAPK pathway, which leads to downstream activation of PTGS2 (COX-2) and to the increase of PGE2 production; this leads to higher survival and faster replication of tumor cells. In a study carried out by Kosumi and colleagues, considering *BRAF*-mutated CRC, the subgroup with higher COX-2 expression presented lower disease-specific survival (DSS) (HR 2.44; 95% CI 1.39–4.28); the association between COX2 and worse survival did not reach statistical significance in the *BRAF*-WT population (HR 0.82; 95%CI). As an explanation of this mechanism, the authors proposed that PGE2 accumulation in the tumor microenvironment resulted in greater resistance to the local immune system [2].

In table 4 the main clinical trials with BRAF inhibitors in CRC BRAF mutant are reported.

Table 4.

Clinical trials with BRAF inhibitors in CRC *BRAF* mutant						
Therapeutic Strategy	**regimen**	**n° of patients**	**ORR**	**PFS**	**OS**	**reference**
Single BRAF inhibitor	vemurafenib	21	5%	2.1	7.7	Kopetz et al
	encorafenib	18	0%	4	\	gomez roca
Doublet combinations						
BRAF + EGFR inhibitors	vemurafenib + cetuximab	27	3.7 %	3.7	7.1	hyman
	vemurafenib + panitumumab	15	13%	3.2	7.6	yaeger
	dabrafenib + panitumumab	20	10	3.5	\	corcoran
	encorafenib + cetuximab	50	22	4.2	\	tabernero
BRAF + MEK inhibitors	dabrafenib + trametinib	43	12	3.5	\	corcoran
Triplet combinations						
BRAF + MEK + EGFR inhibitors	dabrafenib + trametinib + panitumumab	91	21	4.2	9.1	corcoran
BRAF + MEK + EGFR inhibitors	encorafenib + cetuximab ± binimetinib	224 (triplet); 220 (doublet)	26 ; 20	4.3 ; 20	9 ; 8.4	loupakis
BRAF + EGFR + PI3K inhibitors	encorafenib + cetuximab + alpelisib	52	27	5.4	15.2	tabernero
BRAF + EGFR inhibitors + irinotecano	vemurafenib + cetuximab + irinotecano	106	16	4.4	\	Kopetz et al
BRAF + EGFR inhibitors + Wnt inhibitors	encorafenib + cetuximab + WNT974	20	\	\	\	clinicaltrials.gov

RR response rate; mPFS median progression free survival; mOS median overall survival; m: months.

8. Immunotherapy: a new therapeutic promise for *BRAF*-mutated mCRC?

The *BRAF*V600E mutation is strongly associated with MSI and therefore with a high degree of infiltration by tumour-associated lymphocytes, mostly of activated CD8+ cytotoxic T lymphocytes. The cytotoxic T lymphocytes recognize the neo-antigens that are translated because of MSI and presented as peptides on the cell surface by major histocompatibility complex class I molecules (MHC-I). In order to evade an immune response, tumour cells express on their surface the inhibitory programmed cell death 1 ligand (PD-L1) that binds to the PD-1 co-receptor on T lymphocytes, an immune checkpoint, and supresses cytotoxic activity. Thus, although infiltrating T cells are abundantly detected in the *BRAF*/MSI subtype of tumours, their activity is downmodulated by tumour cells, which express PD-L1 in response to the inflammatory microenvironment as an adaptive immune resistance, suggesting the incremented expression of PD-L1 as the main escape mechanism. Blocking the interaction between PD1 and PD-L1 can reactivate cytotoxic T lymphocytes to attack cancer cells. For this reason immune checkpoint inhibition should be considered for CRC *BRAF* mutant/MSI.

Several trials demonstrated that MSI tumors have been shown to be much more sensitive to immunotherapy than proficient MMR tumors [142].

The CHECKMATE 142, phase II trial, evaluated nivolumab (anti PD1) with or without ipilimumab (anti CTLA-4). As expected, no antitumor efficacy was observed for MSS tumors (n = 20). Among patients with MSI mCRC (n = 100), 17 (17%) harboured *BRAF*V600E mutation. Seventy patients received nivolumab 3 mg/kg alone and 30 patients were treated with nivolumab 3 mg/kg plus ipilimumab 1 mg/kg. Objective response rate was 25.5 and 33.3%, respectively (median duration of response not reached in both arms). Median PFS was not reached in the combination arm and reached 5.3 months with nivolumab alone. Thus, nivolumab monotherapy, as well as the combination of nivolumab plus ipilimumab, demonstrated encouraging results with durable responses in patients with MSI mCRC. Interestingly, clinical activity was observed regardless of the *BRAF* and KRAS mutational status and PD-L1 expression [143].

In addition, the recent phase III trial KEYNOTE-177 demonstrated the superiority of pembrolizumab in first-line setting over standard regimens in MSI mCRC, independently from BRAF status [174]. According to these trials, CPIs seem to perform better than standard therapies in BRAFV600E mutant MSI mCRC [174]. The ongoing phase III trial CheckMate 8HW (NCT04008030) is evaluating the combination of nivolumab and ipilimumab in the same setting and it is expected to provide further data for this subset of patients. Summarizing, these studies

support the administration of a CPI as upfront treatment in BRAFV600E mutant MSI mCRC patients. Indeed, following KEYNOTE-177 data, both Food and Drug Administration (FDA) and European Medicines Agency (EMA) recently approved pembrolizumab in the first line setting for MSI mCRC, including those BRAFV600E mutant [175,176]. Today, pembrolizumab is the new standard of care for MSI mCRC harboring BRAFV600E mutation. If immunotherapy is contraindicated or not available, standard cytotoxic treatments remain an option.

9. Literature Review of Colorectal Rhabdoid Carcinoma (CRbC)

For comparison of clinicopathologic data, prognostic parameters, and genetic profiles with those of our tumor series, we performed a thorough review of the MEDLINE literature, for colorectal carcinomas reported as rhabdoid carcinoma, carcinoma with rhabdoid features, rhabdoid tumor, malignant rhabdoid tumor, pleomorphic carcinoma, giant cell carcinoma, and undifferentiated carcinoma. We report in Table 5 (see in appendix) all cases that we have critically reviewed and accepted as they demonstrated similar histology and immunophenotype as the cases reported in the present study. A careful review of the MEDLINE literature revealed 39 cases of CRbCs that should be added to our seven cases for a total of 46 cases (Table 5). The patients were 22 males and 24 females aged 23–87 years (mean age: 64 years; median 69 years). The neoplasms were located in order of frequency in the cecum (26%), sigmoid colon (17.4%), ascending colon and rectum (13% each), right colon unspecified (10.8%), transverse colon (8.7%), hepatic flexure, splenic flexure, and left colon unspecified (2.1% each). The size of the tumors was reported in 33 cases and ranged from 3–15 cm in maximum diameter (mean 7.3 cm, median 7 cm). Positive regional lymph nodes were found in 32/39 (82%) in which detailed data were available. Liver metastases were reported in 31% of the patients and peritoneal metastases in 10% of the cases. Of the 36 patients with available data, 34 received surgery as first or unique treatment and three (8%) of them received adjuvant therapy. The remaining patients underwent palliative treatment because of advanced disease. Fluoropyrimidine- and oxaliplatin-based chemotherapy were the regimens mostly used as first line therapy in a metastatic setting. Other chemotherapy regimens used comprehended irinotecan (FOLFIRI scheme) and anthracycline/platinum (EOX scheme). Only two patients received more than one line of chemotherapy in a metastatic setting and only one patient received monoclonal antibody (both antivascular endothelial growth factor (anti-VEGF) and anti–epidermal growth factor (anti-EGFR)) with no benefit. Follow-up data were available for 41 patients: 31 (75%) died of disease within 1–15 months (mean: 4.5 months, median 4 months).

Ten patients with follow-up ≥6 months (range 6–216 months, mean 59 months, median 29.5 months) were reported as still alive. In the histopathological reports, the rhabdoid cells were the prevalent cells in the majority of cases; however, in 45% of 35 cases an adenocarcinomatous component more frequently focal and at the tumor periphery and often poorly differentiated has been detected. The reported immunohistochemical findings proved in 36/36 cases co-expression of vimentin and pancytokeratin mainly localized to paranuclear cytoplasmic inclusions. Variable positivity for EMA has been found in 15/21 (71.4%) cases. Complete loss or focal loss of SMARCB1 (INI-1) was reported in 9/27 (33.5%) and 3/27 (11.1%) cases, respectively. Nuclear immunoreactivity for TP53 and β-catenin has been found in 12/34 (35.6%) and six of eight (75%) cases, respectively. E-cadherin immunoreactivity was negative in the four cases investigated. CK20 and CDX2 were not expressed in the majority of cases: 24/28 (85.7%) and 29/32 (90.6%), respectively. Actin, desmin, and S-100 were not expressed in all cases investigated (9, 10, and 6 respectively). The Ki-67 labeling index evaluated in 11 cases ranged from 30–90% (mean 57.6%). The number of tumor-associated T lymphocytes (TIL), either CD3 or CD8, reported for cases was registered as low.

As reported in Table 6, BRAF V600E mutation is the prominent molecular feature of CRbCs examined so far, occurring in 13/22 (60%) cases analyzed. By contrast, due to the mutual exclusivity of KRAS and class I BRAF mutations such as BRAF V600E, KRAS variants were observed at low frequency (2/17, 12%) and basically only in BRAF wild-type tumors. Concurrent mutations in both genes were very uncommon (1/17, 0.06% of cases).

Moreover, as in colorectal carcinomas, MSI was strongly associated with BRAF V600E mutation and was observed in seven out of eight BRAF mutant cases. However, although a significant positive association between these two markers is also confirmed in these rare tumors, the frequency of BRAF mutant/microsatellite stable (MSS) CRbC was unexpectedly high (6/13 cases; 46%) compared with the low incidence of this molecular subset among colorectal cancers (CRCs). Molecular features strongly associated with BRAF colorectal cancers such as a high frequency of CIMP and rare loss or mutation of the TP53 gene have been scarcely studied in CRbCs. Finally, recent next-generation target sequencing of SMARCB1 (INI-1) and CROCC genes highlighted point mutations of the CROCC gene in 4/10 (40%) cases and no SMARCB1 (INI-1) genetic alterations.

Table 6. Molecular data of colorectal rhabdoid carcinomas (CRbCs) previously reported.

Studies	MSI status	*BRAF*	*KRAS*	*PIK3CA*	*CROCC*	*INI-1§*	*TP53*	CIMP
Kono et al. 2007 [144]	MSS	-	WT	-	-	-	-	-
Samalavicus et al. 2013 [145]	MSS	V600E	WT	-	-	-	-	-
Lee et al. 2013 (case 1) [146]	MSS	WT	WT	-	WT	WT	-	-
Lee et al. 2013 (case 2) [146]	MSS	V600E	WT	-	WT	WT	-	-
Agaimy et al. 2014 [28]	**MSI**	**V600E**	-	-	-	NEG#	-	CIMP
Moussaly et al. 2015 [147]	MSI	-	-	-	-	-	-	-
Kalyan et al. 2015 [148]	MSS	WT	Q61K	WT	-	WT	R273H	-
Wang et al. 2016 (case 1) [149]	MSS	V600E	-	-	-	POS#	-	-
Wang et al. 2016 (case 2) [149]	**MSI**	**V600E**	-	-	-	NEG#	-	-
Wang et al. 2016 (case 3) [149]	MSS	V600E	-	-	-	POS#	-	-
Agaimy et al. 2016 [150]	MSS	-	-	-	-	POS#	-	-
Remo et al. 2018* [32]	MSS	WT	WT	-	-	-	-	-
Remo et al. 2018 * [32]	MSS	WT	WT	-	-	-	-	-
Remo et al. 2018* [32]	MSS	WT	MUT	-	-	POS#	-	-
Remo et al. 2018* [32]	**MSI**	**V600E**	-	-	-	POS#	-	-
Remo et al. 2018 (RC1) [32]	**MSI**	**V600E**	WT	-	A161S	WT	-	CIMP
Remo et al. 2018 (RC2) ** [32]	**MSI**	**V600E**	-	-	V1885A	WT	-	CIMP
Remo et al. 2018 (RC5) [32]	MSS	V600E	WT	-	WT	WT	-	-
Remo et al. 2018 (RC6) [32]	MSS	V600E	G12V	-	WT	WT	-	-
Remo et al. 2018 (RC7) [32]	**MSI**	**V600E**	WT	-	WT	WT	-	-
Remo et al. 2018 (RC8) [32]	MSS	WT	WT	-	WT	WT	-	-
Remo et al. 2018 (RC9) [32]	**MSI**	**V600E**	WT	-	S1320I	WT	-	-
Remo et al. 2018 (RC10) [32]	MSS	WT	WT	-	WT	WT	-	-
Remo et al. 2018 (RC11) [32]	**MSI**	**WT**	WT	-	A1510T	WT	-	-
Remo et al. 2018 (RC12) [32]	MSS	WT	WT	-	WT	WT	-	-

Legend: -: not available result; MSI presence of microsatellite instability; MSS: absence of microsatellite instability; CIMP: CpG island methylator phenotype; INI§: molecular or immunohistochemical results were indicated: POS or NEG correspond to INI-1 positive expression or negative expression, respectively. WT indicates absence of gene mutation; * CRb quoted by Remo et al. 2018 as personal communication by Sanchez P.A.; ** previously published by Pancione et al. 2011.

AIM

The work of my PhD training in Experimental and Translational Medicine has been carried out at the laboratory of Molecular Pathology of the Anatomic Pathology Unit of Varese Hospital and can be divided in two parts.

In the first part I performed a thorough review of the MEDLINE literature on the role of BRAF mutation in colorectal cancer, from a pathogenetic, molecular and clinical point of view (reported as advanced colorectal cancer, BRAF mutation, CD8 T-cell content, MSI, neuroendocrine differentiation). Moreover, we analysed a selected cohort of BRAFV600E-mCRC in order (1) to compare the clinical-pathological profile of the MSI group with respect to the MSS group, highlighting the most significant differences based on MMR status; (2) to analyse the extent of neuroendocrine differentiation and the types of neuroendocrine neoplasms between MSI and MSS tumors; and (3) to assess intratumoral CD8 + T cell density and tumor cell PD-L1 expression and their prognostic influence. The results obtained from this part of work have been published in the report [13] (1. in appendix section).

In the second part, since BRAF mutation has been reported as a common feature of CRbCs, we report here seven new cases of this rare entity, examining in details their clinical-pathological and molecular characteristics. For comparison, we included four poorly differentiated medullary carcinomas (PDMCs) with focal features mimicking rhabdoid features. Immunohistochemical, genetic and epigenetic analyses were performed to characterize these tumour entities and to correlate BRAF mutation status with the clinico-pathological features of these tumors.

Our results have been integrated and discussed with the literature data available so far on CRbCs. These data have been published in the report (29) (2. in appendix section)

MATERIALS AND METHODS

This chapter is organized in two parts.

Part 1 is a clinico-pathological analysis of 59 mCRC with BRAF mutation and was performed to compare MSI and MSS cases, focusing on the inflammatory profiles and neuroendocrine differentiation of these tumors.

Part 2 is a clinicopathological and molecular study of seven Colorectal rhabdoid carcinomas (CRbCs) compared with poorly differentiated medullary carcinomas (PDMCs) with focal aspects mimicking rhabdoid features

1. Part I

1.1 Patient Cohort

From January 2010 to December 2017, at the pathology department of ASST of Sette Laghi-University of Insubria, we selected 59 patients with mCRC. The inclusion criteria were as follows: (1) stage IV at diagnosis and relapsed early-stage CRC (stage I - III); (2) Presence of *BRAF* p.V600E mutation; (3) availability of histologic samples (ie, surgical samples and/or biopsy specimens); (4) clinical data, including surgery, treatment, and follow-up data; and (5) known MSI status and MMR defect type by using immunohistochemistry.

For each patient, we collected data about sex, age at the diagnosis, Eastern Cooperative Oncology Group performance status [151], colorectal site, disease stage at diagnosis, type of surgery or metastasectomy, metastatic disease sites, and therapy performed. All the patients had undergone treatment for metastatic disease with ≥ 1 regimens of chemotherapy and/or targeted therapy (anti-VEGF or anti – EGFR antibody). *BRAF* p.V600E mutation, MSI status, immunohistochemical expression of MMR proteins, and *MLH1* methylation were evaluated in accordance with previously reported protocols in our lab [152]. The patients were followed up using regular and periodic clinical and instrumental evaluations. The tumor response was assessed using the Response Evaluation Criteria in Solid Tumors [153]. OS was considered as the interval from the diagnosis of metastatic disease to death or the last follow-up evaluation.

The ethics committee of Ospedale di Circolo di Varese approved the present study (approval no. 0008465), which was performed in accordance with the Declaration of Helsinki.

1.2 Histopathologic and Immunophenotypical Study

The histologic diagnosis of CRC was confirmed by 2 gastrointestinal tumor experts in accordance with the criteria of the 2010 World Health Organization classification [154]. The histopathologic

revision evaluated the following features: histologic type (tubular, mucinous, medullary, signet ring cells, undifferentiated, and mixed adenoneuroendocrine histologic features [ie, MANEC]), grade (1-3), mitotic index per 2 mm^2, growth pattern, tumor budding, necrosis, vascular space invasion, perineural invasion, percentage of tumor stroma, residual adjacent adenoma, intratumoral lymphocyte count, and the presence of tertiary lymphoid structures (Crohn-like reaction). For assessment of tumor budding, the Nakamura method was used to score the cases. The degree of tumor budding was categorized into 2 groups: low grade (none or mild) and high grade (moderate or marked) [155]. Using immunohistochemistry, we evaluated the type of lymphocytic infiltrate, both intratumoral lymphatic invasion (ILI) and peritumoral lymphatic invasion (PLI), using anti-CD3 and anti-CD8 antibodies. Tumor-associated inflammation was assessed according to the criteria of Kasajima et al [156] and graded as absent (no inflammatory cells at the tumor margin), weak (mild and patchy inflammatory cells at the tumor margin), moderate (evident bandlike inflammatory reaction at the tumor margin), or high (prominent inflammation at the invasive edges). The number of CD8 and CD3 lymphocytes in the tumor center and tumor periphery was evaluated using a Zeiss Microscope (ocular, x 10; objective, 25 mm) over an average area of 0.882 mm^2. We counted their number at the point at which the inflammatory infiltrate was more intense. PD-L1 expression was assessed on the tumor cells (TCs) and immune cells infiltrating and surrounding the tumor (ILI and PLI, respectively). PD-L1 staining was scored as positive when > 1% of the TCs or immune cells were immunoreactive. Moreover, all cases were evaluated for immunohistochemical expression of p53, CDX2, Ki-67, and synaptophysin. The antibodies, protocols, and criteria for the evaluation of immunohistochemical expression are reported in the Table 7.

Table 7. Antibodies used and immunohistochemical protocols

Primary Antibody	Clone	Working Solution	Treatment	Manufacturer
CD3 Rabbit monoclonal	2GV6	pure	MW 5 min x2 CB, pH 6	Ventana
CD8 Rabbit monoclonal	SP57	1:2	MW 5 min x2 CB, pH 6	Ventana
PDL-1 Rabbit monoclonal	SP142	1:40	MW 5 min x4 EDTA, pH 8	Spring
P53 Mouse monoclonal	DO-7	1:500	MW 5 min x4 CB, pH 6	Dako
CDX2 Mouse monoclonal	CDX2-88	1:50	MW 5 min x4 CB, pH 6	Biocare
Ki67 Mouse monoclonal	MIB-1	1:100	MW 5 min x4 CB, pH 6	Dako
Synaptophysin Rabbit monoclonal	polyclonal	1:2	MW 5 min x4 CB, pH 6	Ventana

Legend. MW: Microwave antigen retrieval solution; CB: citric acid antigen retrieval buffer

Abbreviations: CB= citric acid retrieval buffer; EDTA ethylenediaminetetraacetic acid; MW= microwave antigen retrieval solution.

Immunohistochemistry was performed manually. Formalin-fixed paraffin-embedded sections were mounted on poly-L-lysine coated slides, deparaffinized and hydrated through graded alcohols to water. Endogenous peroxidase activity was quenched in 3% H_2O_2 in water for 20 minutes. Proteolytic treatment was performed using different antigen-retrieval solutions (CB pH6 or EDTA pH8) in a domestic 750-kW microwave oven. Primary antibodies were applied overnight at 4°C, and immunostained using the avidin-biotin peroxidase complex (ABC) method or the MACH4 system. For ABC method, sections were incubated with biotinylated anti-mouse immunoglobulins and ABC peroxidase complex, each for 1 h at room temperature. The immunoreaction was developed with 3.3'-diamonobenzidine tetrahydrochloride (DAB) as chromogen and nuclei were counterstained with hematoxylin. Finally, sections were dehydrated.

1.3 Statistical Analyses

We summarized the major clinical and pathologic features of the tumors using descriptive statistics for the overall case series and stratified by MSI status. We used standard cutoff values to define the expression of stroma (≥20%), synaptophysin (>0), and p53 (≥50) as high. We tested the null hypothesis of no difference in the clinical characteristics when stratified by MSI using either the t test or c2 test for continuous and discrete variables, respectively. We used box plots to represent the distribution of CD3, CD8, and PDL1 when stratified by MSI status. Owing to the skewed nature of these parameters, we tested the difference among the MSI groups using the Wilcoxon rank sum test. We estimated the linear correlation among CD3, CD8, and PD-L1 expression using Pearson's rho coefficient. Of the 58 patients with valid follow-up data, we estimated the hazard ratios (HRs) and 95% confidence intervals (CIs) for mortality when stratified by MSI status (with MSS as reference) and for a 1-standard deviation increase in CD3, CD8, and PD-L1 expression using univariate Cox proportional hazards models. The proportionality of hazards was tested by adding a time*variable interaction; none was significant. We also explored multivariate Cox models, adjusting for age, sex, primary tumor site, and metastasis location (peritoneal vs. other). Finally, to investigate the combined effect of MSI and CD8 expression on OS, we first defined the positivity to CD8 by adopting the sample mean as the cutoff value and created a 4-class exposure variable with MSI status. This was the only independent variable in a univariate Cox regression model, with negative CD8 expression and MSS as the reference class. We used SAS software, release 9.4 (SAS Institute, Cary, NC), for the statistical analyses and R, version 3.2.5 (R Foundation, Vienna, Austria), to create the figures.

2. Part II

2.1 Histopathologic and Immunophenotypical study

Formalin-fixed and paraffin-embedded tissue blocks from 11 colorectal carcinomas were retrieved from our routine surgical pathology files, dating back to 1984. We included seven neoplasms composed (at least 30%) of highly atypical tumor cells with abundant eosinophilic cytoplasm containing hyaline-like globular (rhabdoid) inclusions classified as CRbC. We also included four cases interpreted as poorly differentiated medullary carcinomas (PDMCs) showing areas with discohesive polymorphic cells with abundant eosinophilic cytoplasm and eccentric nuclei, mimicking rhabdoid cells. One of the CRbCs and two of the PDMCs showed a focal glandular component. A combined CRbC and PDMC was found in one case. Tissue sections were stained with hematoxylin–eosin and periodic acid–Schiff/Alcian blue. The histopathologic revision evaluated the following features: Grade, mitotic index per 2 mm^2, growth pattern, tumor budding, necrosis, vascular space invasion, perineural invasion, percentage of tumor stroma, intratumoral and peritumoral lymphocytic infiltration. For assessment of tumor budding, the Nakamura method was used to score the case [155]. The degree of tumor budding was categorized into two groups: Low grade (none or mild) and high grade (moderate or marked). Tumor-associated inflammation at the tumor margin was assessed according to the criteria of Kasajima et al. [156] and graded as absent (0), weak (1+), moderate (2+), or high (3+). The quantity of intratumoral granulocytes was graded as: Absent (0), weak (1+), moderate (2+), or high (3+). The number of intratumoral CD8-positive lymphocytes (CD8-TIL) and peritumoral CD8-positive lymphocytes (CD8-PTL) was assessed using anti-CD8 antibodies and their relative number was evaluated using a Zeiss microscope (ocular x 10; objective 25 mm) over an average of 0.882 mm^2. Moreover, all cases were evaluated for immunohistochemical expression of pancytokeratin, CK7, CK20. EMA, β-catenin, SMARCB1 (INI-1), vimentin, Ki-67, p53, CDX2, and synaptophysin. The antibodies, protocols, and criteria for the evaluation of immunohistochemical expression are reported in Table 8 (see in appendix). Positive and negative controls were used throughout. SMARCB1 (INI-1) staining was interpreted according to the criteria exposed by Wang et al. [149] and categorized as 1) negative staining, 2) focally negative staining, and 3) positive staining.

2.2 Molecular Study

2.2.1 MSI and CpG Island Methylator Phenotype (CIMP) Analysis

MSI status was evaluated in accordance with previously reported protocols [157]. Methylation study was performed using methylation-sensitive multiple ligation-dependent probe amplification (MS-MLPA), that allows the simultaneous assessment of promoter methylation of multiple genes in a single experiment. SALSA MS-MLPA ME042-C1 CIMP Kit (MRC-Holland, Amsterdam, The Netherlands) was used to perform methylation analysis on eight gene promoters frequently methylated in CIMP tumors [69] (details in Table 9, see appendix). In detail, the kit contains 31 MS-MLPA probes which detect the methylation status of promoter regions of CACNA1G, CDKN2A, CRABP1, IGF2, MLH1, NEUROG1, RUNX3, and SOCS1 genes. MS-MLPA reactions were performed according to the manufacturer's instructions. The MS-MLPA products were analyzed on an ABI 310 Automatic DNA Sequencer (Applied Biosystems, Foster City, CA, USA) using GeneMapper 4.0 genotyping software (Applied Biosystems, Foster City, CA, USA). Values corresponding to peak size in base pairs (bp) and peak areas were used for further data processing by Co_alyser V8 software (MRC-Holland, Amsterdam, The Netherlands). All probes were adjusted to reference probes within each sample (intra-sample normalization). The methylation ratio (MR) was calculated by dividing each normalized peak value of the HhaI-digested sample by that of the corresponding undigested sample. Blood-derived DNA samples of three healthy individuals were used as unmethylated reference samples for inter-sample normalization. Sensitivity and specificity of the MS-MLPA assay were determined by a titration experiment, mixing fully methylated DNA (CpGenome Universal Methylated DNA, Millipore) with unmethylated DNA (CpGenome Universal Unmethylated DNA, Millipore) in proportions of 0%, 10%, 25%, 50%, and 100%. Using three replicates for each concentration, we observed MR values between 0 and 0.16 for the probes of fully unmethylated samples, and between 0.28 and 0.47 for the probes of 10% methylated DNAs. MRs obtained in the titration experiment with the 10%-methylated DNA were used as cut off values to determine aberrant methylation Ratio (MR) status of our probes as categorical variables (Table 9 shows the cutoff used for each MS-MLPA probe, see in appendix). To classify a gene promoter as methylated, at least half of the probes had to show methylation. We considered a sample CIMP positive if it showed at least four out of eight methylated promoters.

2.2.2 Targeted Sequencing Libraries and Massively Parallel Sequencing

Tumor DNA was obtained from formalin-fixed paraffin-embedded (FFPE) tissue using three representative 8 µm sections. The sections of every specimen were treated twice with Bio-Clear (Bio-optica, Milan, Italy). Neoplastic areas were manually microdissected for DNA extraction and contained at least 50% of tumor cells to minimize contamination by normal cells. DNA was extracted using the Maxwell®DNA FFPE Kit (Promega, Madison, Wisconsin, USA) and purified using an automatic nucleic acid purification system (Maxwell 16 system, Promega, Madison,Wisconsin, USA) according to the manufacturer's protocol. Each sample was then quantified using Qubit dsDNA High-Sensitivity Assay kit (Invitrogen, Thermo Fisher Scientific, USA). A targeted capture library was constructed according to the protocol Human Actionable Solid Tumor Mutations QIAseq DNA Panel (DHS-101Z, Qiagen, Hilden, Germany) that allows to analyze by NGS, specific exons or hot-spot mutations in 19 oncogenes (*BRAF*, PDGFRA, EGFR, KRAS, NRAS, KIT, AKT1, ALK, CTNNB1, ERBB3, ESR1, FOXL2, GNA11, GNAQ, IDH1, IDH2, MET, RAF1, RET) plus the whole exonic regions of ERBB2, PIK3CA, and TP53. This gene panel covers a total of 15,160 bp with 170 amplicons with a mean of 150 bp. Libraries were generated starting from 40–100 ng of FFPE DNA. Genomic DNA samples were first fragmented, end repaired, and A-tailed within a single multi-enzyme reaction. Prior to target enrichment and library amplification, each original DNA molecule was assigned a unique molecular identifier (UMI) containing a 12-base random sequence. After cleanup of adapter-ligated DNA using QIAseq beads, target enrichment was performed post-UMI assignment through eight cycles of targeted PCR using one region-specific primer and one universal primer complementary to the adapter. After cleanup of target enrichment, a universal PCR and cleanup of the amplicons were ultimately carried out. Equal volumes of individuals libraries were pooled at 4 pm. Bead emulsion for immobilization and clonal amplification were performed with the Ion OneTouch2 System (Thermo Fisher Scientific,Waltham, Massachusetts, USA) and Ion OneTouchES instruments (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Barcoded libraries of 8–10 samples were sequenced on an Ion S5 XL System (A27214, Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instructions using 500 flows.

2.2.3 Next-Generation Sequencing Data Analysis

Upon completion of the sequencing run, unmapped BAM (uBAM) files were imported into the CLC Genomics Workbench (Qiagen Bioinformatics version 12, Hilden, Germany). Sequencing data were

analyzed using the Biomedical Genomics Analysis plugin, which allows to align reads to the reference genome (UCSC build hg19), UMI counting, read trimming, and variant identification.

Data were filtered ensuring a coverage of at least 500x and an allelic fraction of 5%. In order to detect only mutations with a deleterious defect on protein functions, both synonymous mutations and variants described in the 1000 Genome Project were filtered out. All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Varese (Project identification number: 0008465).

RESULTS

1. Part I

1.1 Clinicopathologic Features and Treatment of *BRAF*-mCRC

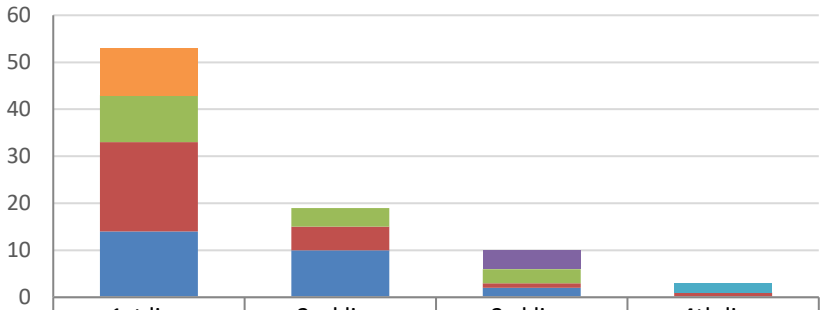
The data from 59 patients with *BRAF*-mCRC were collected. The present series included 22 MSI (37.2%) and 37 MSS (62.7%) neoplasms. Of the 59 specimens, 7 were biopsy samples and 52 were surgical resection samples. The clinicopathologic features of the tumors are summarized in Table 10. In our cohort, the proportion of women was greater than that of men (61% vs. 39%), their mean age was 67 years, and right-sided tumors were more common than left-sided ones (69% vs. 31%). Compared with consecutive cohorts of CRC, we observed a high frequency of tubular and mucinous/signet ring cell cancer types (49.2% and 23.7%, respectively), followed by medullary undifferentiated types (15.3%) and MANEC (12%). Most cases were characterized by an infiltrative growth pattern (79.2%) and high percentage of stroma (61.8%). Clinically, metastatic disease (stage IV) was the initial diagnosis for 63% of the patients and 37% of patients had developed a relapse after a diagnosis of early-stage CRC (stage I-III). Multisite metastatic disease was the prevalent condition, with liver and peritoneum frequently involved (56% and 38%, respectively). Only 5 patients had a single metastasis and underwent metastasectomy. Of these 5 patients, 2 had undergone liver metastasis resection and 3 had undergone pulmonary resection or splenectomy or partial removal of the psoas muscle. As shown in Figure 2, 76% of the patients had undergone first-line chemotherapy with or without a monoclonal antibody (anti-vascular endothelial growth factor and/or anti-EGFR), 33% of the patients had received second-line, 14% third-line, and 5% fourth-line treatment. Only 10 patients (17%) were treated with best supportive care. The chemotherapy regimens were based on schemes containing 5-fluorouracil, irinotecan, and oxaliplatin.

Table 10. Clinicopathologic profiles of MSI and MSS *BRAF*-mCRC

	All patients n (%)	**MSI-CRC n (%)**	**MSS-CRC n (%)**	**p-value****
	n=59	n=22	n=37	
Age, mean±SD, years	66.9±11.8	70±9.6	65.1±12.7	0.1
Women (%)	36 (61%)	12 (54.5%)	24 (64.9%)	0.4
Primary Tumor Side*				
Right-sided	40 (69%)	22 (100%)	18 (50%)	**< .0001**
Left-sided	18 (31%)	0 (0%)	18 (50%)	
Histological Type				
Tubular	29 (49.2%)	8 (36.4%)	21 (56.8%)	
Mucinous/signet ring cells	14 (23.7%)	8 (36.4%)	6 (16.2%)	0.09
Medullary/undifferentiated	9 (15.3%)	5 (22.7%)	4 (10.8%)	

MANEC	7 (11.9%)	1 (4.5%)	6 (16.2%)	
Grading				
G1/G2	30 (50.8%)	8 (36.4%)	22 (59.5%)	0.09
G3	29 (49.2%)	14 (63.6%)	15 (40.5%)	
Stage at diagnosis*				
I-II-III	21 (36.2%)	9 (40.9%)	12 (33.3%)	0.6
IV	37 (63.8%)	13 (59.1%)	24 (66.7%)	
Distant metastasis*				
Liver	28 (55%)	11 (55%)	17 (56%)	
Peritoneum	19 (38%)	10 (50%)	9 (30%)	0.095
Lung	14 (28%)	3 (15%)	11 (36%)	
Lymph node metastasis*				
Presence	14 (28%)	9 (45%)	5 (16.7%)	**0.029**
Absence	36 (72%)	11 (55%)	15 (83.3%)	
Growth pattern*				
Expansive	11 (20.8%)	8 (38.1%)	3 (9.4%)	**0.01**
Infiltrative	42 (79.2%)	13 (61.9%)	29 (90.6%)	
Percentage of stroma ≥20%	34 (61.8%)	6 (30%)	28 (80%)	**0.0002**
Synaptophysin ≥1	16 (29.1%)	1 (4.5%)	15 (45.5%)	**0.001**
Ki67%, mean±SD	66.5±20.5	70±20	64.3±20.7	0.3
p53 ≥50	22 (37.3%)	3 (13.6%)	19 (51.4%)	**0.004**

Legend**:** SD, standard deviation. *data were not available for all patients; ** Student's t-test for continuous variables, chi-square tests for categorical variables.



	1st line	2nd line	3rd line	4th line
Best Supportive Care	10	0	0	0
CLINICAL TRIALS	0	0	0	2
REGORAFENIB	0	0	4	0
CT+ antiEGFR	10	4	3	0
CT+BEVACIZUMAB	19	5	1	1
CT	14	10	2	0

Figure 2: Treatments used in first and following lines: 76% of patients were treated up-front with a chemotherapy combined or not with a monoclonal antibody (anti-VEGF and/or anti-EGFR), 33% of patients received a second line, 14% a third line and 5% a fourth line of treatment. Only ten patients (17%) were treated with best supportive care.

1.2 Clinicopathologic Profiles of *BRAF*-mCRC according to MSI Status

MSI mCRC showed a positive correlation with right-sidedness (P<.0001), poor histologic differentiation (P= .09), an expansive pattern of growth (P = .01), and presence of lymph node metastases (P= .029; Table 10). By contrast, MSS mCRC were characterized by higher stromal component (P = .0002) and positive immunoreactivity for synaptophysin (P = .001) and p53 (P = .004). In this subset of cases, neuroendocrine tumors were more frequent than in the MSI mCRC (16.2% vs. 4.5%; P= .09; Figures 3 and 4). Finally, lung metastases were more common in patients with MSS mCRC compared with patients with MSI mCRC (P= .09). No other significant differences between the 2 groups were found in Ki-67 expression, necrosis, vascular space invasion, perineural invasion, or stage of disease at diagnosis (Table 10; Table 11)

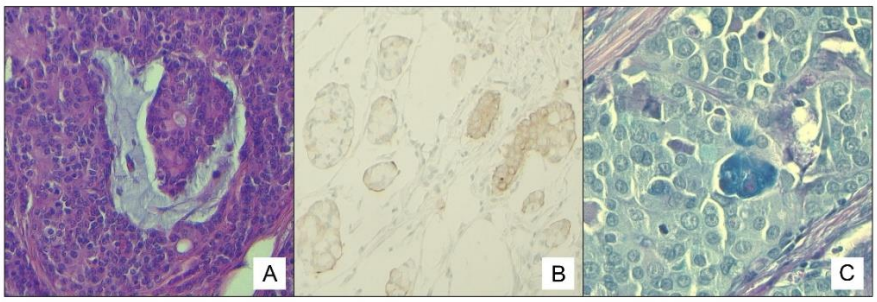


Figure 3 : Histological aspects of a colorectal mixed adenoneuroendocrine carcinoma (MANEC) with *BRAF* mutation. MANECs are neoplasms in which both the neuroendocrine and non-neuroendocrine components are present. **A**: hematoxylin-eosin stained section showing glandular structure with solid mucin production admixed with neuroendocrine proliferation, (×200). **B**: Synaptophysin stained section (x200). The neuroendocrine component express general neuroendocrine markers such as synaptophysin while signet ring exocrine cells are negative. **C**: Acid mucins of non-neuroendocrine cells stained strongly with Alcian Blue (x400).

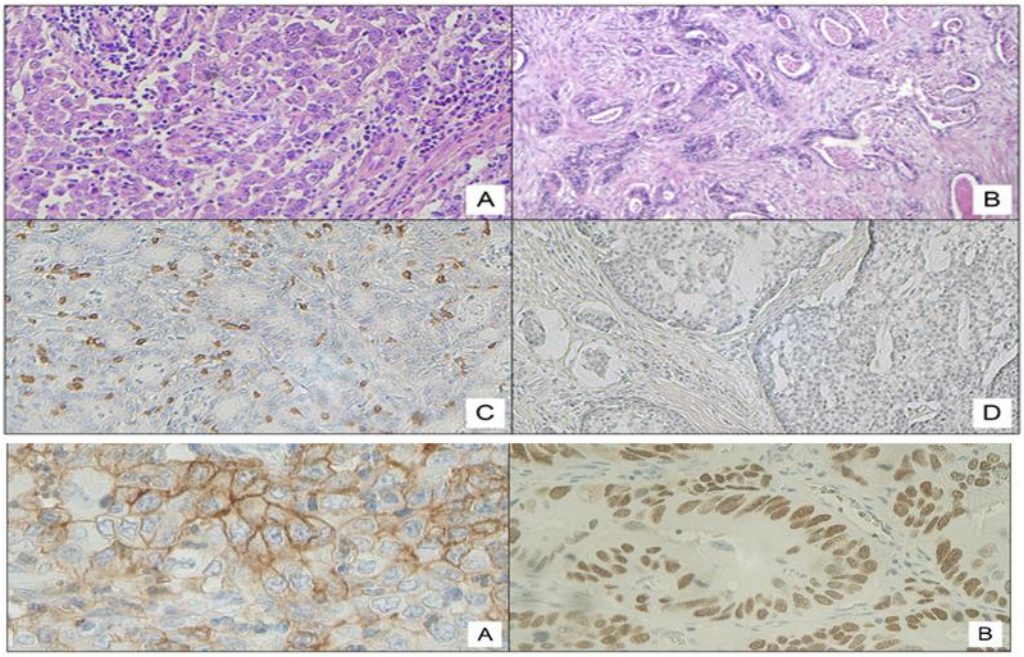


Figure 4 : Comparison of MSI CRCs (panels A, C and E) with MSS CRCs (panels B, D, F). MSI CRC shows more abundant intratumor lymphocytes and has a lower percentage of stroma (A: hematoxylin-eosin stained section, x200) compared to MSS CRC (B: hematoxylin-eosin stained section, x100). CD8 immunostaining highlights the high number of immune cells that infiltrates a MSI CRC (Panel C, x 200) compared to MSS CRC showing no intratumor lymphocytes (Panel D, x 100). PD-L1 expression on the cell membrane of tumor cells in a MSI CRC (Panel E, x400) and intense p53 nuclear immunoreactivity in a MSS CRC (Panel F, x400)

TABLE 11. Histopathological features evaluated in MSI and MSS tumors

	Total patients	**MSI (n=22)**	**MSS (n=37)**	**p-value**
Crohn-like 2+/3+ (%)	12 (25%)	5 (25%)	7 (25%)	1.0
Budding 2+/3+ (%)	13 (25.5%)	4 (20%)	9 (29%)	0.5
Vascular space invasion 2+/3+ (%)	28 (54.9%)	13 (65%)	15 (48.4%)	0.2
Perineural invasion (%)	29 (60.4%)	9 (45%)	20 (71.4%)	0.06
Necrosis (%)				
focal	13 (23.2%)	4 (18.2%)	9 (26.5%)	
geographical	24 (42.9%)	13 (59.1%)	11 (32.4%)	0,1
absent	19 (33.9%)	5 (22.7%)	14 (41.2%)	

Abbreviations: MSI microsatellite instability; MSS microsatellite stable

1.3 Different Immune Infiltration in MSI and MSS *BRAF*-mCRC

An inflammatory infiltrate was strongly associated with MSI tumors. CD8 and CD3 lymphocytes, both ILI and PLI, were prevalent in MSI compared with MSS tumors (CD8 ILI and PLI, P = .0001 and P < .0001; CD3 ILI and PLI, P = .003 and P = .0003, respectively; Figures 4 and 5). The expression of the PD-L1 receptor, on both TCs and lymphocytes, was more positively associated with MSI than with MSS (P < .0001; Figure 2). In addition, we found a significant linear correlation between the expression of PD-L1 on TCs and CD3/CD8 immunoreactivity on ILI lymphocytes. In addition, we observed a positive correlation between CD8 PLI and CD3 PLI cells and between CD8 ILI and CD3 ILI cells (Table 12, see in appendix). No significant differences between the 2 groups were found for the Crohn-like lymphoid reaction.

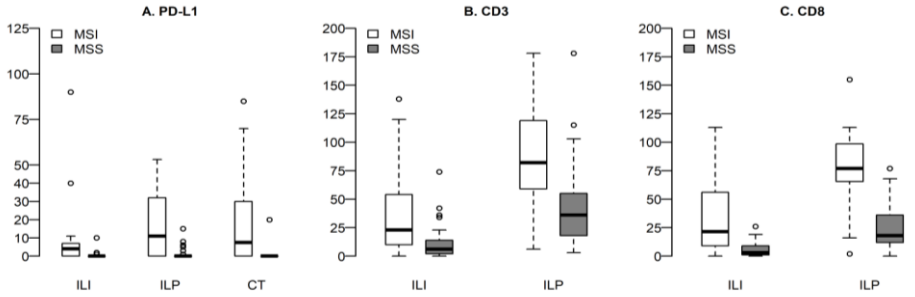


Figure 5: PD-L1 expression and CD3 and CD8 T cell contents in MSI compared with MSS tumors. The expression of PD-L1 receptor, both on TC and on intratumoral (ILI) and peritumoral (PLI) lymphocytes, were significantly associated with MSI than with MSS cases (A panel, p-value <.0001). CD3 (panel B) and CD8 lymphocytes (panel C) were prevalent in MSI compared to MSS tumors (CD8 ILI and PLI: p-value 0.0001 and <0001 respectively; CD3 ILI and PLI: p-value 0.003 and 0.0003 respectively).

1.4 Survival Analysis

The OS of the patients was poor, with a median OS of 9 months. Compared with MSS, the presence of MSI was associated with a 34% decrease in the hazard of mortality, although this was not statistically significant (HR, 0.66; 95% CI, 0.34-1.28; P = .2). The 30-month survival probability was 32% for those with MSI and 14% for those with MSS (P = .3, log-rank test). Intratumoral CD8 as a continuous variable was associated with a 33% decrease in mortality (HR, 0.67; 95% CI, 0.45-0.99; P = .04). The adjustment for covariates, such as gender, age at diagnosis, tumor site, and the

presence of peritoneal metastases, decreased the HR only slightly to 0.72 (95% CI, 0.47-1.10). Peritumoral CD8, CD3, and PD-L1 were also protective with respect to mortality during the follow-up period (HR, < 1 for all), although none of the associations were statistically significant (P > .05 for all). Finally, with respect to tumors with MSS and low intratumoral CD8 expression, the combined presence of MSI and CD8 decreased the hazard of mortality of mortality ≤ 63% (HR, 0.37; 95% CI, 0.14-0.97; P = .2) that decreased only slightly after multivariable adjustment (HR, 0.52; 95% CI, 0.17-1.55).

2. Part II

2.1 Clinicopathological Features

The main clinicopathologic features of the seven patients with CRbC are reported in Table 13). Patients were four males and three females, aged 63–85 years (mean age: 70.5 years; median age: 65 years). Four tumors were localized in the right colon (one in the cecum, one in the ascending colon, and two in the hepatic flexure) and three in the left colon (one in the splenic flexure, one in the sigmoid colon, and one in the rectum). Presenting symptoms included nonspecific abdominal symptoms, weight loss, and evidence of gastrointestinal bleeding. All patients underwent radical surgical procedures (right-sided or left-sided colectomy with node dissection). Five of the six patients, for whom detailed data were available, had positive regional nodes and three of them also had intra-abdominal and/or liver metastases. The stage evaluated after surgical procedures was IIA for one patient, IIIB for two patients, IIIC for two patients, IVA for one patient, and was not defined in one case. Five patients died of disease within 2–11 months (mean 5.5 months) after surgery. Two patients are still alive (May 2019) 186 months and 216 months, respectively, after surgery.

Patients with PDMCs were two males and two females aged 53–94 years (mean: 75 years; median 76 years). Three neoplasms were in the right colon (two in the ascending colon and one in the cecum) and one neoplasm was in the left colon (sigmoid colon). All four patients underwent radical surgical treatment, and all were at stage IIIC. Two patients died of disease within 5–11 months (average 8 months), one patient is still alive after 124 months (May 2019). For one case, the follow-up was not available.

Table 13 Clinicopathological data of CRbCs and poorly differentiated medullary carcinomas (PDMC) included in our study.

Cases	Gender	Age	Site	Size (cm)	Type	Site of metastases	Treatment	Outcome*
CRbC 1	F	63	Right colon	10	Pure	N	Surgery	2 m
CRbC 2	F	76	Left colon	4	Pure	-	Surgery+CT	7 m
CRbC 3	M	85	Left colon	6	Pure	N, L	Surgery	2 m
CRbC 4	M	65	Right colon	6	Pure	N	Surgery	216 m (alive)
CRbC 5	M	63	Left right	6	Pure	N	Surgery	10 m
CRbC 6	M	64	Right colon	6	Composite	N	Surgery	-
CRbC 7	F	77	Right colon	7	Composite	absence	Surgery	187 m (alive)
PDMC1	M	79	Right colon	10	-	N	Surgery	11 m
PDMC2	F	94	Right colon	8	-	N	Surgery	5 m
PDMC3	F	53	Left colon	13	-	N	Surgery	-
PDMC4	M	73	Right colon	8	-	N	Surgery	124 m (alive)

2.2 Pathologic Findings

Grossly, the neoplasms (both CRbCs and PDMCs) were reported as huge ulcerated masses completely replacing the intestinal wall, measuring from 4–13 cm (average size: 7.6 cm). Histologically, CRbCs consisted of sheets of poorly cohesive cells subdivided in clusters by delicate strands of stroma (Figure 6A).

Two types of neoplastic cells were found: large pleomorphic rhabdoid cells and smaller round to polygonal cells. The large cells had pleomorphic eccentrically located nuclei and abundant cytoplasm containing large eosinophilic paranuclear “rhabdoid” inclusions (Figure 6 A, I). The smaller cells were more uniform in size and had less abundant cytoplasm with less evident eosinophilic bodies. The proportion of these cell types varied between tumors.

The rhabdoid cellular aspects were predominant (>75%) in five cases and patchy in two cases. One case had a focal adenocarcinomatous component and another case showed solid medullary areas that blended with rhabdoid areas. Extensive coagulative necrosis was detected in all cases. All

neoplasms contained among the tumor cells many infiltrating neutrophils with several cells showing emperipolesis. Tumor budding was present in all cases and graded as high.

On the contrary, both the intratumoral and peritumoral lymphoid infiltration was absent or inconspicuous in all cases. Prominent vascular space invasion was detected in all cases and perineural invasions in two cases. Mitoses per 2 mm^2 varied from 8–38 (average 16). PDMCs contained solid areas of medullary carcinoma alternating with areas of loosely cohesive medium to large sized cells with eccentric nuclei and eosinophilic cytoplasm, but without well-defined paranuclear cytoplasmic hyaline inclusions (Figure 6B). Focal areas of glandular differentiation with mucin production and groups of signet ring cells were identifiable in two cases.

Compared to CRbCs, PDMCs showed more frequent expansive growth, minor budding, more abundant stromal component, more consistent peritumoral lymphoid infiltration, and comparable tumor necrosis and vascular spaces invasion.

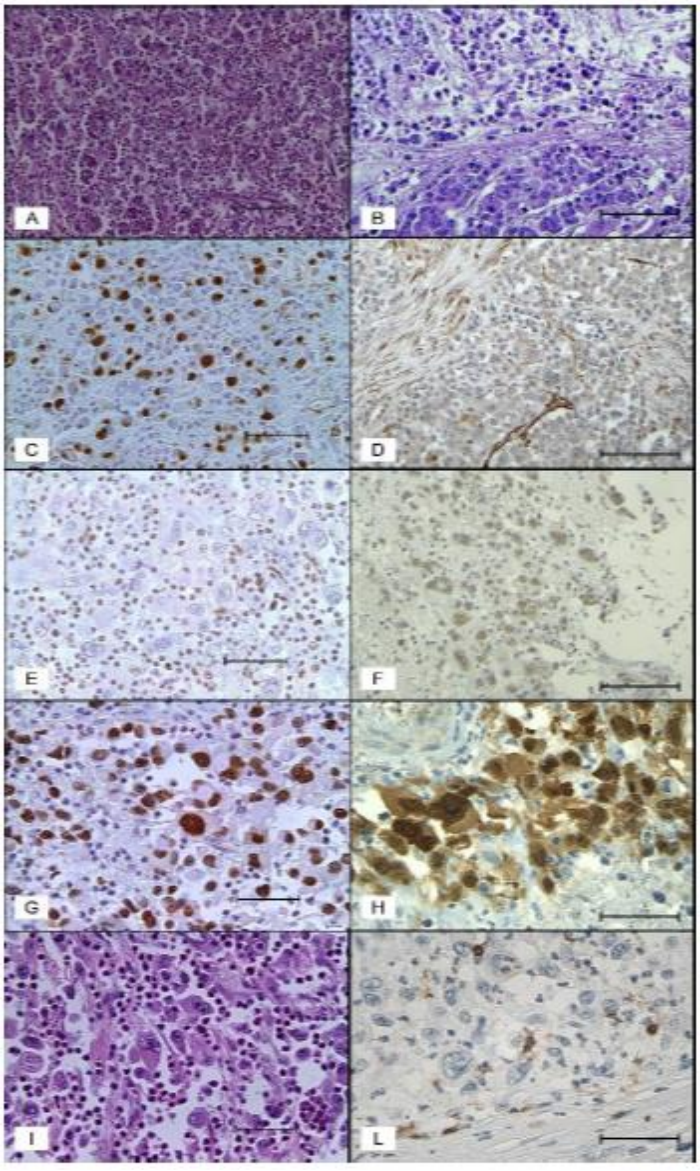


Figure 6. Morphological and immunohistochemical features in CRbCs and PDMCs. (**A**) and (**I**) CRbC showing non-cohesive rhabdoid cells admixed with numerous neutrophils (hematoxylin and eosin stain, 200× and 400×, scale bar 100 and 50 μm); (**B**) PDMC showing a cohesive medullary area adjoining an area of loosely cohesive cells (hematoxylin and eosin stain, 200×, scale bar 100 μm); (**C**) CrbC with strong immunostaining for vimentin predominantly in the paranuclear region of the cytoplasm (vimentin, 200×, scale bar 100 μm); (**D**) PDMC showing negative immunostaining for vimentin (vimentin, 200×, scale bar 100 μm); (**E**) complete loss of SMARCB1 (INI) expression) in a CRbC (INI-1, 400×, scale bar 50 μm); (**F**) loosely cohesive area of a PDMC showing SMARCB1 (INI) nuclear positivity (INI-1, 200×, scale bar 100 μm); (**G**) p53 nuclear expression in a CRbC (p53, 400×, scale bar 50 μm); (**H**) beta catenin nuclear expression in a CRbC (beta catenin, 400×, scale bar 50 μm); (**L**) CRbC showing few CD8-positive tumor infiltrating lymphocytes (CD8, 400x, scale bar 50 μm).

2.3 Immunohistochemical Findings

Immunohistochemistry of CRbCs showed strong cytoplasmic paranuclear positivity for vimentin in the majority (≥ 75%) of tumor cells (Figure 6C) in five cases and in significant areas (≥30%) of twocases (Table 13). Pancytokeratin was variously positive in all cases. Six of the seven cases were positive for epithelial membrane antigen (EMA). Complete loss or reduced expression of nuclear SMARCB1 (INI-1) (Figure 6E) was found in five and two neoplasms, respectively. β-catenin displayed a variable (20% – 100%) nuclear staining (Figure 6H) in five out of seven cases. p53 (Figure 6G) was strongly expressed (≥ 60% of neoplastic cells) in all cases. The proliferative index (Ki-67) varied from 38–90% (average 58%). The average number of CD8+ peritumoral lymphocytes was 31.7 (range 5–73) and that of intratumoral CD8+ lymphocytes 17.1 (range 3–38) (Figure 6L). All the remaining immunohistochemical markers including CK7, CK20, CDX-2, synaptophysin, and desmin were negative. The four PDMCs were positive for pancytokeratin, but negative for vimentin (Figure 6D).

All cases showed intact nuclear SMARCB1 (INI-1) expression. Nuclear-catenin positive staining (in 15–80% of tumor cells) was present in two of four cases. TP53 positivity (in ≥ 60% cells) was observed in two of four neoplasms. The proliferation index (Ki-67) varied from 60–80% (mean 70%). The mean number of CD8+ peritumoral lymphocytes was 52 (range 23–106) and that of intratumoral CD8+ lymphocytes was 6.5 (range 2–15) CK7 and CK20 were negative in all cases. CDX2 immunoreactivity was found in two of four cases.

Table 13. Main immunohistochemical results in CRbCs and PDMCs included in this study

ID	Vim	Pancytokeratin	INI-1	Nuclear β-Catenin	p53	CD8+ (PLI/ILI)
CRbC 1	3+	3+	0	0	3+	5/15
CRbC 2	3+	3+	0	3+	3+	10/3
CRbC 3	3+	1+	0	2+	3+	5/6
CRbC 4	2+	3+	0	0	3+	73/29
CRbC 5	3+	2+	0	3+	2+	53/13
CRbC 6	3+	3+	1+	2+	3+	61/38
CRbC 7	1+	1+	1+	2+	2+	15/16
PDMC1	0	3+	3+	3+	0	23/4
PDMC2	0	3+	3+	3+	3+	26/2
PDMC3	0	3+	3+	1+	0	54/5
PDMC4	0	n.a.	3+	3+	2+	106/15

Legend: 0—negative; 1+—(1–30%); 2+—(31–60%); 3+—(>60%); n.a.—not available; Vim—vimentin; PLI—peritumoral lymphocytic infiltrate (number per 0.882 mm^2); ILI—intratumor lymphocytic infiltrate (number per 0.882 mm^2).

2.4 *Molecular Findings*

MSI was observed in two out of seven (28%) CRbCs and in all four PDMCs (Table 14). CIMP analysis was possible in a total of nine neoplasms including six CRbCs and three PDMCs. CIMP-H was observed only in the three PDMCs while all the CRbCs were classified as CIMP-negative. *MLH1* was methylated in all four MSI cancers in which the CIMP status was assessed. Somatic mutation analysis by next generation sequencing (NGS) sequencing was possible in all cases except for the CRbC 7 sample, showing high levels of DNA fragmentation and degradation. NGS analysis showed an average of 1,306,777 reads per sample with a median read length of 119 bp. The mean number of mapped reads in targeted regions per sample was 183,856, and average coverage per sample was 1689. *BRAF* and TP53 mutations were observed in almost all tumors, occurring in nine out of 10 cases (five CRbCs and four PDMCs) and in nine out of 10 neoplasms (six CRbCs and three PDMCs), respectively. *BRAF* V600E mutation was detected in all cases except for CRbC 3 exhibiting a coexistence of NRAS G12D with a class III *BRAF* variant (i.e., *BRAF* G466A). TP53 mutations were mainly missense pathogenetic variants in 273, 245, 272, and 278 codons, while only two TP53 frameshifts mutations were observed in one case (PDMC 1). Two KRAS mutations were found in two *BRAF* wild-type cases, whereas PIK3CA mutations were detected in one CRbC and in one PDMC (Table 14).

For most cases exhibiting co-occurrence of *BRAF* and TP53 mutations, TP53 mutant allelic fractions (mAFs) were higher than *BRAF* mAFs (Table 14). Interestingly, in three cases showing *BRAF* mAFs higher than TP53 mAFs, we found two simultaneous TP53 mutations (case PDMC 1) or coexistence of TP53 and PIK3CA mutations (cases CRbC 6 and PDMC 3).

These findings are consistent with the "two-hits hypothesis" for tumor-suppressor genes but also suggest a driver role of anti-apoptotic/pro-survival pathways in these tumors that may be likely involved very early and together with the constitutive activation of the mitogen-activated protein kinase (MAP) kinase pathway. In two cases, we observed KRAS or *BRAF* mAFs >50% (CRbC 5 and PDMC 3) that were suggestive of copy number gains in wild-type alleles activation of the mitogen-activated protein kinase (MAP) kinase pathway. In two cases, we observed KRAS or *BRAF* mAFs >50% (CRbC 5 and PDMC 3) that were suggestive of copy number gains in wild-type alleles.

Table 14. Results of MSI, CIMP and mutation analyses in CRbC and PDMC included in this study

ID	MSI	CIMP	*BRAF* (mAF)	*KRAS* (mAF)	*NRAS* (mAF)	*PIK3CA* (mAF)	*TP53* (mAF)
CRbC 1	MSS	no CIMP	**V600E (7.5)**	WT	WT	WT	**R273C (11.9)**
CRbC 2	MSS	no CIMP	**V600E (22.6)**	WT	WT	WT	**R273C (29.7)**
CRbC 3	MSS	no CIMP	**G466A (25.1)**	WT	**G12D (28.2)**	WT	**G245S (55.7)**
CRbC 4	MSS	no CIMP	WT	**Q61K (19.7)**	WT	WT	**R273C (30.9)**
CRbC 5	MSS	no CIMP	WT	**G13D (86.5)**	WT	WT	**P278A (56.9)**
CRbC 6	**MSI**	no CIMP	**V600E (21.2)**	WT	WT	**H1047R (29.1)**	**R273C (22.2)**
CRbC 7	**MSI**	-	**V600E***	-	-	-	-
PDMC1	**MSI**	**CIMP-H**	**V600E (43.31)**	WT	WT	WT	**P152fs*18 (37.9)** **V73fs*50 (34.3)**
PDMC2	**MSI**	**-**	**V600E (21.85)**	WT	WT	WT	WT
PDMC3	**MSI**	**CIMP-H**	**V600E (68.89)**	WT	WT	**R93Q (33.6)** **M772I (39.5)**	**V272M (33.1)**
PDMC4	**MSI**	**CIMP-H**	**V600E (43.27)**	WT	WT	WT	**Y163C (41)**

Legend: -: not available data; mAF: mutated Allelic Fraction; * this mutation was found using Real-Time PCR Easy ® BRAF kit (Diatech Pharmacogenetics, Jesi, Italy).

DISCUSSION

BRAF mutations are reported in about 10% of metastatic CRCs and are almost exclusively non-overlapping with *RAS* mutations. In most cases V600E (1799 T-A nucleotide change) mutations are observed, which confer constitutive kinase activity and lead to aberrant activation of MAPK pathway [2, 4, 24].

BRAF-mutated CRCs constitute a distinct subgroup with specific characteristics as underlined by their peculiar gene expression signature and have also been associated with MSI and CIMP phenotype. Frequent clinicopathological features of these tumors include: female sex, right side, advanced age at diagnosis, stage IV at onset, poor prognosis with less than 1 year life expectancy, higher rate of nodal and peritoneal metastases and low rate of lung involvement, mucinous histology and poor differentiation [2,3,40,41].

Although it is clinically considered an unique entity, increasing evidences by literature report a significant clinical and biological heterogeneity.

Around 20% of patients with BRAFV600E mCRC patients survives beyond 24 months from the initial diagnosis. The reason for this prognostic heterogeneity has not been identified yet. According to molecular consensus subtypes (CMS), BRAFV600E mutant mCRC are identified for the vast majority in the CMS1 subgroup while the few remaining are scattered across the other CMS subtypes. However, CMS classification does not explain this prognostic heterogeneity.

Barras and coworkers from a cohort of 218 BRAFV600E mutant CRC identified two subtypes of disease with different prognosis: BM1 (BRAF mutant 1) and BM2 (BRAF mutant 2) [34]. These two subgroups were characterized by substantial differences both at transcriptomic and proteomic level and they are independent from patients' gender, sidedness, MMR status and PI3K status. BM1 is less common (1/3 of cases) and is characterized by strong activation of AKT/mTOR, KRAS, 4EBP1 and epithelial-mesenchymal transition features. On the other hand, BM2 represent most of cases and it is characterized by cell cycle deregulation, high level of CDK1 and low level of cyclin D1. Despite prognostic subdivision, this classification has no direct implication for the BRAFV600E treatment decision algorithm. In addition to molecular characterization, a retrospective platform of 395 BRAFV600E mutant mCRC led to the identification of three different prognostic subgroups based on the use of clinical data [177].

Even if this classification Even if this classification might have potential implication for treatment decision and for guiding translational research, its integration with molecular classification such as BM1/BM2 or CMS is warranted [177].

BRAF mutations do not cause the same functional effect and they do not have the same clinical impact [5]. Little is known about non-V600E mutations but studies conducted on these uncommon mutations (especially at 594 and 596 codons, detected in <1% of mCRC) identify a rare and unexplored molecular subtype of mCRC with clinical and pathological features very different from *BRAF* V600E mutated and with surprisingly better clinical outcome even when compared with non-mutated ones. Unlike *BRAF*V600E-mutated CRC, tumors with mutations in codons 594 and 596 exhibited better overall survival, were less likely to have high-grade or right-sided tumors, are not associated with microsatellite instability [5, 46].

Moreover, based on MMR status, it seems that MSI/*BRAF*-CRC and MSS/*BRAF* CRC represent two different subgroups of the same disease. Indeed, although both subsets likely derive from the serrated pathway in which *BRAF* mutation acts as a driver, MSS/*BRAF* tumors share many typical aspects of traditional colorectal tumors which develop from the conventional pathway [6,7,45]

The conventional pathway is characterized by *KRAS* mutations, CIN, absence of CIMP and of MMR defects.

At present two main serrated pathways have been described, namely sessile and traditional ones, which differ in the lesions encountered along the oncogenic process: SSA (sessile serrated adenoma) and TSA, (traditional serrated adenoma), respectively. On the one hand, SSAs are characterized by *BRAF* mutations, CIMP-H and *MLH1* promoter methylation. On the other hand, TSA lesions harbour KRAS or *BRAF* mutations and can be MSS, CIMP-H or CIMP-L [42].

From a clinical point of view, MSI is usually considered a molecular marker of a favourable prognosis regardless of *BRAF*/KRAS mutations and CIMP status. It is associated with a lower frequency of late-stage diseases [9, 42]. While MSI CRCs at early stage usually show a better prognosis and longer DFS compared to MSS tumors, related to the increased immune response in MSI tumor, the prognostic benefit appears lost in advanced stage [10,11,12]. It suggests that the detrimental prognosis seen in late stage MSI cancers is driven by presence of *BRAF* mutation [45]. MSI test is recommended for patients with stage II CRC to evaluate chemotherapy-based strategies. In fact, 5-fluorouracil treatment has no positive effect on survival in patients with MSI CRCs.

MSS cancers, developing from traditional serrated adenomas (TSA) or sessile serrated adenomas (SSA) is usually related to a poor prognosis in both early and metastatic setting. Several studies reported that MSS CRCs, associated with *BRAF* mutations, show higher mortality and decreased OS with respect to both MSI/*BRAF* mutated and MSS/KRAS mutated cancers. Thus, MSI-H CRC

patients without the *BRAF* mutation demonstrated the best prognosis, while MSS/*BRAF* V600E patients exhibited the worst; MSS/*BRAF*-WT and MSI/*BRAF* V600E CRCs seems to have an intermediate prognosis [42, 45].

To our knowledge, no clinical-pathological studies have been reported in the literature that have comprehensively characterized *BRAF* CRC according to MMR status, highlighting the histopathological, molecular and clinical characteristics of the *BRAF* MSI groups compared to MSS CRCs.

We performed a comprehensive clinicopathologic analysis of a well-characterized series of 59 *BRAF*-mCRC cases to highlight any differences between MSI and MSS tumors. Our series included 22 MSI and 37 MSS neoplasms. As expected, we found that MSI was significantly more frequent in CRC from the right colon, with a poor grade of differentiation and abundant TILs. In contrast, MSS *BRAF*-mCRC cases were characterized by a striking stromal reaction, immunohistochemical accumulation of p53, and a high percentage of neuroendocrine marker expression (17%). Neuroendocrine differentiation, detected by greater synaptophysin expression, was significantly more frequent in MSS tumors than in the MSI tumors. This finding is new and highlights the importance of evaluating the potential for neuroendocrine differentiation in *BRAF*-mCRC to identify a distinct subset of tumors with different prognosis and to define tailored treatments for these patients. To date, neuroendocrine differentiation has been reported in *BRAF*-CRC at widely variable frequencies, ranging from 5% to 51.5% and is considered a negative prognostic marker. Although no association between the outcome and MANEC histologic type was observed in our series, likely for the limited number of these tumors (7 cases), our data are in line with the recent hypothesis that the *BRAF* mutation might be an oncogenic driver of neuro endocrine carcinoma of the gastrointestinal tract [18, 19, 20]

Since the prognostic and predictive implications of TILs and inhibitory PD-1/PD-L1 proteins in mCRC, especially in *BRAF*-CRCs, are poorly understood and the clinical relevance of a pronounced host immune reaction remains elusive including MSI and MSS, a second purpose of our study was to characterize PD-L1 expression in the context of TILs and Crohn-like lymphoid reaction by comparing MSI and MSS *BRAF*-mCRC. In our study, MSI tumors exhibited a "hot phenotype," with rich intra- and peritumoral TILs mainly composed of a high CD8 T-cell content, demonstrated by a strong positive correlation between CD3 and CD8 immunoreactivity with both ILI and PLI. In contrast, no significant differences between the 2 groups were found for the Crohn-like lymphoid reaction. Moreover, most PD-L1+ tumors also contained TILs, and MSI cases were more likely to be

associated with PD-L1 expression in the TCs than were MSS tumors. Univariate analysis demonstrated that the presence of MSI and high CD8 T-cell content were associated with a 34% and 33% decrease in the hazard of mortality, respectively. Also, the combined presence of MSI and a high CD8 T-cell content decreased the hazard of mortality by ≤ 63%, which was only slightly decreased after multivariable adjustment. No other variable was associated with improved prognosis on univariate or multivariate analysis. Although further studies are needed to confirm our data, these results have demonstrated that a pronounced host immune reaction, intratumoral CD8 T-cell, and the presence of MSI might have an independent prognostic role. These results are in agreement with previous data obtained from large series of patients with stage I to III CRC and suggest the importance of a simultaneous evaluation of MSI status and CD8 T-cell content in *BRAF*-mCRC to identify a subgroup of biologically less aggressive tumors. At present, conflicting data have been reported regarding the prognostic role of PD-L1 expression in *BRAF*-mCRC and no information is available regarding a possible association between the outcomes and CD3+/CD8+ lymphocytes in these tumors. Some investigators have shown that the expression of PD-L1 on tumor cells and lymphocytes was associated with better outcomes [158 – 160] but others have reported that PD-L1 expression is a negative prognostic marker [161]. Masugi et al [162] analyzed PD-L1 expression in a series of 823 CRC cases at all stages. *BRAF* mutation was present in 15% of the cases, and they found that PD-L1 expression was greater in MSS than in MSI tumors. However, its expression did not correlate with CD3+/CD8+ lymphocytes or with prognosis. In contrast, 2 recent studies analyzed 454 and 181 CRC tumors and found that PD-L1 expression was associated with *BRAF* mutation and several histological features, including medullary histotype, a poor degree of differentiation, and the presence of a rich inflammatory infiltrate [162].

The *BRAF*-mCRC is clinically characterized by dismal prognosis and poor response to standard treatment. All the published series recognised that *BRAF*V600E mutation is a strong negative prognostic determinant in mCRC and *BRAF*-mutated metastatic patients have an extremely poor life-expectancy of around 12 months [2, 40,41,43, 76].

Although numerous studies show that *BRAF* V600E mutation is associated with reduced survival (OS, DFS or cancer specific survival) especially in MSS tumors. Its role in MSI is not clearly defined.

Intensified strategies of first line chemotherapy have been invastigated for *BRAF* CRC patients who mostly do not receive second line treatments.

The TRIBE trial showed that the combination of FOLFOXIRI regimen (5-fluorouracil, leucovorin, oxaliplatin, irinotecan) plus bevacizumab is associated with a better outcome than the

combination of FOLFIRI and bevacizumab (leucovorin, 5-fluorouracil, irinotecan) as first line therapy: 16 patients with *BRAF*-mutant tumors treated with FOLFOXIRI + bevacizumab had a median OS of 19 months, with better outcomes, although not statistically significant, in comparison of the combination of FOLFIRI + bevacizumab (10.7 months). Thus, FOLFOXIRI plus bevacizumab is now considered the standard of care for the first line treatment for *BRAF* CRC patients with good performance status [22]. Howewer, the TRIBE-2 study and a meta analysis have recently demonstrated no benefit from this regimen if compared to standard doublet cytotoxic combinations [172, 173].

In our series, the prognosis of the patients was poor with a median survival of 9 months. As expected, among the patients who had received active treatment, the median survival was 12.5 months. In contrast, the patients who had received palliative care at diagnosis had a median survival of 4 months. Only a few patients had received a triplet regimen plus bevacizumab, which according to the TRIBE study results seems to be the best treatment for these patients. Furthermore, many patients had received anti-EGFR therapy during the first or subsequent treatment lines.

Regarding response to anti-EGFR, the predictive role of *BRAF* mutation is still debated in literature [2]. In particular, in the meta-analysis that included two second-line trials and two trials involving chemorefractory patients, the lack of a significant efficacy benefit by anti-EGFR monoclonal antibodies (mAbs) over standard chemotherapy alone in patients with *BRAF* mutated tumours was considered to support the assessment of tumour *BRAF* mutation status before the initiation of anti-EGFR therapy. Conversely, other recent meta-analysis, concluded that there is currently insufficient evidence to definitively consider *BRAF* mutation a negative predictive biomarker of survival benefit from anti-EGFR mAbs for mCRC. The benefit in OS and PFS for *BRAF* mutated tumours treated with anti-EGFR mAbs may be smaller, but further data are required to clarify this observation [126, 127].

While *BRAF* inhibitors have produced impressive response rates of ~60–80% in melanoma, disappointing results were obtained in *BRAF* mutant mCRC patients. [2]

These differences suggest that several resistance mechanisms, primitive or acquired, rapidly emerge in CRC. Preclinical data suggests that *BRAF* inhibitors alone have limited efficacy because of a compensatory feedback reactivation of EGFR and its downstream pathways such as MEK and ERK. Thus, combination strategies have been developed using combinations of *BRAF* inhibitors in

combination with MEK and EGFR inhibition, and in some cases conventional cytotoxic therapy [2, 131].

Recently, the results of the phase III BEACON trial were published, in which 665 patients with pre-treated *BRAF*-mutated mCRC were randomized to receive a triple combination of encorafenib, cetuximab, and binimetinib (anti-*BRAF*, anti EGFR and MEK inhibitor, respectively) vs. encorafenib and cetuximab vs. irinotecan/FOLFIRI and cetuximab. The mOS was 9.0 months for the triplet combination vs. 5.4 months for standard therapy and 8.4 months for doublet. In the triplet and doublet arms the ORR was 26% and 20%, while mPFS was 4.3 months and 4.2 months, respectively. This is the largest cohort that has ever been studied and the first phase III trial to demonstrate a survival and response advantage in the setting of pre-treated *BRAF*-mutated CRCs [27]. An ongoing study (ANCHOR-CRC) is investigating the effects of the same triplet therapy as a first-line treatment for patients with *BRAF*-mutated CRC [2, 138].

Since targeting *BRAF* V600E seems challenging and multiple mechanisms of acquired resistance are rapidly emerging, including activation of the PI3K / AKT and WNT pathways, novel combination strategies with target therapies are ongoing and results are awaited [2]

Moreover, given the strong association of *BRAF*-V600E mutation with MSI, immunotherapy (such as immune-checkpoint inhibitors like pembrolizumab or nivolumab) could play an important role in this particular setting. In the phase II study CHECKMATE 142 treatment with nivolumab monotherapy, as well as the combination of nivolumab plus ipilimumab, demonstrated encouraging results with durable responses in patients with dMMR mCRC (objective response rate was 25.5 and 33.3%, respectively). As expected, no antitumor efficacy was observed for pMMR tumors (n = 20). Among patients with dMMR mCRC (n = 100), 17 (17%) harbored BRAFV600E mutation. Interestingly, clinical activity was observed regardless of the BRAF and KRAS mutational statuses and PD-L1 expression [143].

In our series, no patient had received immunotherapy. Although the Food and Drug Administration has approved pembrolizumab and nivolumab for MSI mCRC, this promising treatment option has not yet been approved in Italy and is not currently available except for in clinical trials.

Over the past decade, *BRAF* mutations have garnered a great deal of attention both because the *BRAF*/MEK inhibitors have revolutionized the melanoma treatment, obtaining a magnificent clinical success, and because *BRAF* mutation is involved as a driver in several kinds of cancers such as NSCLC, thyroid cancer, hairy cell leukemia and other very rare tumors. Thus, targeting *BRAF*

appears a potential therapeutic chance for aggressive and unknown neoplasms without efficacy standard treatments [14]

The molecular landscape of colorectal cancer is a very heterogeneous and includes different subtypes characterized by specific morphological and molecular alterations [2]. Interestingly, *BRAF* mutation has been found in a very rare and lethal entity of CRC, known as colorectal rabhoid carcinoma (CRbC) of which only 39 cases have been reported in literature [28]. In addition, CRbCs are often misclassified as poorly differentiated CRC and consequently underdiagnosed.

The diagnostic hallmark of these neoplasms is the presence of rhabdoid cells characterized by round eosinophilic aggregates of intermediate filaments that displace the nucleus to the cell periphery. Other consistent morphologic features are: the non-cohesive growth of tumor cells, the scarcity of tumor stroma, the abundance of tumor infiltrating neutrophils, and the scarcity of lymphocytic infiltration. The main immunohistochemical findings are: the expression of vimentin and pancytokeratin within filamentous cytoplasmic inclusions, the loss of membranous E-cadherin, the nuclear dislocation of β-catenin, the lack or reduced expression of important markers of colonocyte differentiation such as CK20 and CDX2, the marked nuclear p53 accumulation, and the high proliferative Ki-67 index [30].

The majority of CRbCs consist solely of rhabdoid cells and are indicated as “pure”, while other CRbCs combine a rhabdoid component with an adenocarcinoma component most frequently focal and confined to the tumor periphery and are designated as “combined”. The presence of a transitional zone in combined CRbCs with a continuum between rhabdoid and non-rhabdoid cells indicates that rhabdoid cancers cells (RbCs) might have originated from dedifferentiated primary colorectal cancer [144].

The most relevant immunohistochemical finding for the diagnosis of CRbC is the coexpression in tumor cells of pancytokeratin and vimentin. This was found in all CRbCs but not in PDMCs examined. Coexpression of a mesenchymal marker such as vimentin and epithelial markers is one of the phenomena that characterize the process of epithelial–mesenchymal transition (EMT) [163]. This is a unique process in which cells lose epithelial features and acquire mesenchymal properties [164]. The process of EMT is characterized by the reduction of epithelial markers and increase of mesenchymal markers [165]. E-cadherin is the most important mediator of cell adhesion in epithelial tissues and loss of E-cadherin is a crucial step in EMT. During EMT, loss of E-cadherin is associated with the release of βcatenin, which is consequently translocated to the nucleus where it activates the WNT signaling pathway. In colorectal cancer, altered expression of

E-cadherin and β-catenin and progressive increase of vimentin in late stages are significantly associated with aggressive tumor behaviour and, furthermore, confer resistance to cancer drugs [166]. In addition, in a recent study [167] it has been demonstrated that the gene expression profile of tumor budding regions in CRC closely matches with consensus molecular subtypes 4 (CMS4) mesenchymal subtype, while the bulk presents a CMS2 epithelial profile.

Previous immunohistochemical results demonstrating loss of membranous E-cadherin in CRbC [28] and our results showing β-catenin nuclear localization and loss of colonic epithelial markers (i.e., CK20 and CDX2) support the pathogenetic involvement of EMT as an essential player in the heterogeneous make-up of CRbC. In addition, the loss of membranous E-cadherin and β-catenin suitably explains the discohesive histologic pattern of CRbC. Cells undergoing EMT maintain the same genomic background in both mesenchymal and epithelial states, but during the progression of EMT, the gene expression profile significantly changes. A series of protein complexes, known as chromatin remodelers, are crucial to mediate this event as they can slide, destabilize, or relocate nucleosomes in an ATP-dependent manner [168]. The SWI/SNF mating-type switching (SWI) and sucrose nonfermenting (SNF) subfamily has specifically been investigated in malignant pediatric rhabdoid. These tumors are highly lethal neoplasms of the kidney and brain where *SMARCB1* (INI-1) is frequently mutated either at germline or at somatic level [169]. To date, the role of *SMARCB1* (INI-1) inactivation remains to be determined in CRbC and only few studies reported SMARCB1 (INI-1) immunohistochemical loss in a small subset of CRbCs that were frequently *BRAF*-mutant, MSI, and CIMP [28, 30, 150]. These data allowed to hypothesize that SMARCB1 (INI-1) may occur as a secondary molecular event during EMT in a subset of CRCs characterized by *BRAF* V600E mutation, MSI, and CIMP, virtually conferring a rhabdoid phenotype [30]. In line with this hypothesis, Wang et al. [149]. demonstrated that loss of SMARCB1 (INI-1) expression occurs at least focally in 0.46% of 3051 CRCs and is associated with higher grade, larger tumor size, poorer survival, MSI, and *BRAF* V600E mutation.

In this context, our study sheds some light on the biological features of this rare entity thanks to a genetic/epigenetic comparative analysis of CRbCs and PDMCs showing *BRAF* V600E as a common prominent genetic feature. A first important finding of our analysis was that CRbC mainly included *BRAF* mutant/MSS cancers without CIMP. By contrast, PDMC only comprised *BRAF* mutant/MSI and CIMP cancers. Two *BRAF* mutant/MSI cases were observed among CRbCs and in one case we could exclude CIMP. Interestingly, both these cases showed a reduced SMARCB1 (INI-1) expression but not a complete loss of the protein as we found in the remaining five CRbCs.

For the first time, with this work, we suggest that *BRAF* mutant/MSS cancers include the rare entity of CRbCs, characterized by a strong activation of EMT and complete loss or reduced expression of SMARCB1 (INI-1). Moreover, a recurrent finding of CRbCs in this study, was the abundance of tumor-infiltrating neutrophils which contribute to the formation of the tumor microenvironment. Although neutrophils were at first considered to possess defensive functions against cancerous cells, it has been demonstrated that some subtypes of neutrophils, known as tumor-associated neutrophils (TANs) possess a tumor-supporting function [170]. TANs contribute to tumor invasion and angiogenesis through production of matrix metalloproteinases, vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF). Interestingly, intratumoral neutrophils in CRCs have been found to correlate closely with a malignant phenotype and to represent an independent factor of poor prognosis for the patients [171].

Although *BRAF* mutant/MSS cancers display hypermethylation events that commonly characterize all *BRAF* mutant cancers, this subset of tumor shows lower frequency of CIMP than *BRAF*/MSI cancers [15, 17]. In line with this observation, CIMP was not found in CRbC in contrast to PDMC, analyzing the conventional panel of genes suggested to identified CIMP in tumors of the serrated pathways. Although this result does not preclude the presence of gene hypermethylation in CRbCs, the use of this gene panel may be useful to distinguish them from tumors of the classical serrated pathway.

Finally, TP53 mutation has been correlated with advanced stages and with conventional pathway in CRCs. *BRAF* mutant/MSS cancers have been found to have a comparably high rate of TP53 mutation as the *BRAF* wild-type cancers, whereas *BRAF* mutant/MSI were confirmed to have a low rate of mutation [15]. In our study, all but one tumor showed TP53 mutation and no specific differences we observed comparing CRbCs and PDMCs. An interesting observation was that TP53 mAFs were often higher than *BRAF* mAF in most of the tumors analyzed. These data emphasize a driver role of TP53 in the early phases of the development of these tumors suggesting that in addition to the constitutive activation of the MAP kinase pathway through *BRAF*/RAS mutations, simultaneous upregulation of anti-apoptotic pathways may be crucial for the rapid and aggressive growth of these tumors.

Rhabdoid carcinomas seem to be resistant to conventional therapy used for gastrointestinal neoplasms (FOLFOX, FOLFIRI scheme associated with monoclonal antibody). Moreover, anthracycline based regimes generally used in sarcoma do not seem effective. The co-presence of

BRAF and P53 mutations in CRbCs suggests the possible therapeutic role of a double block acting on *BRAF* and p53.

In summary, CRbCs are characterized by *BRAF* and less frequently KRAS mutations co-occurring with TP53 mutations. Coexpression of pancytokeratin and vimentin, dense neutrophilic infiltration, loss/reduced expression of nuclear of SMARCB1/INI, and low frequency of CIMP are useful markers to recognize these rare aggressive tumors. Elucidation of the genetic and epigenetic landscape alterations of these tumors is crucial to hypothesize specific treatments with novel biological agents such as MAPK inhibitors and small molecules blocking p53 degradation and epigenetic drugs.

CONCLUSIONS

The *BRAF*V600E mutation is a strong predictor of poor prognosis in mCRC and associated with resistance to standard chemotherapeutic regimens. Although the best treatment has not yet been identified, an aggressive strategy involving triplet chemotherapy and anti VEGFR is currently the standard of care for *BRAF* CRC patients with good performance status. *BRAF*-targeted therapies have shown insufficient efficacy when used alone, but their combination with other targeted therapies such as anti-EGFRs, MEK inhibitors or PI3K inhibitors seems promising.

The results from our study supports the idea that *BRAF*-mCRC tumors are not a single entity but that different clinical, histologic, immunophenotypical, and molecular characteristics allow for the recognition of distinct tumor subgroups. The significant heterogeneity of this subtype justifies the efforts for increasingly personalized therapeutic approaches.

Although our results require validation against independent data, the present study has demonstrated a high frequency of MANECs among MSS *BRAF*mCRC cases, supporting previous data on *BRAF* mutation as oncogenic driver of neuroendocrine carcinoma, and suggests that simultaneous evaluation of MSI status and CD8 T-cell content could be a useful strategy for identifying a subgroup of patients with a better prognosis and potential eligibility for cancer immunotherapy drugs.

The *BRAF* mutation has been detected in several types of cancer, representing a potential target of tailored therapies for aggressive neoplasms. It has been found in CRbCs, which are highly aggressive and very rare cancers with no specific protocols available with proven efficacy.

Although CRbCs show a wide phenotypic heterogeneity and molecular complexity, our study suggests that an integrated analysis of morphological, immunohistochemical, and molecular traits helps to recognize these uncommon tumors. Specifically, co-occurrence of *BRAF* and TP53 mutations, simultaneous expression of pancytokeratin and vimentin, dense neutrophilic infiltration, loss/reduced expression of nuclear of SMARCB1 (INI), and low frequency of CIMP are valuable markers to identify CRbCs. Elucidation of their genetic and epigenetic landscape will be critical in guiding the clinical development of personalized therapeutic treatments.

Finally, the place of each of the therapeutic combinations described and the way to sequence these new options remains an open question today. Further investigations are therefore justified, hence the need to promote the enrolment of *BRAF*-mt mCRC patients in clinical trials.

REFERENCES

1. Bray, F.; Ferlay, J.; Soerjomataram, I.; Siegel, R.L.; Torre, L.A.; Jemal, A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin*. **2018**, 68, 394–424.

2. Francesco Caputo, Chiara Santini, Camilla Bardasi, Krisida Cerma, Andrea Casadei-Gardini , Andrea Spallanzani , Kalliopi Andrikou , Stefano Cascinu and Fabio Gelsomino. BRAF-Mutated Colorectal Cancer: Clinical and Molecular Insights. Review ; International Journal Molecular Sciences.

3. Wang, J.; Shen, J.; Huang, C.; Cao, M.; Shen, L. Clinocopathological significance of BRAFV600E mutation in colorectal cancer: An updated meta-analysis. *J. Cancer* 2019, 10, 2332–2341.

4. Cantwell-Dorris, E.R.; O'Leary, J.J.; Sheils, O.M. BRAFV600E: Implications for carcinogenesis and molecular therapy. *Mol. Cancer Ther*. 2011, 10, 385–394

5. Jones, J.C.; Renfro, L.A.; Al-Shamsi, H.O.; Schrock, A.B.; Rankin, A.; Zhang, B.Y.; Kasi, P.M.; Voss, J.S.; Leal, A.D.; Sun, J.; et al. Non-V600BRAF mutations define a clinically distinct molecular subtype of metastatic colorectal cancer. *J. Clin. Oncol*. 2017, 35, 2624–2630.

6. Rajagopalan H, Bardelli A, Lengauer C, Kinzler KW, Vogelstein B, Velculescu VE. Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status. Nature 2002; 418:934.

7. Thiel A, Heinonen M, Kantonen J, et al. BRAF mutation in sporadic colorectal cancer and Lynch syndrome. Virchows Arch 2013; 463:613-21.

8. Jass JR. Diagnosis of hereditary non-polyposis colorectal cancer. *Histopathology* 1998; 32:491-7.

9. Koopman M, Kortman GA, Mekenkamp L, et al. Deficient mismatch repair system in patients with sporadic advanced colorectal cancer. Br J Cancer 2009; 100: 266-73.

10. Lothe RA, Peltomaki P, Meling GI, et al. Genomic instability in colorectal cancer: relationship to clinicopathological variables and family history. *Cancer Res* 1993; 53:5849-52.

11. Thibodeau SN, Bren G, Schaid D. Microsatellite instability in cancer of the proximal colon. *Science* 1993; 260:816-9.

12. Smyrk TC, Watson P, Kaul K, Lynch HT. Tumor-infiltrating lymphocytes are a marker for microsatellite instability in colorectal carcinoma. *Cancer* 2001; 91: 2417-22.

13. Nunzio Digiacomo, Elena Bolzacchini, Giovanni Veronesi, Roberta Cerutti, Nora Sahnane, Graziella Pinotti, Marco Bregni, Salvatore Artale, Claudio Verusio, Filippo Crivelli, Carlo Capella, Fausto Sessa, Daniela Furlan. Neuroendocrine Differentiation, Microsatellite Instability, and Tumor-infiltrating Lymphocytes in Advanced Colorectal Cancer With BRAF Mutation. *Clinical Colorectal Cancer*, Vol. 18, No. 2, e251-60, 2018 Elsevier.

14. Robert et al. Improved overall survival in melanoma with combined dabrafenib and trametinib. NEJM 2015.

15. C. E. Bond, A. Umapathy, I. Ramsnes et al., "p53 mutation is common in microsatellite stable, BRAF mutant colorectal cancers," International Journal of Cancer, vol. 130, no. 7, pp. 1567–1576, 2012.

16. C. E. Bond, A. Umapathy, R. L. Buttenshaw, L. Wockner, B. A. Leggett, and V. L. J. Whitehall, “Chromosomal instability in BRAF mutant, microsatellite stable colorectal cancers,”PLoS One, vol. 7, no. 10, article e47483, 2012.

17. C. E. Bond, D. J. Nancarrow, L. F. Wockner et al., “Microsatellite stable colorectal cancers stratified by the BRAF V600E mutation show distinct patterns of chromosomal instability,” PLoS One, vol. 9, no. 3, article e91739, 2014.

18. Idrees K, Padmanabhan C, Liu E, et al. Frequent BRAF mutations suggest a novel oncogenic driver in colonic neuroendocrine carcinoma. J Surg Oncol 2018; 117: 284-9.

19. Klempner SJ, Gershenhorn B, Tran P, et al. BRAFV600E mutations in high-grade colorectal neuroendocrine tumors may predict responsiveness to BRAF-MEK combination therapy. Cancer Discov 2016; 6:594-600.

20. Olevian DC, Nikiforova MN, Chiosea S, et al. Colorectal poorly differentiated neuroendocrine carcinomas frequently exhibit BRAF mutations and are associated with poor overall survival. Hum Pathol 2016; 49:124-34.

21. Romain Cohen, Pascale Cervera, Magali Svrcek, Anna Pellat, Chantal Dreyer, Aimery de Gramont, Thierry Andre´; BRAF-Mutated Colorectal Cancer: What Is the Optimal Strategy for Treatment? Curr. Treat. Options in Oncol. (2017) 18: 9.

22. Cremolini C, Loupakis F, Antoniotti C, et al. FOLFOXIRI plus bevacizumab versus FOLFIRI plus bevacizumab as first-line treatment of patients with metastatic colorectal cancer: updated overall survival and molecular subgroup analyses of the open-label, phase 3 TRIBE study. Lancet Oncol. 2015;16:1306–15.

23. Van Cutsem E, Cervantes A, Adam R, et al. ESMO consensus guidelines for the management of patients with metastatic colorectal cancer. Ann Oncol. 2016;27:1386–422.

24. Davies H, Bignell GR, Cox C, et al: Mutations of the BRAF gene in human cancer. Nature 417:949-954, 2002.

25. Robert C, Karaszewska B, Schachter J, et al: Improved overall survival in melanoma with combined dabrafenib and trametinib. N Engl J Med 372:30-39, 2015

26. Cohn AL, Day BM, Abhyankar S, et al: BRAFV600 mutations in solid tumors, other than metastatic melanoma and papillary thyroid cancer, or multiple myeloma: A screening study. OncoTargets Ther 10:965-971, 2017

27. Kopetz, S.; Grothey, A.; Yaeger, R.; Van Cutsem, E.; Desai, J.; Yoshino, T.;Wasan, H.; Ciardiello, F.; Loupakis, F.; Hong, Y.S.; et al. Encorafenib, Binimetinib, and Cetuximab in BRAFV600E-Mutated Colorectal Cancer. *N. Engl. J. Med*. 2019, 381, 1632–1643

28. Agaimy, A.; Rau, T.T.; Hartmann, A.; Stoehr, R. SMARCB1 (INI1)-negative rhabdoid carcinomas of the gastrointestinal tract: Clinicopathologic and molecular study of a highly aggressive variant with literature review. Am. J. Surg. Pathol. 2014, 38, 910–920.

29. Elena Bolzacchini, Nunzio Digiacomo, Cristina Marrazzo , Nora Sahnane, Roberta Maragliano , Anthony Gill , Luca Albarello , Fausto Sessa , Daniela Furlan and Carlo Capella. BRAF Mutation in Colorectal

Rhabdoid and Poorly Differentiated Medullary Carcinomas. Cancers, 2019

30. Agaimy, A. The expanding family of SMARCB1(INI1)-deficient neoplasia: Implications of phenotypic, biological, and molecular heterogeneity. Adv. Anat. Pathol. 2014, 21, 394–410.

31. Pancione, M.; Remo, A.; Sabatino, L.; Zanella, C.; Votino, C.; Fucci, A.; Di Blasi, A.; Lepore, G.; Daniele, B.; Fenizia, F.; et al. Right-sided rhabdoid colorectal tumors might be related to the serrated pathway. Diagn. Pathol. 2013, 20, 31

32. Remo, A.; Manfrin, E.; Parcesepe, P.; Ferrarini, A.; Han, H.S.; Mickys, U.; Laudanna, C.; Simbolo, M.; Malanga, D.; Oliveira, D.M.; et al. Centrosome Linker-induced Tetraploid Segregation Errors Link Rhabdoid Phenotypes and Lethal Colorectal Cancers. Mol. Cancer Res. 2018, 16, 1385–1395.

33. Michel Ducreux, Ali Chamseddine , Pierre Laurent-Puig, Cristina Smolenschi, Antoine Hollebecque, Peggy Dartigues, Emmanuelle Samallin, Valérie Boige, David Malka and Maximiliano Gelli. Molecular targeted therapy of BRAF-mutant colorectal cancer. Therapeutic Advances in Medical Oncology, 2019.

34. David Barras. BRAF Mutation in Colorectal Cancer: An Update. 2015

35. Seligmann JF, Fisher D, Smith CG, et al. Investigating the poor outcomes of BRAF-mutant advanced colorectal cancer: analysis from 2530 patients in randomised clinical trials. Ann Oncol. 2017; 28: 562–568.

36. Seppala TT, Bohm JP, Friman M, et al. Combination of microsatellite instability and BRAF mutation status for subtyping colorectal cancer. Br J Cancer 2015; 112: 1966–1975.

37. Sorbye H, Dragomir A, Sundstrom M, et al. High BRAF mutation frequency and marked survival differences in subgroups according to KRAS/BRAF mutation status and tumor tissue availability in a prospective population-based metastatic colorectal cancer cohort. PLoS One, 2015; 10: e0131046.

38. Sahin IH, Kazmi SM, Yorio JT, et al. Rare though not mutually exclusive: a report of three cases of concomitant KRAS and BRAF mutation and a review of the literature. J Cancer 2013; 4: 320–322.

39. Dienstmann R. Tumor side as model of integrative molecular classification of colorectal cancer. Clin Cancer Res 2018; 24: 989–990.

40. Tran B, Kopetz S, Tie J, et al. Impact of BRAF mutation and microsatellite instability on the pattern of metastatic spread and prognosis in metastatic colorectal cancer. Cancer 2011;117:4623–4632.

41. Venderbosch S, Nagtegaal ID, Maughan TS, et al. Mismatch repair status and BRAF mutation status in metastatic colorectal cancer patients: a pooled analysis of the CAIRO, CAIRO2, COIN, and FOCUS studies. Clin Cancer Res 2014;20:5322–5330.

42. Fatima Domenica Elisa De Palma, Valeria D'Argenio , Jonathan Pol, Guido Kroemer Maria Chiara Maiuri and Francesco Salvatore. The Molecular Hallmarks of the Serrated Pathway in Colorectal Cancer. Cancers, 2019.

43. Fotios Loupakis, Roberto Moretto, Giuseppe Aprile, Marta Muntoni, Chiara Cremolini, Donatella Iacono, Mariaelena Casagrande, Laura Ferrari, Lisa Salvatore, Marta Schirripa,

Daniele Rossini, Giovanna De Maglio, Gianpiero Fasola, Lorenzo Calvetti, Sara Pilotto, Luisa Carbognin, Gabriella Fontanini , Giampaolo Tortora, Alfredo Falcone, Isabella Sperduti and Emilio Bria. Clinico-pathological nomogram for predicting BRAF mutational status of metastatic colorectal cancer. BJC, 2016 114, 30-36.

44. Barras, D.; Missiaglia, E.; Wirapati, P.; Sieber, O.M.; Jorissen, R.N.; Love, C.; Molloy, P.L.; Jones, I.T.; McLaughlin, S.; Gibbs, P.; et al. BRAFV600E mutant colorectal cancer subtypes based on gene expression. Clin. Cancer Res. 2017, 23, 104–115.

45. Catherine E. Bond and Vicki L. J. Whitehall. How the BRAF V600E Mutation Defines a Distinct Subgroup of Colorectal Cancer: Molecular and Clinical Implications. Gastroenterology Research and Practice, 2018.

46. Cremolini C, Di Bartolomeo M, Amatu A, et al. BRAF codons 594 and 596 mutations identify a new molecular subtype of metastatic colorectal cancer at favorable prognosis. Ann Oncol 2015;26:2092–2097.

47. Jones JC, Kipp B, Leal AD, Voss JS, Hubbard JM, McWilliams RR, Grothey A. Commonality and clinical, pathological, and prognostic characteristics of non-V600E BRAF mutations (BRAFMut) in metastatic colorectal cancers (mCRC) compared to V600 BRAFMut CRCs. J Clin Oncol. 2016; 34:abstr 3529.

48. Yao Z, Yaeger R, Rodrik-Outmezguine VS, et al. Tumours with class 3 BRAF mutants are sensitive to the inhibition of activated RAS. Nature 2017; 548: 234–238.

49. Zhan Yao, Neilawattie M. Torres, Anthony Tao, Yijun Gao, Lusong Luo, Qi Li, Elisa de Stanchina, Omar Abdel-Wahab, David B. Solit, Poulikos Poulikakos, and Neal Rosen. BRAF mutants evade ERK dependent feedback by different mechanisms that determine their sensitivity to pharmacologic inhibition. Cancer Cell; 2016.

50. Schirripa, M.; Biason, P.; Lonardi, S.; Pella, N.; Pino, M.S.; Urbano, F.; Antoniotti, C.; Cremolini, C.; Corallo, S.; Pietrantonio, F.; et al. Class 1, 2 and 3 BRAF mutated metastatic colorectal cancer: A detailed clinical, pathological and molecular characterization. Clin. Cancer Res. 2019, 25, 3954–3961.

51. Sinicrope FA. Lynch syndrome-associated colorectal cancer. N Engl J Med 2018; 379:764-73.

52. J. H. Kim, Y. Y. Rhee, J. M. Bae, N. Y. Cho, and G. H. Kang. Loss of CDX2/CK20 expression is associated with poorly differentiated carcinoma, the CpG island methylator phenotype and adverse prognosis in microsatellite-unstable colorectal cancer. The American Journal of Surgical Pathology, vol. 37, no. 10, pp. 1532–1541, 2013.

53. I. Zlobec, M. Bihl, A. Foerster, A. Rufle, and A. Lugli. Comprehensive analysis of CpG island methylator phenotype (CIMP)-high, -low, and -negative colorectal cancers based on protein marker expression and molecular features. The Journal of Pathology, vol. 225, no. 3, pp. 336–343, 2011.

54. R. Rad, J. Cadiñanos, L. Rad et al. A genetic progression model of BrafV600E-induced intestinal tumorigenesis reveals targets for therapeutic intervention. Cancer Cell, vol. 24, no. 1, pp. 15–29, 2013

55. J. Guinney, R. Dienstmann, X. Wang et al., “The consensus molecular subtypes of

colorectal cancer," Nature Medicine, vol. 21, no. 11, pp. 1350–1356, 2015.

56. Javier Molina-Cerrillo, María San Román, Javier Pozas, Teresa Alonso-Gordoa, Miguel Pozas, Elisa Conde, Marta Rosas, Enrique Grande, María Laura García-Bermejo 4 and Alfredo Carrato. BRAF Mutated Colorectal Cancer: New Treatment Approaches. Cancers.

57. Lenz, H.J.; Ou, F.S.; Venook, A.P.; Hochster, H.S.; Niedzwiecki, D.; Goldberg, R.M.; Mayer, R.J.; Bertagnolli, M.M.; Blanke, C.D.; Zemla, T.; et al. Impact of consensus molecular subtype on survival in patients with metastatic colorectal cancer: Results from CALGB/SWOG 80405 (Alliance). J. Clin. Oncol. 2019, 37, 1876–1885

58. Stintzing, S.; Wirapati, P.; Lenz, H.J.; Neureiter, D.; Fischer vonWeikersthal, L.; Decker, T.; Kiani, A.; Kaiser, F.; Al-Batran, S.; Heintges, T.; et al. Consensus molecular subgroups (CMS) of colorectal cancer (CRC) and first-line e cacy of FOLFIRI plus cetuximab or bevacizumab in the FIRE3 (AIO KRK-0306) trial. Ann. Oncol. 2019, 30, 1796–1803.

59. Barbara Leggett, Vicki Whitehall. Role of the Serrated Pathway in Colorectal Cancer Pathogenesis. Gastroenterology 2010;138:2088–2100.

60. Wajapeyee, N.; Serra, R.W.; Zhu, X.; Mahalingam, M.; Green, M.R. Role for IGFBP7 in senescence induction by BRAF. Cell 2010, 141, 746–747.

61. Kriegl, L.; Neumann, J.; Vieth, M.; Greten, F.R.; Reu, S.; Jung, A.; Kirchner, T. Up and downregulation of p16(Ink4a) expression in BRAF-mutated polyps/adenomas indicates a senescence barrier in the serrated route to colon cancer. Mod. Pathol. 2011, 24, 1015–1022.

62. Patai, Á.V.; Molnár, B.; Tulassay, Z.; Sipos, F. Serrated pathway: Alternative route to colorectal cancer. World J. Gastroenterol. 2013, 19, 607–615

63. Bettington, M.; Walker, N.; Clouston, A.; Brown, I.; Leggett, B.; Whitehall, V. The serrated pathway to colorectal carcinoma: Current concepts and challenges. Histopathology 2013, 62, 367–386.

64. M. L. Bettington, N. I. Walker, C. Rosty et al., "A clinicopathological and molecular analysis of 200 traditional serrated adenomas," Modern Pathology, vol. 28, no. 3, pp. 414–427, 2015.

65. Tsai, J.-H.; Liau, J.-Y.; Lin, Y.-L.; Lin, L.-I.; Cheng, Y.-C.; Cheng, M.-L.; Jeng, Y.-M. Traditional serrated adenoma has two pathways of neoplastic progression that are distinct from the sessile serrated pathway of colorectal carcinogenesis. Mod. Pathol. 2014, 27, 1375–1385.

66. Ehsan Nazemalhosseini Mojarad, Peter JK Kuppen, Hamid Asadzadeh Aghdaei, Mohammad Reza Zali. The CpG island methylator phenotype (CIMP) in colorectal cancer. Review, Gastroenterology and Hepatology From Bed to Bench; 2013.

67. Lao VV, Grady WM. Epigenetics and colorectal cancer. Nat Rev Gastroenterol Hepatol 2011; 8:686-700.

68. Yagi, K.; Akagi, K.; Hayashi, H.; Nagae, G.; Tsuji, S.; Isagawa, T.; Midorikawa, Y.; Nishimura, Y.; Sakamoto, H.; Seto, Y.; et al. Three DNA methylation epigenotypes in human colorectal cancer. Clin. Cancer Res. 2010, 16, 21–33.

69. Ogino, S.; Kawasaki, T.; Kirkner, G.J.; Kraft, P.; Loda, M.; Fuchs, C.S. Evaluation of markers for CpG island methylator phenotype (CIMP) in colorectal cancer by a large population-based sample. J. Mol. Diagn. 2007, 9, 305–314.

70. Ogino, S.; Cantor, M.; Kawasaki, T.; Brahmandam, M.; Kirkner, G.J.; Weisenberger, D.J.; Campan, M.; Laird, P.W.; Loda, M.; Fuchs, C.S. CpG island methylator phenotype (CIMP) of colorectal cancer is best characterised by quantitative DNA methylation analysis and prospective cohort studies. Gut 2006, 55, 1000–1006.

71. Hawkins, N.; Norrie, M.; Cheong, K.; Mokany, E.; Ku, S.-L.; Meagher, A.; O'Connor, T.; Ward, R. CpG island methylation in sporadic colorectal cancers and its relationship to microsatellite instability. Gastroenterology 2002, 122, 1376–1387.

72. Toyota, M.; Ahuja, N.; Ohe-Toyota, M.; Herman, J.G.; Baylin, S.B.; Issa, J.P. CpG island methylator phenotype in colorectal cancer. Proc. Natl. Acad. Sci. USA 1999, 96, 8681–8686.

73. Weisenberger, D.J.; Siegmund, K.D.; Campan, M.; Young, J.; Long, T.I.; Faasse, M.A.; Kang, G.H.; Widschwendter, M.;Weener, D.; Buchanan, D.; et al. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. Nat. Genet. 2006, 38, 787–793.

74. Minggang Fang, Jianhong Ou, Lloyd Hutchinson, and Michael R. Green. The BRAF Oncoprotein Functions Through the Transcriptional Repressor MAFG to Mediate the CpG Island Methylator Phenotype. Molecular Cell, 2014.

75. Shiovitz, S.; Bertagnolli, M.M.; Renfro, L.A.; Nam, E.; Foster, N.R.; Dzieciatkowski, S.; Luo, Y.; Lao, V.V.; Monnat, R.J.; Emond, M.J.; et al. CpG island methylator phenotype is associated with response to adjuvant irinotecan-based therapy for stage III colon cancer. Gastroenterology 2014, 147, 637–645.

76. Richman SD, Seymour MT, Chambers P, et al. KRAS and BRAF mutations in advanced colorectal cancer are associated with poor prognosis but do not preclude benefit from oxaliplatin or irinotecan: results from the MRC FOCUS trial. J Clin Oncol 2009;27:5931–5937.

77. Venderbosch S, Nagtegaal ID, Maughan TS, et al. Mismatch repair status and BRAF mutation status in metastatic colorectal cancer patients: a pooled analysis of the CAIRO, CAIRO2, COIN, and FOCUS studies. Clin Cancer Res 2014;20:5322–5330.

78. Innocenti, F.; Ou, F.S.; Qu, X.; Zemla, T.J.; Niedzwiecki, D.; Tam, R.; Mahajan, S.; Goldberg, R.M.; Bertagnolli, M.M.; Blanke, C.D.; et al. Mutational analysis of patients with colorectal cancer in CALGB/SWOG 80405 identifies new roles of microsatellite instability and tumor mutational burden for patient outcome. J. Clin. Oncol. 2019, 37, 1217–1227.

79. Modest, D. P., Ricard, I., Heinemann, V., Hegewisch-Becker, S., Schmiegel, W., Porschen, R. et al. Outcome according to KRAS-, NRAS-and BRAF-mutation as well as KRAS mutation variants: pooled analysis of five randomized trials in metastatic colorectal cancer by the AIO colorectal cancer study group. Ann. Oncol. 27, 1746–1753 (2016).

80. Seligmann J, Fisher D, Elliott F, et al. Exploring the poor outcomes of BRAFmutant (BRAF Mut) advanced colorectal cancer (aCRC): analysis from 2,530 patients (pts) in randomized clinical trials (RCTs). J Clin Oncol. 2015;33:abstr 3509.

81. Tran B, Kopetz S, Tie J, et al. Impact of BRAF mutation and microsatellite instability on the pattern of metastatic spread and prognosis in metastatic colorectal cancer. Cancer 2011;117:4623–4632.

82. Hegewisch-Becker, S.; Nopel-Dunnebacke, S.; Hinke, A.; Graeven, U.; Reinacher-Schick, A.; Hertel, J.; Lerchenmuller, C.A.; Killing, B.; Depenbusch, R.; Al-Batran, S.E.; et al. Impact of primary tumour location and RAS/BRAF mutational status in metastatic colorectal cancer treated with first-line regimens containing oxaliplatin and bevacizumab: Prognostic factors from the AIO KRK0207 first-line and maintenance therapy trial. Eur. J. Cancer 2018, 101, 105–113.

83. Gavin PG, Colangelo LH, Fumagalli D, et al. Mutation profiling and microsatellite instability in stage II and III Colon cancer: an assessment of their prognostic and oxaliplatin predictive value. Clin Cancer Res. 2012;18:6531–41.

84. Hutchins G, Southward K, Handley K, et al. Value of mismatch repair, KRAS, and BRAF mutations in predicting recurrence and benefits from chemotherapy in colorectal cancer. J Clin Oncol. 2011;29:1261–70.

85. French AJ, Sargent DJ, Burgart LJ, Foster NR, Kabat BF, Goldberg R, Shepherd L, Windschitl HE, Thibodeau SN. Prognostic significance of defective mismatch repair and BRAF V600E in patients with colon cancer. Clin Cancer Res. 2008;14:3408–15.

86. André T, de Gramont A, Vernerey D, et al. Adjuvant fluorouracil, leucovorin, and oxaliplatin in stage II to III Colon cancer: updated 10-year survival and outcomes according to BRAF mutation and mismatch repair status of the MOSAIC study. J Clin Oncol. 2015;33:4176–87.

87. Farina-Sarasqueta, A.; van Lijnschoten, G.; Moerland, E.; Creemers, G.J.; Lemmens, V.E.; Rutten, H.J.; van den Brule, A.J. The BRAFV600E mutation is an independent prognostic factor for survival in stage II and stage III colon cancer patients. Ann. Oncol. 2010, 21, 2396–2402.

88. RothAD, Tejpar S, DelorenziM, et al. 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. 2010;28:466–74.

89. Taieb J, ZaananA, LeMalicot K, et al. Prognostic effect of BRAF and KRAS mutations in patients with stage III colon cancer treated with leucovorin, fluorouracil, and oxaliplatin with or without cetuximab: a post hoc analysis of the PETACC-8 trial. JAMA Oncol. 2016; 2:643.

90. Ogino S, Shima K, Meyerhardt JA, McCleary NJ, NgK, Hollis D,et al. Predictive and prognostic roles of BRAF mutation in stage III colon cancer: Results from intergroup trial CALGB89803. Clin CancerRes (2012) 18:890–900.

91. Kalady MF, Dejulius KL, Sanchez JA, Jarrar A, Liu X, Manilich E, Skacel M, Church JM. BRAF mutations in colorectal cancer are associated with distinct clinical characteristics and worse prognosis. Dis. Colon Rectum (2012) 55:128–33.

92. Sinicrope, F.A.; Mahoney, M.R.; Smyrk, T.C.; Thibodeau, S.N.; Warren, R.S.; Bertagnolli, M.M.; Nelson, G.D.; Goldberg, R.M.; Sargent, D.J.; Alberts, S.R. Prognostic impact of deficient DNA mismatch repair in patients with stage III colon cancer from randomized trial of FOLFOX-based adjuvant chemotherapy. J. Clin. Oncol. 2013, 31, 3664–3672.

93. Samowitz WS, Sweeney C, Herrick J, Albertsen H, Levin TR, Murtaugh MA, et al. Poor survival associated with the BRAFV600E mutation in microsatellite-stable Colon cancers. CancerRes (2005) 65: 6063–9.

94. V Eklof, M L Wikberg, S Edin, A M Dahlin, B-A Jonsson, Å O¨ berg, J Rutegård and R Palmqvist. The prognostic role of KRAS, BRAF, PIK3CA and PTEN in colorectal cancer. British Journal of Cancer (2013) 108, 2153–2163.

95. Lochhead P,Kuchiba A, Imamura Y, Liao X, Yamauchi M, Nishihara R,etal. Microsatellite instability and BRAFmutation testing in colorectal cancer prognostication. J Natl Cancer Inst (2013) 105:1151–6.

96. Reetesh K. Pai, Priya Jayachandran, Albert C. Koong, Daniel T. Chang, Shirley Kwok, Lisa Ma, Daniel A. Arber, Raymond R. Balise, Raymond R. Tubbs, Bonnie Shadrach, and Rish K. Pai. BRAF-mutated, Microsatellite-stable Adenocarcinoma of the Proximal Colon: An Aggressive Adenocarcinoma With Poor Survival, Mucinous Differentiation, and Adverse Morphologic Features. Am J Surg Pathol. 2012 May ; 36(5): 744–752.

97. Gholamreza Safaee Ardekani, Seyed Mehdi Jafarnejad, Larry Tan, Ardavan Saeedi, Gang Li. The Prognostic Value of BRAF Mutation in Colorectal Cancer and Melanoma: A Systematic Review and Meta-Analysis. PLos One 2012.

98. Yaeger R, Cercek A, Chou JF, et al. BRAF mutation predicts for poor outcomes after metastasectomy in patients with metastatic colorectal cancer. Cancer 2014; 120: 2316–2324.

99. Schirripa M, Bergamo F, Cremolini C, et al. BRAF and RAS mutations as prognostic factors in metastatic colorectal cancer patients undergoing liver resection. Br J Cancer 2015; 112: 1921–1928.

100. Cremolini C, Casagrande M, Loupakis F, et al. Efficacy of FOLFOXIRI plus bevacizumab in liver-limited metastatic colorectal cancer: a pooled analysis of clinical studies by Gruppo Oncologico del Nord Ovest. Eur J Cancer 2017; 73: 74–84.

101. Johnson, B.; Jin, Z.; Truty, M.J.; Smoot, R.L.; Nagorney, D.M.; Kendrick, M.L.; Kipp, B.R.; Grothey, A. Impact of metastasectomy in the multimodality approach for BRAFV600E metastatic colorectal cancer: The Mayo Clinic experience. Oncologist 2018, 23, 128–134.

102. Bachet JB, Moreno-Lopez N, Viganò L, et al. What is the prognostic impact of BRAF mutation in patients undergoing resection of colorectal liver metastases? Results of nationwide intergroup (ACHBT, FRENCH, AGEO) cohort of 249 patients. J Clin Oncol 2018; 36: 3554.

103. Gagniere J, Dupre A, Gholami SS, et al. Is hepatectomy justified for BRAF mutant colorectal liver metastases? a multi-institutional analysis of 1497 patients. Ann Surg. 2018.

104. Margonis GA, Buettner S, Andreatos N, et al. Association of BRAF mutations with survival and recurrence in surgically treated patients with metastatic colorectal liver cancer. JAMA Surg. 2018.

105. Julien Taieb, Alexandra Lapeyre-Prost, Pierre Laurent Puig and Aziz Zaanan. Exploring the best treatment options for BRAF-mutant metastatic colon cancer. Review. BJC 2019.

106. Morris, V., Overman, M. J., Jiang, Z.-Q., Garrett, C., Agarwal, S., Eng, C. et al. Progression free survival remains poor over sequential lines of systemic therapy in patients with BRAF-mutated colorectal cancer. Clin. Colorectal Cancer 13, 164–171 (2014).

107. Seligmann J, Fisher D, Elliott F, et al. Exploring the poor outcomes of BRAFmutant (BRAF Mut) advanced colorectal cancer (aCRC): analysis from 2,530 patients (pts) in randomized clinical trials (RCTs). J Clin Oncol. 2015.

108. Falcone A. Biweekly chemotherapy with oxaliplatin, irinotecan, infusional fluorouracil, and leucovorin: a pilot study in patients with metastatic colorectal cancer. J Clin Oncol. 2002;20:4006–14.

109. Masi G, Allegrini G, Cupini S, Marcucci L, Cerri E, Brunetti I, Fontana E, Ricci S, Andreuccetti M, Falcone A. First-line treatment of metastatic colorectal cancer with irinotecan, oxaliplatin and 5-fluorouracil/leucovorin (FOLFOXIRI): results of a phase II study with a simplified biweekly schedule. Ann Oncol. 2004;15:1766–72.

110. Falcone A, Ricci S, Brunetti I, et al. Phase III trial of Infusional fluorouracil, leucovorin, oxaliplatin and irinotecan (FOLFOXIRI) compared with Infusional fluorouracil, leucovorin, and irinotecan (FOLFIRI) as first-line treatment for metastatic colorectal cancer: the Gruppo Oncologico Nord Ovest. J Clin Oncol. 2007.

111. Masi G, Loupakis F, Salvatore L, et al. Bevacizumab with FOLFOXIRI (irinotecan, oxaliplatin, fluorouracil, and folinate) as first-line treatment for metastatic colorectal cancer: a phase 2 trial. Lancet Oncol. 2010

112. Loupakis F, Cremolini C, Salvatore L, et al. FOLFOXIRI plus bevacizumab as first-line treatment in BRAF mutant metastatic colorectal cancer. Eur J Cancer. 2014;50:57–63.

113. Loupakis F, Cremolini C, Masi G, et al. Initial therapy with FOLFOXIRI and bevacizumab for metastatic colorectal cancer. N Engl J Med. 2014.

114. Cremolini C, Loupakis F, Antoniotti C, et al. FOLFOXIRI plus bevacizumab versus FOLFIRI plus bevacizumab as first-line treatment of patients with metastatic colorectal cancer: updated overall survival and molecular subgroup analyses of the open-label, phase 3 TRIBE study. Lancet Oncol. 2015.

115. Van Cutsem E, Cervantes A, Adam R, et al. ESMO consensus guidelines for the management of patients with metastatic colorectal cancer. Ann Oncol. 2016.

116. Ince, W. L., Jubb, A. M., Holden, S. N., Holmgren, E. B., Tobin, P., Sridhar, M. et al. Association of k-ras, b-raf, and p53 status with the treatment effect of bevacizumab. JNCI J. Natl Cancer Inst. 97, 981–989 (2005).

117. Price, T. J., Hardingham, J. E., Lee, C. K., Weickhardt, A., Townsend, A. R., Wrin, J. W. et al. Impact of KRAS and BRAF gene mutation status on outcomes from the phase III AGITG MAX trial of capecitabine alone or in combination with bevacizumab and mitomycin in advanced colorectal cancer. J. Clin. Oncol. 29, 2675–2682 (2011).

118. Wirapati, P., Pomella, V., Vandenbosch, B., Kerr, P., Maiello, E., Jeffery, G. M. et al. Velour trial biomarkers update: Impact of RAS, BRAF, and sidedness on aflibercept activity. J. Clin. Oncol. (2017).

119. Yoshino, T., Obermannova, R., Bodoky, G., Prausová, J., Garcia-Carbonero, R., Ciuleanu, T.-E. et al. Are BRAF mutated metastatic colorectal cancer (mCRC) tumors more responsive to VEGFR-2 blockage? Analysis of patient outcomes by RAS/RAF mutation status in the RAISE study—a global, randomized, double-blind, phase III study. J. Clin. Oncol. (2018).

120. Van Cutsem, E.; Köhne, C.H.; Láng, I.; Folprecht, G.; Nowacki, M.P.; Cascinu, S.; Shchepotin, I.; Maurel, J.; Cunningham, D.; Tejpar, S.; et al. 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. 2011, 29, 2011–2019.

121. Bokemeyer, C.; van Cutsem, E.; Rougier, P.; Ciardiello, F.; Heeger, S.; Schlichting, M.; Celik, I.; Kohne, C.H. Addition of cetuximab to chemotherapy as first-line treatment for KRAS wild-type metastatic colorectal cancer: Pooled analysis of the CRYSTAL and OPUS randomised clinical trials. Eur. J. Cancer 2012, 48, 1466–1475.

122. Douillard, J.-Y., Oliner, K. S., Siena, S., Tabernero, J., Burkes, R., Barugel, M. et al. Panitumumab–FOLFOX4 treatment and RAS mutations in colorectal cancer. N. Engl. J. Med. 369, 1023–1034 (2013).

123. Geissler, M.; Martens, U.; Knorrenschield, R.; Greeve, J.; Florschuetz, A.; Tannapfel, A.; Wessendorf, F.; Seuerlein, T.; Kanzler, S.; Heinemann, V.; et al. 475O-mFOLFOXIRI + Panitumumab versus FOLFOXIRI as first-line treatment in patients with RAS wild-type metastatic colorectal cancer m(CRC): A randomized phase II VOLFI trial of the AIO (AIO-KRK0109). Ann. Oncol. 2017.

124. Peeters, M., Oliner, K. S., Price, T. J., Cervantes, A., Sobrero, A. F., Ducreux, M. et al. Analysis of KRAS/NRAS mutations in a phase III study of panitumumab with FOLFIRI compared with FOLFIRI alone as second-line treatment for metastatic colorectal cancer. Clin. Cancer Res. 21, 5469–5479 (2015).

125. Seymour, M. T., Brown, S. R., Middleton, G., Maughan, T., Richman, S., Gwyther, S. et al. Panitumumab and irinotecan versus irinotecan alone for patients with KRAS wild-type, fluorouracil-resistant advanced colorectal cancer (PICCOLO): a prospectively stratified randomised trial. Lancet Oncol. 14, 749–759 (2013).

126. Pietrantonio F, Petrelli F, Coinu A, et al. Predictive role of BRAF mutations in patients with advanced colorectal cancer receiving cetuximab and panitumumab: a meta-analysis. Eur J Cancer 2015;51:587–594.

127. Rowland A, Dias MM, Wiese MD, et al. Meta-analysis of BRAF mutation as a

predictive biomarker of benefit from anti-EGFR monoclonal antibody therapy for RAS wild-type metastatic colorectal cancer. Br J Cancer 2015;112:1888–1894.

128. Stintzing, S., Miller-Phillips, L., Modest, D. P., Fischer von Weikersthal, L., Decker, T., Kiani, A. et al. Impact of BRAF and RAS mutations on first-line efficacy of FOLFIRI plus cetuximab versus FOLFIRI plus bevacizumab: analysis of the FIRE-3 (AIO KRK-0306) study. Eur. J. Cancer 79, 50–60 (2017).

129. Kopetz, S.; Desai, J.; Chan, E.; Hecht, J.R.; O'Dwyer, P.J.; Maru, D.; Morris, V.; Janku, F.; Dasari, A.; Chung,W.; et al. Phase II pilot study of vemurafenib in patients with metastatic BRAF-mutated colorectal cancer. J. Clin. Oncol. 2015, 33, 4032–4038.

130. Gomez-Roca, C. A., Delord, J., Robert, C., Hidalgo, M., von Moos, R., Arance, A. et al. 535P Encorafenib (LGX818), an oral BRAF inhibitor, in patients with BRAF V600E metastatic colorectal cancer: results of dose expansion in an open-label, phase I study. Ann. Oncol. 33, 4032–4038 (2015).

131. Hyman, D. M., Puzanov, I., Subbiah, V., Faris, J. E., Chau, I., Blay, J.-Y. et al. Vemurafenib in multiple nonmelanoma cancers with BRAF V600 mutations. N. Engl. J. Med. 373, 726–736 (2015).

132. Yaeger, R.; Cercek, A.; O'Reilly, E.M.; Reidy, D.L.; Kemeny, M.; Wolinsky, T.; Capanu, M.; Gollub, M.J.; Rosen, N.; Berger, M.F.; et al. Pilot trial of combined BRAF and EGFR inhibition in BRAF-mutant metastatic colorectal cancer patients. Clin. Cancer Res. 2015, 21, 1313–1320.

133. Desai, J.; Markman, B.; Ananda, S.; Tebbutt, N.C.; Michael, M.; Solomon, B.J.; McArthur, G.A.; Tie, J.; Gibbs, P.; Ritchie, D.; et al. A phase I/II trial of combined BRAF and EGFR inhibition in patients (pts) with BRAFV600E mutated (BRAFm) metastatic colorectal (mCRC): The EViCT (erlotinib and vemurafenib in combination trial) study. J. Clin. Oncol. 2017.

134. Tabernero, J.; Geel, R.V.; Guren, T.K.; Yaeger, R.D.; Spreafico, A.; Faris, J.E.; Yoshino, T.; Yamada, Y.; Kim, T.W.; Bendell, J.C.; et al. Phase 2 results: Encorafenib (ENCO) and cetuximab (CETUX) with or without alpelisib (ALP) in patients with advanced BRAF-mutant colorectal cancer (BRAFm CRC). J. Clin. Oncol. 2016, 34 (Suppl. 15), 3544.

135. Corcoran, R.B.; André, T.; Yoshino, T.; Bendell, J.C.; Atreya, C.E.; Schellens, J.H.M.; Ducreux, M.P.; McRee, A.; Siena, S.; Middleton, G.; et al. Efficacy and circulating tumor DNA (ctDNA) analysis of the BRAF inhibitor dabrafenib (D), MEK inhibitor trametinib (T), and anti-EGFR antibody panitumumab (P) in patients (pts) with BRAFV600E–mutated (BRAFm) metastatic colorectal cancer (mCRC). Ann. Oncol. 2016.

136. Corcoran, R.B.; Atreya, C.E.; Falchook, G.S.; Kwak, E.L.; Ryan, D.P.; Bendell, J.C.; Hamid, O.; Messersmith, W.A.; Daud, A.; Kurzrock, R.; et al. Combined BRAF and MEK inhibition with dabrafenib and trametinib in BRAF V600-mutant colorectal cancer. J. Clin. Oncol. 2015.

137. Corcoran, R.B.; Andre, T.; Atreya, C.E.; Schellens, J.H.M.; Yoshino, T.; Bendell, J.C.; Hollebecque, A.; McRee, A.J.; Siena, S.; Middleton, G.; et al. Combined BRAF, EGFR, and MEK inhibition in patients with

BRAFV600E-mutant colorectal cancer. Cancer Discov. 2018.

138. ClinicalTrials.gov. Encorafenib, Binimetinib and Cetuximab in Subjects with Previously Untreated BRAF-Mutant Colorectal Cancer (ANCHOR-CRC).

139. ClinicalTrials.gov FOLFOXIRI Plus Cetuximab vs. FOLFOXIRI Plus Bevacizumab 1st-line in BRAF-mutated mCRC (AIO-KRK-0116).

140. ClinicalTrials.gov. Study of WNT974 in Combination with LGX818 and Cetuximab in Patients with BRAF-Mutant Metastatic Colorectal Cancer (mCRC) and Wnt Pathway Mutations.

141. Virginia Alonso-Espinaco, Miriam Cuatrecasas, Vicente Alonso, Pilar Escudero, Maribel Marmol, Carlos Horndler, Javier Ortego, Rosa Gallego, Jordi Codony-Servat, Xabier Garcia-Albeniz, Pedro Jares , Antoni Castells, Juan Jose´ Lozano, Rafael Rosell, Joan Maurel. RAC1b overexpression correlates with poor prognosis in KRAS/BRAF WT metastatic colorectal cancer patients treated with first-line FOLFOX/XELOX chemotherapy. Elsevier 2014.

142. D.T. Le, J.N. Uram, H. Wang, B.R. Bartlett, H. Kemberling, A.D. Eyring et al. PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. NEJM, 2015.

143. Michael J. Overman, Ray McDermott, Joseph L. Leach, Sara Lonardi, et al. Overman Nivolumab in patients with metastatic DNA mismatch repair deficient/microsatellite instability–high colorectal cancer (CheckMate 142): results of an open-label, multicentre, phase 2 study. Lancet Oncology; 2017.

144. Kono, T.; Imai, Y.; Imura, J.; Ono, Y.; Hagiwara, S.; Taira, K.; Fujimori, T. Cecal adenocarcinoma with prominent rhabdoid feature: Report of a case with immunohistochemical, ultrastructural, and molecular analyses. Int. J. Surg. Pathol. 2007, 15, 414–420.

145. Samalavicius, N.E.; Stulpinas, R.; Gasilionis, V.; Baltruskeviciene, E.; Aleknavicius, E.; Mickys, U. Rhabdoid carcinoma of the rectum. Ann. Coloproctol. 2013, 29, 252–255.

146. Lee, S.H.; Seol, H.; Kim, W.Y.; Lim, S.D.; Kim, W.S.; Hwang, T.S.; Han, H.S. Rhabdoid colorectal carcinomas: Reports of two cases. Korean. J. Pathol. 2013, 47, 372–377.

147. Moussaly, E.; Atallah, J.P. A rare case of undi erentiated carcinoma of the colon with rhabdoid features: A case report and review of the literature. Case Rep. Oncol. Med. 2015, 2015, 531348.

148. Kalyan, A.; Pasricha, G.; Monga, D.; Singhi, A.; Bahary, N. Case report of rhabdoid colon cancer and review of literature. Clin. Colorectal. Cancer 2015, 14, e5–e8.

149. Wang, J.; Andrici, J.; Sioson, L.; Clarkson, A.; Sheen, A.; Farzin, M.; Toon, C.W.; Turchini, J.; Gill, A.J. Loss of INI1 expression in colorectal carcinoma is associated with high tumor grade, poor survival, BRAFV600E mutation, and mismatch repair deficiency. Hum. Pathol. 2016, 55, 83–90

150. Agaimy, A.; Daum, O.; Märkl, B.; Lichtmannegger, I.; Michal, M.; Hartmann, A. SWI/SNF Complex-deficient

Undifferentiated/Rhabdoid Carcinomas of the Gastrointestinal Tract: A Series of 13 Cases Highlighting Mutually Exclusive Loss of SMARCA4 and SMARCA2 and Frequent Co-inactivation of SMARCB1 and SMARCA2. Am. J. Surg. Pathol. 2016, 40, 544–553.

151. Oken MM, Creech RH, Tormey DC, et al. Toxicity and response criteria of the Eastern Cooperative Oncology Group. Am J Clin Oncol 1982; 5:649-55.

152. Sahnane N, Magnoli F, Bernasconi B, et al. Aberrant DNA methylation profiles of inherited and sporadic colorectal cancer. Clin Epigenetics 2015; 7:131.

153. Eisenhauer EA, Therasse P, Bogaerts J, et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). Eur J Cancer 2009; 45: 228-47

154. Bosman FT, Carneiro F, Hruban RH, Theise ND. WHO Classification of Tumours of the Digestive System. 4th ed. France: IARC Press; 2010.

155. Nakamura T, Mitomi H, Kanazawa H, Ohkura Y, Watanabe M. Tumor budding as an index to identify high-risk patients with stage II colon cancer. Dis Colon Rectum 2008; 51:568-72.

156. Kasajima A, Sers C, Sasano H, et al. Down-regulation of the antigen processing machinery is linked to a loss of inflammatory response in colorectal cancer. Hum Pathol 2010; 41:1758-69.

157. Sahnane, N.; Furlan, D.; Monti, M.; Romualdi, C.; Vanoli, A.; Vicari, E.; Solcia, E.; Capella, C.; Sessa, F.; La Rosa, S. Microsatellite unstable gastrointestinal neuroendocrine carcinomas: A new clinicopathologic entity. Endocr. Relat. Cancer 2015, 22, 35–45.

158. Droeser RA, Hirt C, Viehl CT, et al. Clinical impact of programmed cell death ligand 1 expression in colorectal cancer. Eur J Cancer 2013.

159. Song M, Chen D, Lu B, et al. PTEN loss increases PD-L1 protein expression and affects the correlation between PD-L1 expression and clinical parameters in colorectal cancer. PLoS One 2013.

160. Lee KS, Kwak Y, Ahn S, et al. Prognostic implication of CD274 (PD-L1) protein expression in tumor-infiltrating immune cells for microsatellite unstable and stable colorectal cancer. Cancer Immunol Immunother 2017; 66:927-39.

161. Lee LH, Cavalcanti MS, Segal NH, et al. Patterns and prognostic relevance of PD-1 and PD-L1 expression in colorectal carcinoma. Mod Pathol 2016.

162. Masugi Y, Nishihara R, Yang J, et al. Tumour CD274 (PD-L1) expression and T cells in colorectal cancer. Gut 2017.

163. Geiger, T.R.; Peeper, D.S. Metastasis mechanisms. Biochim. Biophys. Acta 2009, 1796, 293–308.

164. Thiery, J.P.; Sleeman, J.P. Complex networks orchestrate epithelial-mesenchymal transitions. Nat. Rev. Mol. Cell Biol. 2006, 7, 131–142

165. Ryu, H.S.; Chung, J.H.; Lee, K.; Shin, E.; Jing, J.; Choe, G.; Kim, H.; Xu, X.; Lee, H.E.; Kim, D.G.; et al. Overexpression of epithelial-mesenchymal transition-related markers according to cell dedifferentiation: Clinical implications as an independent predictor of

poor prognosis in cholangiocarcinoma. Hum. Pathol. 2012, 43, 2360–2370

166. Choi, J.E.; Bae, J.S.; Kang, M.J.; Chung, M.J.; Jang, K.Y.; Park, H.S.; Moon, W.S. Expression of epithelial-mesenchymal transition and cancer stem cell markers in colorectal adenocarcinoma: Clinicopathological significance. Oncol. Rep. 2017.

167. De Smedt, L.; Palmans, S.; Andel, D.; Govaere, O.; Boeckx, B.; Smeets, D.; Galle, E.; Wouters, J.; Barras, D.; Sotti, M.; et al. Expression profiling of budding cells incolorectal cancer reveals an EMT-like phenotype and molecular subtype switching. Br. J. Cancer 2017, 116, 58–65.

168. Harikrishnan, K.N.; Chow, M.Z.; Baker, E.K.; Pal, S.; Bassal, S.; Brasacchio, D.; Wang, L.; Craig, J.M.; Jones, P.L.; Sif, S.; et al. Brahma links the SWI/SNF chromatin-remodeling complex with MeCP2-dependent transcriptional silencing. Nat. Genet. 2005

169. Sévenet, N.; Lellouch-Tubiana, A.; Schofield, D.; Hoang-Xuan, K.; Gessler, M.; Birnbaum, D.; Jeanpierre, C.; Jouvet, A.; Delattre, O. Spectrum of hSNF5/INI1 somatic mutations in human cancer and genotype-phenotype correlations. Hum. Mol. Genet. 1999

170. Mizuno, R.; Kawada, K.; Itatani, Y.; Ogawa, R.; Kiyasu, Y.; Sakai, Y. The Role of Tumor-Associated Neutrophils in Colorectal Cancer. Int. J. Mol. Sci. 2019, 20, 529.

171. Rao, H.L.; Chen, J.W.; Li, M.; Xiao, Y.B.; Fu, J.; Zeng, Y.X.; Cai, M.Y.; Xie, D. Increased intratumoral neutrophil in colorectal carcinomas correlates closely with malignant phenotype and predicts patients' adverse prognosis. PLoS ONE 2012,

172. Cremolini, C.; Antoniotti, C.; Rossini, D.; Lonardi, S.; Loupakis, F.; Pietrantonio, F.; Bordonaro, R.; Latiano, T.P.; Tamburini, E.; Santini, D.; et al. 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, randomised, controlled trial. Lancet Oncol. 2020, 21, 497–507.

173. Cremolini, C.; Antoniotti, C.; Stein, A.; Bendell, J.; Gruenberger, T.; Rossini, D.; Masi, G.; Ongaro, E.; Hurwitz, H.; Falcone, A.; et al. Individual Patient Data Meta-Analysis of FOLFOXIRI Plus Bevacizumab Versus Doublets Plus Bevacizumab as Initial Therapy of Unresectable Metastatic Colorectal Cancer. J. Clin. Oncol. 2020, 38, JCO2001225.

174. André, T.; Shiu, K.-K.; Kim, T.W.; Jensen, B.V.; Jensen, L.H.; Punt, C.; Smith, D.; Garcia-Carbonero, R.; Benavides, M.; Gibbs, P.; et al. Pembrolizumab in Microsatellite-Instability-High Advanced Colorectal Cancer. N. Engl. J. Med. 2020, 383, 2207–2218.

175. FDA Approves Pembrolizumab for First-Line Treatment of MSI-H/dMMR Colorectal Cancer. Available online: https://www.fda. gov/drugs/drug-approvals-

and-databases/fda-approves-pembrolizumab-first-line-treatment-msi-hdmmr-colorectal-cancer (accessed on 23 December 2020).

176. New Indication Concerns the First-Line Treatment of Metastatic MSI-H or dMMR Colorectal Cancer. Available online: https://www.esmo.org/oncology-news/ema-recommends-extension-of-indications-for-pembrolizumab4 (accessed on 23 December 2020).

177. Loupakis, F.; Intini, R.; Cremolini, C.; Orlandi, A.; Sartore-Bianchi, A.; Pietrantonio, F.; Pella, N.; Spallanzani, A.; Dell'Aquila, E.; Scartozzi, M.; et al. A validated prognostic classifier for V600EBRAF-mutated metastatic colorectal cancer: The "BRAF BeCool" study. Eur. J. Cancer 2019, 118, 121–130.

178. Gianluca Mauri et al. The Evolutionary Landscape of Treatment for BRAFV600E Mutant Metastatic Colorectal Cancer. Cancer 2020. Review.

APPENDIX

Table 5: Reported colorectal carcinomas with Rhabdoid features

N°	References	Year of publication	Age	Gender	Size (cm)	Site	Clinical presentation	Type	Site of metastasis	Treatment	Outcome*
1	Bak and Teglbjaerg (Case 1)	1989	86	F	12	C	retrocecal abscess	Pure	L	Palliative	1 w
2	Bak and Teglbjaerg (Case 2)	1989	82	M	5	LC	weight loss; abdominal pain	Pure	-	Surgery	1 m
3	Chetty and Bhathal	1993	72	F	6	C	abdominal mass	Composite	L, N	Surgery	3 m
4	Yang et al.	1994	75	M	15	RC	abdominal mass	Pure	N	Surgery	2 w
5	Macak and Kodet	1995	50	M	6	R	rectal bleeding; constipation	Composite	N	Surgery+RCT	6 m
6	Marcus et al.	1996	84	F	7	LC	abdominal mass	Pure	None	Surgery	12 m (alive)
7	Nakamura et al.	1999	76	M	14	C	Abdominal pain	Pure	L, N	Surgery	3 m
8	Kono et al.	2007	66	M	13	C	Abdominal mass, anemia	Composite	P, N	Surgery	1.5 m
9	Oh et al.	2008	69	F	3.5	LC	Blood in stools	Composite	N, L	-	6 m
10	Mastoraki et al.	2009	62	F	10	LC	Abdominal pain	Pure	L	Surgery	4 m
11	Pancione et al.	2011	71	F	10	C	Abdominal pain	Pure	P, L, N	Surgery+CT+Bev+Cet	8 m
12	Han et al.	2010	23	F	6	R	diarrhea	Pure	None	Surgery+CT	17 m (alive)
13	Remo et al.	2012	73	F	10	RC	Rectal bleeding	Composite	N	CT	6 m
14	Lee et al. case 1	2013	62	M	4.5	LC	Occult blood in stool	Composite	N	Surgery+CT	36 m (alive)
15	Lee et al. case 2	2013	83	M	6.5	R	Rectal mass	Composite	L, LU, N	Surgery	1 m
16	Samalavicus et al.	2013	49	M	7	R	loss	Composite	N, L	Surgery+CT	7 m
17	Baba et al.	2014	45	F	-	LC	Abdominal pain	-	P	Surgery	6 w
18	Romera Barba et al.	2014	77	M	-	LC	Abdominal pain	Pure	P, N, L	Surgery	2 m
19	Agaimy et al.	2014	79	M	9	C	-	Pure	N		6 m
20	Moussaly et al.	2015	87	F	12	RC	Abdominal mass	Pure	P, N, SP, Lu	Surgery	2 m
21	Cho et al.	2015	73	M	4	C	Abdominal pain	Composite	P, L, N, B		2 m
22	Kalyan et al.	2015	31	F	7	C	Pain, weight loss	Composite	L, N, B	Surgery+RCT	4 m
23	Dhavaleshwar et al	2015	31	F	9	C	Abdominal pain	Pure	L, N	Surgery+CT	4 m
24	Agaimy et al. (Case1)	2016	32	M	-	-	-	Composite	-	Surgery	-
25	Agaimy et al. (Case2)	2016	34	F	-	RC	-	Composite	L	Biopsy	-
26	Agaimy et al. (Case3)	2016	50	M	-	RC	-	Composite	-	Surgery	-
27	Wang et al. (Case 1)	2016	56	F	7,5	C	-	Composite	N		15 m
28	Wang et al. (Case 2)	2016	76	M	8	RC	-	Composite	N		13 m (alive)
29	Wang et al. (Case 3)	2016	72	F	6,5	LC	-	Pure	N		5 m
30	D'Amico et al.	2018	65	M	10	RC	Abdominal pain, weakness	Pure	None	Surgery	48 m (alive)
31	Remo et al. Case 1 (RC6)	2018	63	M	-	LC	-	-	N	-	1 m
32	Remo et al. Case 2 (RC7)	2018	71	F	-	C	-	-	N		8 m
33	Remo et al. Case 3 (RC8)	2018	71	F	-	R	-	-	N		26 m (alive)
34	Remo et al. Case 4 (RC9)	2018	76	F	-	RC	-	-	None		6 m (alive)
35	Remo et al. Case 5 (RC10)	2018	65	F	-	RC	-	-	None		7 m
36	Remo et al. Case 6 (RC11)	2018	81	M	-	RC	-	-	N		33 m (alive)
37	Remo et al. Case 7 (RC12)	2018	73	M	-	RC	-	-	N		6 m (alive)
38	Remo (Seok)	2018	63	M	-	C	-	Pure	NS		-
39	Remo (Hoon-kuy)	2018	69	F	-	LC	-	Composite	None		6 m
40	This study (84-1522)	2019	63	F	10	RC	-	Pure	N	Surgery	2 m
41	This study (86-3722)	2019	76	F	4	LC	-	Pure	-	Surgery+CT	7 m
42	This study (87-3203)	2019	85	M	6	LC	-	Pure	N, L	Surgery	2 m
43	This study (99-17491)	2019	65	M	6	C	-	Pure	N	Surgery	216 m (alive)
44	This study (08-1679)	2019	63	M	6	LC	-	Pure	N	Surgery	10 m
45	This study (05-2235)	2019	64	M	6	RC	-	Composite	-	Surgery	-
46	This study (HSR)	2019	77	F	7	RC	-	Composite	None	Surgery	187 m (alive)

Legend: -: Not Available; Not Specified: NS; Cecum: C; Right colon: RC; Left colon: LC; Rectum: R; Lymph nodes: N: lymph nodes; Liver: L; Bone:B; Peritoneum:P; Spleen:SP; Lung: Lu; Month: m; Week: w; CRT: Chemioradiotherapy adjuvant; CT: Chemiotherapy; Bev: Bevacizumab; Cet: Cetuximab. *, time from diagnosis to death was reported (or to the last follow-up if the patient was alive)

Table 8. Antibodies Used and Immunohistochemical Protocols

Primary Antibody	Clone	Working Solution	Treatment	Manufacturer
CD3 (rabbit monoclonal)	2GV6	Pure	MW 5 min'×2 CB, pH 6	Ventana
CD8 (rabbit monoclonal)	SP57	1:2	MW 5 min'×2 CB, pH 6	Ventana
PD-L1 (rabbit monoclonal)	SP142	1:40	MW 5 min'×4 EDTA, pH 8	Spring
p53 (mouse monoclonal)	DO-7	1:500	MW 5 min'×4 CB, pH 6	Dako
CDX2 (mouse monoclonal)	CDX2-88	1:50	MW 5 min'×4 CB, pH 6	Biocare
Ki-67 (mouse monoclonal)	MIB-1	1:100	MW 5 min'×4 CB, pH 6	Dako
Synaptophysin (rabbit polyclonal)	Polyclonal	1:2	MW 5 min'×4 CB, pH 6	Ventana
EMA	E29	1:100	MW 5 min'×2 CB, pH 6	Dako
CK AE1/AE3	PCK26	1:2	MW 5 min'×4 CB, pH 6	Ventana
CK7	SP42	1:2	MW 5 min'×2 CB, pH 6	Ventana
CK20	KS20.8	1:100	MW 5 min'×2 CB, pH 6	Dako
b-catenin	14	Pure	MW 5 min'×4 CB, pH 6	Ventana
SMARCB1(INI-1)	MRQ27	Pure	MW 5 min'×6 CB, pH 6	Ventana
Vimentin	V9	Pure	MW 5 min'×2 CB, pH 6	Ventana

Abbreviations: CB=citric acid antigen retrieval buffer; EDTA =ethylenediaminetetraacetic acid; MW= microwave antigen retrieval solution.

Immunohistochemistry was performed manually; formalin-fixed paraffin-embedded sections were mounted on poly-L-lysine–coated slides, deparaffinized, and hydrated through graded alcohol to water. Endogenous peroxidase activity was quenched in 3% hydrogen peroxide in water for 20 minutes; proteolytic treatment was performed using different antigen-retrieval solutions (CB, pH 6; or EDTA, pH 8) in a domestic 750-kW microwave oven. Primary antibodies were applied overnight at 4°C and immunostained using the avidin-biotin-peroxidase complex (ABC) method or the MACH4 system. For ABC method, the sections were incubated with biotinylated anti-mouse immunoglobulins and ABC complex, each for 1 hour at room temperature. The immunoreaction was developed with 3.3'-diaminobenzidine tetrahydrochloride as chromogen and nuclei were counterstained with hematoxylin. Finally, the sections were dehydrated.

Table 9. List of the eight gene promoters analyzed for hypermethylation status using SALSA MS-MLPA ME042-C1 CIMP Kit

ME042-C1 PROBES	Length	Chr band	hg18	CUT-OFF FOR METHYLATION
RUNX3-2	346	01p36.11	01-025.128720	0.39
RUNX3-2	371	01p36.11	01-025.128920	0.29
RUNX3-2	258	01p36.11	01-025.129596	0.41
MLH1-1	355	03p22.2	03-037.009361	0.41
MLH1-1	463	03p22.2	03-037.009621	0.36
MLH1-1	130	03p22.2	03-037.009769	0.4
MLH1-1	178	03p22.2	03-037.010228	0.38
NEUROG1-1	166	05q31.1	05-134.898938	0.45
NEUROG1-1	283	05q31.1	05-134.899244	0.41
NEUROG1-1	212	05q31.1	05-134.899351	0.45
NEUROG1-1	391	05q31.1	05-134.899479	0.37
NEUROG1-1	364	05q31.1	05-134.899537	0.32
NEUROG1-1	201	05q31.1	05-134.899663	0.34
CDKN2A-2	232	09p21.3	09-021.964676	0.34
CDKN2A-2	184	09p21.3	09-021.965200	0.4
CDKN2A-1	335	09p21.3	09-021.984269	0.36
CDKN2A-up	195	09p21.3	09-021.985277	0.37
IGF2-4	172	11p15.5	11-002.117590	0.39
IGF2-3	418	11p15.5	11-002.118681	0.34
IGF2-3	141	11p15.5	11-002.118895	0.47
CRABP1-1	206	15q25.1	15-076.419820	0.37
CRABP1-1	310	15q25.1	15-076.420033	0.36
CRABP1-2	265	15q25.1	15-076.420493	0.41
CRABP1-2	318	15q25.1	15-076.420701	0.36
SOCS1-2	238	16p13.13	16-011.256544	0.32
SOCS1-2	155	16p13.13	16-011.256960	0.33
SOCS1-1	399	16p13.13	16-011.257200	0.33
SOCS1-1	300	16p13.13	16-011.257552	0.29
CACNA1G-1	273	17q21.33	17-045.993509	0.39
CACNA1G-1	250	17q21.33	17-045.993745	0.29
CACNA1G-1	218	17q21.33	17-045.993972	0.38

Table 12. Pearson correlation coefficient for PDL-1, CD3 and CD8 at diagnosis

Variable	PD-L1 TC	PD-L1 PLI	PD-L1 ILI	CD3 PLI	CD3 ILI	CD3 II	CD8 PLI	CD8 ILI
PD-L1 TC	1,000	NA	NA	NA	NA	NA	NA	NA
PD-L1 PLI	0,371	1,000	NA	NA	NA	NA	NA	NA
PD-L1 ILI	**0,603**	**0,632**	1,000	NA	NA	NA	NA	NA
CD3 PLI	**0,504**	0,261	0,210	1,000	NA	NA	NA	NA
CD3 ILI	**0,726**	0,370	**0,546**	**0,605**	1,000	NA	NA	NA
CD3 II	0,228	0,105	0,236	0,372	0,238	1,000	NA	NA
CD8 PLI	**0,645**	0,234	0,265	**0,822**	**0,727**	0,387	1,000	NA
CD8 ILI	**0,813**	0,364	**0,539**	**0,591**	**0,935**	0,265	**0,740**	1,000

Abbreviations: ILI = intratumoral lymphoid infiltrate; NA =not applicable; PD-L1 = programmed cell death ligand 1; PLI = peritumoral lymphoid infiltrate; TC = tumor cell. aStatistically significant correlation.

Reports published during my PhD training in Experimental and Translational Medicine at the laboratory of Molecular Pathology of the Anatomic Pathology Unit of Varese Hospital.

1. Nunzio Digiacomo, Elena Bolzacchini, Giovanni Veronesi, Roberta Cerutti, Nora Sahnane, Graziella Pinotti, Marco Bregni, Salvatore Artale, Claudio Verusio, Filippo Crivelli, Carlo Capella, Fausto Sessa, Daniela Furlan. Neuroendocrine Differentiation, Microsatellite Instability, and Tumor-infiltrating Lymphocytes in Advanced Colorectal Cancer With BRAF Mutation. Clinical Colorectal Cancer, Vol. 18, No. 2, e251-60, 2018 Elsevier.

2. Elena Bolzacchini, Nunzio Digiacomo, Cristina Marrazzo , Nora Sahnane, Roberta Maragliano , Anthony Gill , Luca Albarello , Fausto Sessa , Daniela Furlan and Carlo Capella. BRAF Mutation in Colorectal Rhabdoid and Poorly Differentiated Medullary Carcinomas. Cancers, 2019