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Class 1, 2 and 3 *BRAF* mutated metastatic colorectal cancer: a detailed clinical, pathological and molecular characterization

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Conflict of interest

Sara Lonardi had roles as consultant or advisor for Amgen, Bayer, Merck-Serono, Lilly. She received research funding from Amgen, Merck-Serono and she is part of speakers bureau of Lilly, BMS.

Filippo Pietrantonio received honoraria and had roles as consultant or advisor for Amgen, Merck-Serono, Roche, Sanofi, Bayer, Servier, Lilly.

Angelo Paolo Dei Tos had roles as consultant or advisor for Roche, Pfizer, PharmaMar, Lilly.

Vittorina Zagonel received honoraria and had roles as consultant or advisor for Bristol-Mayers Squibb, Bayer, Roche, Pfizer, Janssen, Novartis, Astellas, Servier. She had roles as consultant or advisor for Celgene, Merck-Serono.

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This study focused on two rare and distinct subgroups of $^{non\text{-}V600E}$*BRAF* mutated mCRC patients while comparing them to ^{V600E}*BRAF* mutated and a control set of wild-type patients. A detailed description of clinical and pathological features, including Consensus Molecular Subtypes (CMS) and BM1/BM2 categorization according to Barras et al. is reported, along with outcome data. Results underline the importance of $^{non\text{-}V600E}$*BRAF* mutation detection and point out the need for focusing therapeutic research effort staking into account the specificities of these peculiar CRC subtypes.

Abstract

Purpose

BRAF mutations 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). While clinical, pathological and molecular features of ^{V600E}*BRAF* mutated metastatic colorectal cancer (mCRC) are well known, limited data are available from the two other classes.

Experimental Design

Data from 117 *BRAF* (92 class 1, 12 class 2, and 13 class 3) mutated mCRC patients were collected. 540 *BRAF* wt mCRC were included as control. Immunohistochemical profiling was performed to determine the consensus molecular subtypes (CMS), cytokeratins 7/20 profiles, tumor infiltrating lymphocytes (TILs) infiltration and BM1/BM2 categorization. OS and PFS were evaluated by Kaplan-Meier and log-rank test.

Results

Class 3 *BRAF* mutated mCRC were more frequently left sided (p=0.0028), pN0 (p=0.0159), and with no peritoneal metastases (p=0.0176) compared to class 1, whereas class 2 cases were similar to class 1. HR for OS, as compared to *BRAF* wt, was 2.38 (95%CI 1.61-3.54) for class 1, 1.90 (95% CI 0.85-4.26) for class 2 and 0.93 (95% CI 0.51-1.69) for class 3 (p<0.0001). Class 2 and 3 tumors were all assigned to CMS2-3. A higher median CD3/CD8 positive lymphocytes infiltration was observed in *BRAF* mutated class 2 (p= 0.033) compared to class 3 cases.

Conclusions

For the first time different clinical, pathological features and outcome data are reported according to the 3 *BRAF* mutation classes in mCRC. Specific targeted treatment strategies should be identified in the next future for such patients.

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Introduction

Analysis of molecular alteration like *RAS,* ^{V600E}*BRAF* mutation and microsatellite instability are nowadays a consolidated routine in the assessment of metastatic colorectal cancer (mCRC) patients[1] since all of them have a clear prognostic and/or predictive role.

^{V600E}*BRAF* mutated mCRC patients share specific clinical and pathological features such as older age at diagnosis, female sex, right sided location of primary tumors, poor differentiation, mucinous histology and microsatellite instability[2-4]. In the metastatic setting, ^{V600E}*BRAF* mutation occurs in approximately 10% of cases, and it is associated with poor prognosis and scarce overall benefit from standard therapeutic approaches[5,6]. Recently, specific gene expression profiles were described for distinguishing 2 subgroups among ^{V600E}*BRAF* mutated cancers, named BM1 (showing activation of KRAS/mTOR/AKT/4EBP1 pathway) and BM2 (with deregulation in the cell-cycle)[7].

In recent years, thanks to the adoption of more accurate techniques for mutational status evaluation such as next generation sequencing and mass spectrometry other rare *BRAF* mutations have been identified. Overall $^{non\text{-}V600E}$*BRAF* mutations occur in only 2% of mCRC patients and cover 19 different codons[8,9]. The clinical significance of these mutations is largely unknown, due to the rarity of this condition.

Intriguing data on clinical features of $^{non\text{-}V600E}$*BRAF* mutations in CRC patients emerged from 2 recent retrospective cohort studies. Those reports agree in defining the $^{non\text{-}V600E}$*BRAF* mutated population (mainly including mutations in codons 594 and 596) as a distinct subgroup with its own features, in particular longer overall survival compared to ^{V600E}*BRAF* mutated patients, no association with older age, female sex, right sided tumor, mucinous histology, peritoneal spread and microsatellite instability[8,10].

A deeper knowledge of $^{non\text{-}V600E}$*BRAF* mutation derived from functional studies on non-colorectal preclinical models which led to identify 3 classes of *BRAF* mutations: activating *RAS*-independent *BRAF* mutations signaling as monomers (class 1) or as dimers (class 2) and *RAS*-dependent *BRAF* mutations with impaired kinase activity or kinase-dead (class 3)[9]. ^{V600E}*BRAF* mutation belongs to class 1 whereas among $^{non\text{-}V600E}$*BRAF* mutations those affecting codons 601 and 597 are assigned to class 2; while those in codons 594 and 596 to class 3. Preliminary data, mainly derived from melanoma models, suggested also a different sensitivity to *BRAF* inhibitors based on the 3 classes[5,9,11].

Moving from all the above considerations, the simple distinction in V600E and non-V600E mutations may be too simplistic, and a specific characterization of *BRAF* class 2 and 3 mutations is needed.

The present study aimed to provide a specific clinical, pathological, molecular and prognostic characterization of $^{non\text{-}V600E}$*BRAF* mutated mCRC patients.

Materials and Methods

Study design

Patients were categorized as follows: i) *BRAF* wild-type, ii) *BRAF* mutant class 1 (i.e. harboring the V600E alteration), iii) *BRAF* mutant class 2 (i.e. harboring codons 601 or 597 alterations) and iv) *BRAF* mutant class 3 (i.e. harboring codons 594 or 596 alterations). For all the *BRAF* mutated cases, tissue specimens (paraffin embedded block or, as an alternative, 5 micron thick slides for IHC analyses) of primary and/or metastases were collected from the archives of the referral Pathology Departments.

Data on availability of CRC samples diagnosed with $^{non\text{-}V600E}$*BRAF* mutations were firstly retrieved from the 9 Italian Surgical Pathology Units involved in the study. Clinical and survival data of identified patients were retrieved from 8 Italian Oncology Units and matched with available molecular and pathological information. Main inclusion criteria were: diagnosis of metastatic CRC and available clinical data. Clinico-pathological and survival data of ^{V600E}*BRAF* mutated and *BRAF* wild-type cases were collected from patients referred to the Veneto Institute of Oncology, Padua, from January 2010 to December 2016. Clinical and pathological features are described in **Table 1**. The study was approved by the Ethics Committee of Veneto Institute of Oncology and was conducted according to ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. Written informed consent was obtained from all the patients.

Mutational status analyses

RAS and *BRAF* mutational profiling were carried out on formalin fixed paraffin-embedded (FFPE) samples from primary tumors and/or paired metastases by means of Sanger Sequencing, Sequenom MassArray technology (Myriapod® Colon status, Diatech Pharmacogenetics, Jesi, Italy) or Ion Torrent PGM sequencing (SiRe® next generation sequencing panel).

Pathological evaluation and IHC Analysis

Two experienced gastrointestinal pathologists, who were blinded to mutational status and patients' outcome, revised specific pathological features of each sample.

Available primary and/or metastatic FFPE surgical samples were processed using the Galileo CK3500 Arrayer, a semiautomatic and computer-assisted Tissue microarray (TMA) platform. Four and three tissue cores (1 mm in diameter) were obtained from each primary and metastatic lesion, respectively. Small biopsy samples were processed separately. Immunohistochemical stainings were automatically performed using the Bond Polymer Refine Detection kit (Leica Biosystems, Newcastle Upon Tyne, UK) in the BOND-MAX system (Leica Biosystems) on 4 µm-thick sections. Primary antibodies, dilutions and scoring evaluation are available upon request.

DNA mismatch repair machinery deficient tumours (MMRd) were defined in the absence of nuclear

immunostaining for one of the couples MLH1/PMS2 or MSH2/MSH6 in tumor cells[12].

Consensus molecular subgroups (CMS) were qualified according to Ten Hoorn and coll.[13] by assessing 4 IHC markers (FRMD6, ZEB1, HTR2B, CDX2) in combination with pan-cytokeratin (KER) to normalize results. Primary tumors and/or metastasis were then categorized into the 3 CMS classes (CMS1, CMS2/3 or CMS4) using the online classification tool[14],[15] (**Supplementary Figure 1**).

In *BRAF* class 2 and 3 cases, histological type and grading was revised according to the last updated WHO classification[16]. Cytokeratin expression pattern was evaluated by CK7 and CK20 expression, while tumor infiltrating lymphocytes (TILs) by means of positive intratumoral CD3/CD8 elements. To stratify class 2 and 3 tumors according to Barras et al.[7] in BM1 and BM2 groups, an immunohistochemical profiling for CDK1, ATM, Phospho-AKT (Ser473), Cyclin D1, and Phospho-4E-BP1 (Thr70) expression was performed (**Supplementary Figure 2**). According to the Barras' paper, retained expression of ATM, activation of the AKT/4EB-P1 cascade (considering both the phosphorylated forms of AKT and 4E-BP1), low CDK1 expression and high Cyclin D1 expression are BM1 markers. Since no *a priori* criteria have been defined, we exploratively categorized each tumor based on their IHC profiles as follows.

To stratify class 2 and 3 tumors according to Barras et al. in BM1 and BM2 groups, we exploratively categorized each tumor based on the presence/absence of these 5 markers: CDK1, ATM, Phospho-Akt (Ser473), Cyclin D1, and Phospho-4E-BP1 (Thr70). Since BM1 is characterized by activation of PI3K/mTOR/AKT pathway, while BM2 of cell cycle pathway, we assigned samples to BM1 or BM2 based on the coherence of the following parameters. Overexpression of Phospho-Akt, Phospho-4E-BP1, ATM and Cyclin D1 and downregulation of CDK1 were consistent with a BM1 profile. On the other hand, BM2 cases were characterized by overexpression of CDK1 and downregulation of the remaining markers. A tumor was considered positive in ATM if >10% of tumor cells were positive for nuclear ATM staining. The activation of the AKT/4E-BP1 cascade was defined in the presence of high expression levels of the phosphorylated forms of AKT and/or 4E-BP1. High levels of Cyclin D1 and CDK1 expression were defined in the presence of at least 50% of positive cancer cells (Cyclin D1 in the nucleus, CDK1 both in the nucleus and cytoplasm). Samples with 4 or 5 coherent parameters were defined as BM1 or BM2, whereas tumors in which 3 out of 5 parameters were coherent with the hypothesis were defined as borderline BM1 or BM2. Tumors with only 1 or 2 parameters coherent with the original classification were defined as not evaluable.

Statistical analysis

Fisher's exact test or chi square test were used when appropriate to compare clinical, pathological and molecular features according to *BRAF* mutational status (*BRAF* wild-type versus (vs) *BRAF* mutant class 1 vs *BRAF* mutant class 2 vs *BRAF* mutant class 3; *BRAF* mutant class 1 vs *BRAF* mutant class 2; *BRAF* mutant class 1 vs *BRAF* mutant class 3 and *BRAF* mutant class 2 vs *BRAF* mutant class 3). Overall survival (OS) was defined as the time from the diagnosis of metastatic disease to death due to any cause whereas progression free survival (PFS) was calculated from the date of first-line systemic treatment start to the first observed progression or death due to any cause. OS and PFS analyses were performed according to

the Kaplan–Meier method and survival curves were compared using the log-rank test. Statistical significance was set at p = 0.05 for a bilateral test. All analyses were carried out by means of MedCalc Software (Ostend, Belgium).

Results

Patients' characteristics, clinical outcome and treatments

Class 1, 2 and 3 *BRAF* mutated included 92, 12 and 13 patients respectively. *BRAF* wild-type patients were 540. Female patients were 49%, 50%, 46% and 37% in the 4 groups respectively. Median age was 69, 60, 56 and 62 years (p=0.004). Right-sided primary tumor occurred in 79%, 33.3%, 0% and 26% of cases (p<0.001) (**Table 1**).

Class 3 *BRAF* mutated patients were more frequently left-sided (46% vs 15%, p=0.003), with no loco-regional nodal involvement (56% vs 17%, p=0.016) and no peritoneal spread (100% vs 69%, p=0.018) compared to class 1 ones. No differences were observed comparing class 2 with class 1 or class 3 cases (**Tables 1** and **2**).

Median OS was 21.0 vs 23.4 vs 44.5 vs 42.2 months, in *BRAF* mutated class 1, 2, 3 and *BRAF* wild-type, respectively (p<0.0001). Hazard ratio (HR) for OS was 2.38 (95% CI 1.61-3.54) for class 1 *BRAF* mutated, 1.90 (95% CI 0.85-4.26) for class 2 *BRAF* mutated and 0.93 (95% CI 0.51-1.69) for class 3 *BRAF* mutated compared to *BRAF* wild-type patients (**Figure 1**).

Median PFS from the beginning of the first-line treatment was 7.3 vs 7.0 vs 13.8 vs 10.1 months, in the 4 groups, respectively (p<0.0001). HR for PFS was 2.02 (95% CI 1.39-2.94) for class 1 *BRAF* mutated, 2.49 (95% CI 0.92-6.74) for class 2 *BRAF* mutated and 0.85 (95% CI 0.47-1.54) for class 3 *BRAF* mutated compared to *BRAF* wild-type patients (**Figure 2**).

In the group of *BRAF* wild type patients, among 463 patients undergoing first-line chemotherapy, 49 received a monotherapy plus/minus a biologic agent, 288 received a doublet plus/minus a biologic agent and 124 received a three drugs combination plus/minus a biologic agent, 2 received other treatments. Overall, 292 of them received a bevacizumab-based treatment and 123 an anti-EGFR antibody.

Among 73 class 1 *BRAF* mutated patients receiving first-line treatment, 7 underwent to a monotherapy plus/minus a biologic agent, 43 received a doublet plus/minus a biologic agent and 14 received a three drugs combination plus/minus a biologic agent, 9 received other treatments. Overall, 50 of them received a bevacizumab-based treatment, 7 received a treatment with an anti-EGFR antibody.

Nine out of 12 class 2 *BRAF* mutated patients received a first-line treatment. In particular, 2 underwent to a monotherapy and 7 to a doublet plus/minus a biologic agent. None of them received an anti-EGFR antibody.

Nine of 13 class 3 *BRAF* mutated patients received a first-line treatment. In particular, 1 underwent to a

monotherapy, 5 to a doublet plus/minus a biologic agent, 3 to a three drugs combination plus/minus a biologic agent. Overall, 4 of them received a bevacizumab-based treatment and 5 received treatment with an anti-EGFR antibody.

Mutational status and CMS in BRAF mutant patients

Among class 1 *BRAF* mutated patients, adequate tissue specimens were available for 74 out of 92 patients. In 40 out of 74 cases both primary and metastatic samples were available (9 cases had multiple metastatic sites available for IHC analysis). Overall, as indicated in **Table 3**, 39% of class 1 tumors were defined as CMS1 (immune-like subtype), 44% of patients as CMS 2-3 (epithelial-like subtype) and 17% of patients as CMS4 (mesenchymal-like subtype). Three cases were deemed as not evaluable due to lack of concordance between primary and metastatic samples.

Among class 2 *BRAF* mutated patients, all tumors carried a *BRAF* p.K601E mutation. Of note, one tumor was characterized by a concomitant *NRAS* p.G12C mutation. Adequate tissue specimens were available for 11 out of 12 patients. A total of 20 specimens were considered (8 primary and 12 metastatic); three cases had multiple metastatic sites available for IHC analysis and in three cases both primary and metastatic samples were available. All analyzed samples were categorized as CMS2-3.

Among class 3 *BRAF* mutated patients, 2 tumors carried a *BRAF* p.D594N mutation and 11 a *BRAF* p.D594G mutation. No other concomitant *RAS* mutation was identified. Adequate tissue specimens were available for 10 out of 13 patients. Overall 18 samples were analyzed (8 primary and 10 metastatic); 3 cases had multiple metastatic sites available for IHC analysis and in 3 cases both primary and metastatic samples were available. All analyzed samples were categorized as CMS2-3.

Pathological features, lymphocyte infiltration and BM1/BM2 Classification in class 2 and 3 BRAF mutated patients

Based on the WHO classification, among class 2 *BRAF* mutated cases, 1 tumor had mucinous histology, 1 micropapillary histology and 9 were not otherwise specified (NOS) adenocarcinomas. Tumor grade was high (i.e. grade 3 and 4) in 5 cases and low (i.e. grade 1 and 2) in the remaining 6 tumors. Intratumor phenotypic heterogeneity was evident only in a NOS adenocarcinoma, which showed intermingled areas of well-to-poor differentiation. No MMRd tumor was identified (**Table 3**). All cases showed a high CDX2 expression. In one case, metastatic samples showed a significant loss of CDX2 expression in comparison to the matched primary tumor, however those metastatic lesions retained the same histologic grade and a strong CK20 positivity, as observed in primary samples. The analysis of cytokeratins expression and infiltrating lymphocytes evaluation was possible in 9 samples. Two tumors (29%) were characterized by a low CK20 expression and two metastatic samples showed a significant loss in CK20 expression in

comparison to their matched primary/metastatic samples. All samples showed loss/low CK7 expression, in particular 3 cases (33.3%) showed a faint (1+) CK7 immunoreactivity. Median CD3 and CD8 positive infiltrating lymphocytes per high power field were high in 4 cases (**Table 4** and **Supplementary Figure 3**).

Among class 3 *BRAF* mutated patients, based on the WHO classification, 2 tumors had micropapillary histology, 1 cribriform histology and 7 were NOS adenocarcinomas. Intratumor phenotypic heterogeneity was evident in the 2 cases with micropapillary histology, which showed areas of poorly differentiated NOS histology. Tumor grade was high in 3 tumors and low in the remaining 7. As observed in class 2 lesions, no MMRd tumor was identified. Three cases (30%) showed a low expression of CDX2. Four tumors (40%) showed a low CK20 expression, all samples showed loss/low CK7 expression, whereas 2 samples showed a faint CK7 immunoreactivity. No significant intratumor heterogeneity for CMS, CDX2 and cytokeratins expression was observed in the 3 cases with multiple metastatic biopsies, nor among matched primary and metastatic samples. Median CD3 and CD8 positive infiltrating lymphocytes per high power field were low in all cases (**Table 4** and **Supplementary Figure 3**).

Overall no differences were observed among class 2 and 3 cases, with the exception of a higher median CD3 and CD8 positive lymphocytes infiltration in class 2 *BRAF* mutated samples (p= 0.033) (**Table 4**).

In class 2 *BRAF* mutated tumors, 6 cases were positive for ATM, 7 showed an activation in AKT/4E-BP1, 2 were characterized by high levels of CDK1 and 3 by high levels of Cyclin D1. Overall, 5 cases were classified as BM1 (56%), 4 as BM2 (44%) whereas 2 tumors were not classifiable according to BM status (**Table 4**).

In class 3 *BRAF* mutated tumors, 4 cases were positive for ATM, 5 showed an activation in AKT/4E-BP1, 5 were characterized by high levels of CDK1 and 5 by high levels of Cyclin D1. Overall, 2 cases were classified as BM1 (33%), 4 as BM2 (67%), whereas 4 tumors were not classifiable according to BM status (**Table 4**).

Discussion

Several clinical and pathological descriptions of class 1 *BRAF* mutated mCRC patients (i.e. V600E) have been published indicating specific features and overall poor life expectancy[5,6]. Our work characterized from a clinical, prognostic and biological perspective the complete panel of known *BRAF* mutations according to functional classes, showing specific features for class 2 *BRAF* mutated mCRC that have never been so extensively reported before, mainly due to their rarity. Moreover, we confirmed previous findings on class 1 and class 3 *BRAF* mutated[8,10] and cross compared all the categories.

Looking at OS and PFS data, we observed that class 2 *BRAF* mutated patients have worse prognosis compared to class 3 and wild type patients, and class 1 - class 2 *BRAF* mutated patients share similar poor median OS and PFS.

From a practical point of view, the identification of new subgroups of mCRC patients with specific and rare *BRAF* mutations underlines the importance of the extensive adoption of modern techniques such as mass spectrometry or NGS in the daily clinical assessment of mCRC patients.

In the present study, we applied the CMS classification to the 3 *BRAF* classes adopting the practical and rapid immunohistochemical method proposed by Trinh et al.[15] which allow to distinguish CMS2-3 from CMS4 cases analyzing four specific markers (CDX2, FRMD6, HTR2B and ZEB1). We defined CMS1 based on MMRd status, testing for lack of expression of PMS2 and/or MSH6. All class 2 and 3 patients were classified as CMS2-3 and no heterogeneity was observed when pairing primary and/or metastatic samples. So that, disease development and progression might not be dependent on immune related mechanisms and/or mesenchymal related pathways. Looking at class 1 *BRAF* mutated patients, a wider heterogeneity has been detected since CMS1, 2-3 and 4 occurred respectively in 39%, 44% and 17% of cases. Among this group, such classification might provide further helpful information for patients' stratification and treatment decision making and would need specific studies. Recently Sveen et al.[17], investigated drug sensitivity according to CMS classification in preclinical models showing that CMS2 cell lines and PDX are more sensitive to EGFR and HER2 inhibition compared to other CMS subgroups. Unfortunately, IHC evaluation of CMS does not allow distinguishing CMS2 and 3 patients, so that to this extent further studies are needed.

To better characterize differences among class 2 and 3 patients we performed a deep IHC characterization. CK7 and CK20 profiling revealed low rate of heterogeneity when looking at primaries and paired metastases and none of our cases showed a complete inversion of the CK7 and CK20 expression, already described elsewhere in some class 1 *BRAF* mutated cases [18,19].

Loss of CDX2 expression has been proposed as a negative prognostic feature in mCRC patients and it is frequently associated with ^{V600E}*BRAF* mutation and MMRd[18-20]. In our series, loss of CDX2 was observed among 3 class 3 *BRAF* mutated cases. Due to the small numbers we were not able to further speculate on the prognostic impact of such feature.

Of note, CD3 and CD8 infiltration, already known as a prognostic feature in CRC[21-23],was higher in class 2

BRAF mutated compared to class 3 patients (p= 0.033). Such finding might contribute to explain the outcome differences observed in our series.

From a therapeutic point of view, we can't drive any definitive conclusion on anti-EGFR sensitivity of *BRAF* class 2 and 3 patients. A recent case report described a class 2 *BRAF* mutated mCRC patient achieving a durable response after treatment with panitumumab single agent[24]. In our series, among class 2 *BRAF* mutated only 3 patients received an anti-EGFR with one responder, among class 3 *BRAF* mutated 6 patients received an anti-EGFR with 4 responders. Although definitive conclusions cannot be drawn, taking into account the kinase signaling mechanisms in class 2 and 3 patients, it could be hypothesized that class 3 patients might derive some benefit from anti-EGFRs due to their impaired kinase activity and to their RAS dependency. On the other hand, no reliable and specific data are available regarding systemic treatments of class 2 and 3 *BRAF* mutated patients.

International guidelines recommend as first line treatment for *BRAF* class 1 patients with good performance status and younger than 75 years a 4-drugs combination (the triplet FOLFOXIRI plus bevacizumab regimen)[1]. Many trials have been performed and/or are ongoing in order to identify targeted strategies able to block the hyper activated ^{V600E}*BRAF* signals. Disappointing results have been obtained with *BRAF* inhibitors alone[24-26] and currently, new therapeutic options are under evaluation for this subgroup of patients adopting *BRAF* plus *MEK* inhibitors together with anti-EGFRs monoclonal antibodies[27]. The recent stratification of class 1 *BRAF* mutated patients into BM1 and BM2 upon gene expression laid the basis for a more tailored biologic approach in the development of targeted therapies. Specifically, BM1 subgroup is characterized by activation of KRAS/mTOR/AKT/4EBP1 pathway, whereas the BM2 had a deregulation in the cell-cycle[7]. In our study, we assigned samples to BM1 or BM2 category adopting a specific IHC expression analyses. Of note, 2 and 4 cases among class 2 and 3 patients, respectively, were not assigned to either BM1 or BM2 since they presented a peculiar IHC profile not properly matching with BM1/BM2 categorization. One can hypothesize the presence of peculiar activation pathways for $^{non\text{-}V600E}$*BRAF* mutated cases, however we should also consider the intrinsic limitations of our results due to the application of a different technique (IHC instead of gene expression) and the limited sample size of our work.

Interestingly, *in vitro* sensitivity of class 2 and 3 *BRAF* mutations to *MEK* and *BRAF* inhibition has been proven in melanoma, and a small number of patients with these mutations showed responses to treatment with MEK inhibitors[28-31]. The largest cohort of patients was collected by Boweyer et al. describing the antitumor activity of trametinib in 5 patients bearing the rare class 2 or class 3 *BRAF* mutations[32]. Moreover, in order to elucidate these differences, functional studies were conducted in melanoma models showing that the activation of *RAS* and *ERK* pathways by these 3 classes through different mechanisms can explain their different sensitivity to therapeutic inhibitors: class 1 is sensitive to RAF "monomer" inhibitors (i.e. vemurafenib), class 2 is resistant to vemurafenib and could be sensitive to novel RAS dimer inhibitors (i.e. LY3009120) or MEK inhibitors (i.e. trametinib), class 3 is potentially sensitive to RTKs inhibitors (i.e. dasatinib)[3,5,9,11]. Further studies are needed to clarify such mechanisms in CRC models and patients in the

next future.

In conclusion, our study extensively described for the first time the $^{non\text{-}V600E}$*BRAF* mutations as two different subtypes of rare mCRC with specific clinical and prognostic and pathological features that might be taken into account when planning new basic research initiatives and innovative clinical trials in this setting.

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Tables

Table 1. Baseline characteristics and major clinical parameters•

Characteristics		*BRAF wt* TOT=540 *N (%)*	*BRAF* mut class 1 TOT=92 N (%)	*BRAF* mut class 2 TOT=12 N (%)	*BRAF* mut class 3 TOT=13 N (%)	*p*	*p**	*p§*	*p#*
Sex	Female	200 (37)	45 (49)	6 (50)	6 (46)	**0.0458**	*1.0000*	*1.0000*	*1.0000*
	Male	340 (63)	47 (51)	6 (50)	7 (54)				
Age	Median (range)	62 (21-91)	69 (35-85)	60 (45-89)	56(45-83)	***0.004***	*0.115*	*0.759*	*0.251*
Age	> 70	132 (24)	39 (42)	5 (42)	3 (23)	***0.0007***	*0.4110*	*1.0000*	*0.2351*
	≤ 70	408 (76)	53 (58)	7 (58)	10 (77)				
Baseline ECOG PS	0	373 (69)	64 (70)	6 (55)	11 (85)	*0.8875*	*0.2506*	*1.0000*	*0.167*
	1	139 (26)	22 (24)	3 (18)	0 (0)				
	≥ 2	28 (5)	6 (6)	3 (27)	2 (15)				
Primary tumor resected	Yes	314 (58)	75 (82)	9 (75)	10 (77)	***<.0001***	*1.0000*	0.6965	0.7097
	No	226 (42)	17 (18)	3 (25)	3 (23)				
Primary tumor location	Right	137 (26)	73 (79)	4 (33.3)	0 (0)	***<.0001***	*0.4666*	*0.1294*	***0.0028***
	Left	265 (49)	14 (15)	4 (33.3)	6 (46)				
	Rectum	136 (25)	5 (5)	4 (33.3)	7 (54)				
	NA	*2*	*0*	*0*	*0*				
Presentation of mets	Synchronous	383 (71)	60 (65)	7 (58)	7 (54)	*0.3401*	*1.0000*	*0.7508*	0.5395
	Metachronous	157 (29)	32 (35)	5 (42)	6 (46)				
Mucinous histology	Yes	34 (7)	26 (31)	2 (17)	3 (23)	**<.0001**	*1.0000*	*0.4993*	*1.0000*
	No	497 (93)	58 (69)	10 (83)	10 (77)				
	NA	*9*	*8*	*0*	*0*				
pT	1-2	58 (14)	3 (4)	2 (20)	2 (20)	**0.013**	*1.0000*	*0.0828*	*0.0828*
	3-4	350 (86)	83 (96)	8 (80)	8 (80)				
	NA	*132*	*6*	*2*	*3*				
pN	0	104 (26)	14 (17)	4 (40)	5 (56)	*0.0973*	*0.6563*	*0.0944*	***0.0159***
	≥1	292 (74)	70 (83)	6 (60)	4 (44)				
	NA	144	8	2	4				
N° of metastatic sites	1	358 (66)	61 (66)	9 (75)	10 (77)	*0.9203*	*1.0000*	*0.7467*	*0.5406*
	≥ 2	182 (34)	31 (34)	3 (25)	3 (23)				

** class 2 vs class 3*

§ class 1 vs class 2

[#] class 1 vs class 3

[•] i.e. at the time of first-line treatment start or, for candidates to BSC only, at the first visit for metastatic disease

Table 2. Sites of metastasis at diagnosis

Sites of mets at diagnosis		*BRAF* wt TOT= 540 *N (%)*	*BRAF* mut class 1 TOT=92 N (%)	*BRAF* mut class 2 TOT=12 N (%)	*BRAF* mut class 3 TOT=13 N (%)	*p*	*p**	*p*§	*p*#
Liver	Yes	387 (72)	44 (48)	8 (67)	10 (77)	***<.0001***	0.6728	0.3579	0.0740
	No	153 (28)	48 (52)	4 (33)	3 (23)				
Lung	Yes	104 (19)	15 (16)	2 (18)	2 (15)	*0.6101*	*1.0000*	*1.0000*	*1.0000*
	No	436 (81)	77 (84)	10 (82)	11 (85)				
Distant Nodes	Yes	109 (20)	38 (41)	2 (18)	2 (15)	***<.0001***	*1.0000*	*0.1234*	*0.1245*
	No	431 (80)	54 (59)	10 (82)	11 (85)				
Peritoneum	Yes	102 (19)	29 (31)	3 (27)	0 (0)	***0.0098***	*0.0957*	*0.7510*	***0.0176***
	No	438 (81)	63 (69)	9 (73)	13 (100)				
Other	Yes	60 (11)	12 (13)	1 (10)	3 (23)	*0.729*	0.5930	1.0000	0.3928
	No	480 (89)	80 (87)	11 (90)	10 (77)				

** class 2 vs class 3*

§ class 1 vs class 2

 # class 1 vs class 3

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Table 3. Molecular stratification according to CMS

	BRAF mut class 1 TOT=74 N (%)	*BRAF* mut class 2 TOT=11 N (%)	*BRAF* mut class 3 TOT=10 N (%)	*p*	*p**	*p*§	*p*#
CMS 1 - Immune-like	28 (39%)	0 (0%)	0 (0%)	*0.0146*	*1*	*0.0015*	*0.0009*
CMS 2/3 - Epithelial-like	31 (44%)	11 (100%)	10 (100%)				
CMS 4 - Mesenchymal-like	12 (17%)	0 (0%)	0 (0%)				
NA	3	0	0				

** class 2 vs class 3*

§ class 1 vs class 2

 # class 1 vs class 3

Table 4. Histopathological features, tumor infiltrating lymphocytes immunophenotype and BM1/BM2 classification in *BRAF* mutated class 2 and 3 patients

	BRAF mut class 2 TOT=11 N (%)	*BRAF* mut class 3 TOT=10 N (%)	p-value
Grading			
Low	6 (54)	7 (70)	0.659
High	5 (46)	3 (30)	
CK7			
Low (0-1)	9 (100)	10 (100)	1
High (2-3)	0 (0)	0 (0)	
NE	2	0	
CK20			
Low (0-1)	2 (29)	4 (40)	1
High (2-3)	5 (71)	6 (60)	
Discordant	2	0	
NE	2	0	
CDX2			
Low (0-150)	0 (0)	3 (30)	0.211
High (≥150)	10 (100)	7 (70)	
Discordant	1	0	
NE	0	0	
CD3/CD8			
Low (0-20)	5 (56)	10 (100)	**0.033**
High (>20)	4 (44)	0 (0)	
NE	2	0	
BM1/BM2 Classification			
BM1	5 (56)	2 (33)	0.608
BM2	4 (44)	4 (67)	
NA	2	4	

NE = not evaluable; NA = not assessable

Figure 1. Median OS according to *BRAF* mutation classes

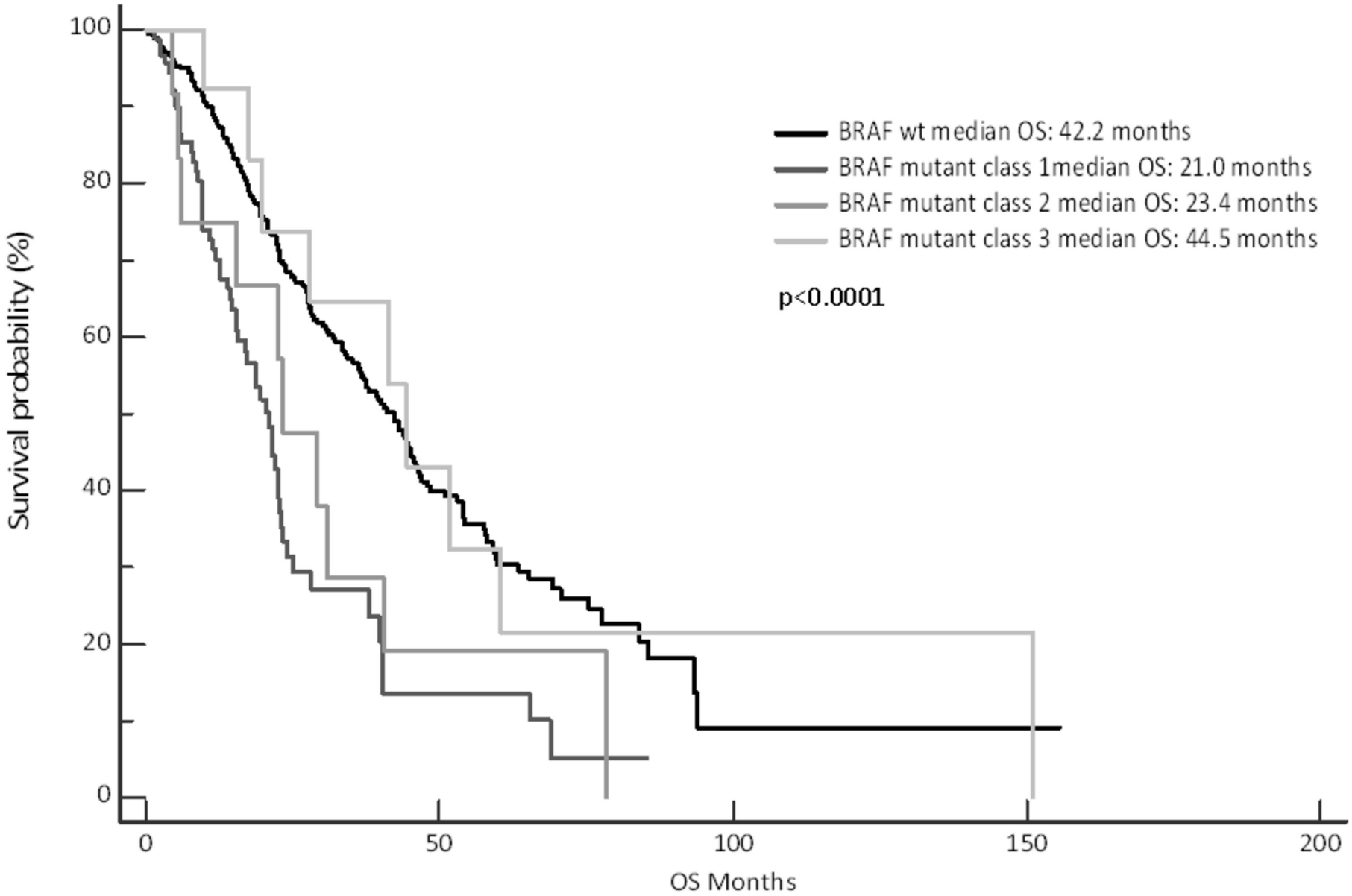
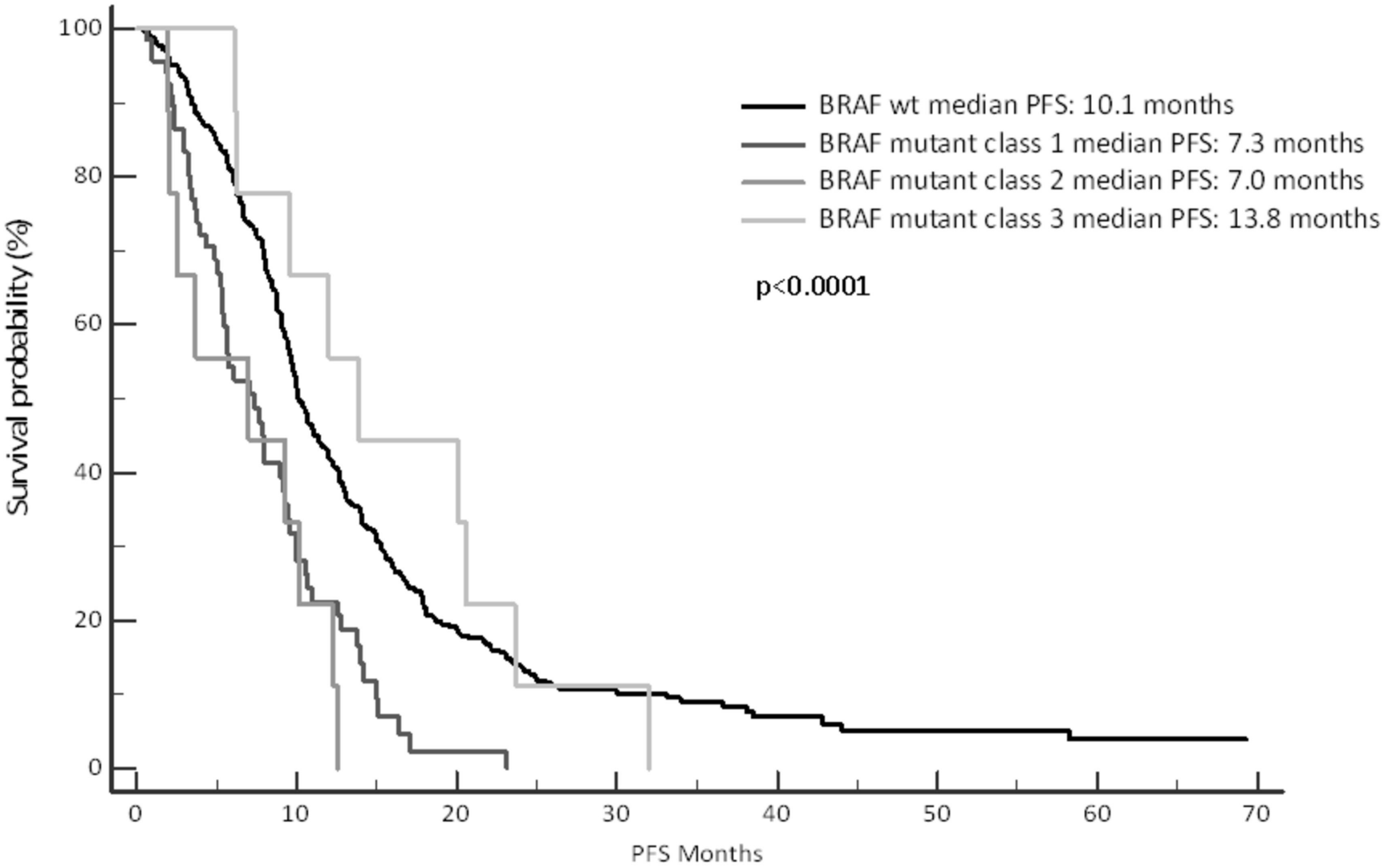


Figure 2. Median PFS according to *BRAF* mutation classes



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Clinical Cancer Research

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