Abstract

Purpose: Mutation of BRAF at the valine 600 residue occurs in approximately 10% of colorectal cancers, a group with particularly poor prognosis. The response of BRAF mutant colorectal cancer to recent targeted strategies such as anti-BRAF or combinations with MEK and EGFR inhibitors remained limited and highly heterogeneous within BRAF V600E cohorts. There is clearly an unmet need in understanding the biology of BRAF V600E colorectal cancers and potential subgroups within this population.

Experimental Design: In the biggest yet reported cohort of 218 BRAF V600E with gene expression data, we performed unsupervised clustering using non-negative matrix factorization to identify gene expression–based subgroups and characterized pathway activation.

Results: We found strong support for a split into two distinct groups, called BM1 and BM2. These subtypes are independent of MSI status, PI3K mutation, gender, and sidedness. Pathway analyses revealed that BM1 is characterized by KRAS/akt pathway activation, mTOR/4EBP deregulation, and EMT whereas BM2 displays important deregulation of the cell cycle. Proteomics data validated these observations as BM1 is characterized by high phosphorylation levels of AKT and 4EBP1, and BM2 patients display high CKD1 and low cyclin D1 levels. We provide a global assessment of gene expression motifs that differentiate BRAF V600E subtypes from other colorectal cancers.

Conclusions: We suggest that BRAF mutant patients should not be considered as having a unique biology and provide an in-depth characterization of heterogeneous motifs that may be exploited for drug targeting.

Introduction

Colorectal cancer is the third leading cause of cancer-related death in the United States (1). The BRAF gene is activated by mutation in 50% of melanomas and 10% of colorectal cancer (2). In the MAPK pathway, KRAS activates BRAF, which then activates MEK kinase (MEK1 and MEK2) and finally ERK (3). However, the nonrandom occurrence of BRAF mutations in certain disease subtypes such as hypermethylated right-sided colorectal cancer or specific thyroid carcinoma subtypes suggests that additional tumor features and alterations are associated to the presence of V600 BRAF and will determine the final signal output (2). Moreover, the gene expression patterns of KRAS mutant and BRAF mutant colorectal cancer are typically very different from each other (4, 5). Of interest, the oncogenic contribution of mutated BRAF may vary between tumor types, as suggested by the very heterogeneous clinical benefit seen for BRAF inhibition strategies between melanoma and colon (6, 7). This heterogeneity in drug resistance indicates cancer-type specificity of the biology underlying the BRAF mutation. In addition, the recent outcome data of trials targeting selected V600 BRAF mutant colorectal cancer cohorts with monotherapy (8, 9), double-targeted therapy (8, 10–13), and triple-targeted therapeutics (13–15) have shown a surprisingly strong heterogeneity in response, suggesting that further biological subdivisions may exist even within BRAF V600E mutant colorectal cancer.

The BRAF V600E mutation (from now one referred to as BM) results in a constitutively active protein (2) and accounts for 96%
Translational Relevance

Colorectal cancer BRAF V600E mutant patients have a poor prognosis and do not respond efficiently to standard chemotherapy, BRAF-targeted therapeutic approaches and even to recently reported combination with MEK and EGFR inhibitors. There is emerging clinical evidence of further heterogeneity within this population; however, the low percentage of BRAF mutation in colorectal cancer has prevented its systematic characterization. We have collected and investigated gene expression of 218 BRAF mutant colorectal cancer patients, the largest yet reported cohort. Unsupervised subtyping identified a previously unrecognized heterogeneity of this cohort at the gene expression level, indicating two subgroups of V600E BRAF mutant colorectal cancer. These two subtypes display distinct molecular patterns with one exhibiting high KRAS/mTOR/akt/4EBP1, EMT activation, and immune infiltration, whereas the other displaying cell-cycle checkpoints dysregulation. In addition, a cell drug screen indicates that these two subtypes may have different responses to some drugs including BRAF and MEK inhibitors.

of BRAF V600 mutations (16). More than 30 other oncogenic BRAF mutations were found. Although most of them also cause constitutive kinase activity, other mutations produce kinase-dead alterations that nevertheless can lead to oncogenic signals via CRAF activation (17,18). The prognostic role of non-V600E BRAF mutations is still uncertain as they are not routinely tested for in clinical trials. The BRAF mutation is associated with other clinical factors such as the microsatellite instability (MSI) status (58% of BM patients are MSI), tumor site (81% of BM patients have rightsided tumors), and gender (62% of BM patients are females; refs. 19,20).

In general, colorectal cancer patients have so far been treated irrespectively of clinical or molecular features (except exclusion of anti-EGFR therapy for RAS-activated tumors) and display heterogeneous treatment response. Major efforts have been devoted to defining molecular colorectal cancer subtypes with the increasing use of transcriptomics data (21-28), to which we have extensively contributed (22,29). Colorectal cancer classification is a highly active field of research as it aims to stratify patients for therapeutic interventions and provides more precise assessment of heterogeneous trial outcomes. Recently, a concerted effort by the Colorectal Cancer Subtyping Consortium (CRCSC) succeeded in unifying colorectal cancer subtyping (29). This led to subdivision of colorectal cancer tumors into four consensus molecular subtypes (CMS), capturing the main overall gene expression variability in colorectal cancer (29). The CRCSC system aimed at consolidating the variation in expression, basic mutation, and outcome data for further study.

Here we report that BM patients can be separated into two different gene expression subtypes with distinct molecular patterns and potential different therapeutic targets.

Materials and Methods

BRAF V600E mutant patient cohort

The database used in this study was constituted by combining the following independent cohorts: colorectal adenocarcinomas from The Cancer Genome Atlas (TCGA; ref. 21), the Pan-European Trials in Alimentary Tract Cancers (PETACC-3; refs. 5,30), three different datasets from Agenda (GSE42284; ref. 31), ICO (4,29), and VHB (4,29). Marisa and colleagues (GSE39582; ref. 24), Schlicker and colleagues (GSE35896; ref. 28), and two datasets from the Ludwig Institute for Cancer Research in Melbourne, GSE75315 and GSE75316. The number and origin of patients with some of their clinicopathologic characteristics are highlighted in Figure 1. Detection of clinicopathologic features, MSI status, KRAS, and BRAF mutation status was performed as described in the original reports describing the cohorts (4,5,21,24,28,30,31). The mutational analysis collected by each study is summarized in Supplementary Table S1. Briefly, KRAS mutants include mutations at residue G12, G13, and Q61; BRAF mutants only include the V600E mutation and PIK3CA mutants include all known activating mutations.

Gene expression data and scripting

Unless otherwise mentioned, all analyses in this study were performed using R software (version 3.2.2). A reproducible research script and data can be found at our institutional website (32). Level 3 TCGA RNA-sequencing expression data were obtained from the TCGA portal. GSE39582 and GSE35896 were obtained preprocessed from the Gene Expression Omnibus ( GEO) website. PETACC-3 expression data had been processed as described earlier (5). Probe levels expression datasets were processed as follows: for probes mapping to a unique gene, only the probe displaying the largest variation was conserved. Gene expression data from all cohorts were merged at the gene level, which resulted in a dataset of 9,328 genes for 218 BRAF V600E mutant patients. The ComBat function from the sva package was used to normalize and correct for batch effects across datasets (33). The similarity structure of the tumor profiles was then assessed by principal component analysis (PCA) and hierarchical clustering.

BRAF V600E mutant patient clustering

Cluster analyses were performed using correlation distance metrics and the average linkage agglomeration algorithm (R package cluster version 1.09; ref. 34). Non-negative matrix factorization (NMF) was done with the NMF package (version 0.20.5) and standard strategies. Detailed descriptions of the NMF-based subtyping is described in the Supplementary Methods. Statistical analyses assessing the association between BM subtypes and clinicopathologic factors were performed using Fisher exact tests. P-values were corrected for multiple comparisons using the Bonferroni method. The MSI-adjusted clustering analysis was performed as described in the Supplementary Methods.

BM classifier and genes specific for BM subtypes

We constructed a classifier for the two BM groups by training a penalized logistic model with the subtype labels
given by the selected two subgroups NMF solution, using the glmnet package (version 2.0.2). We obtained a classifier with nonzero coefficients for 44 genes listed in the Supplementary Table S2. Methodological details are described in the Supplementary Methods. To determine genes that characterizes the difference between BM1 and BM2 without being confounded by relevant clinical factors (MSI status, site, and gender), which were not perfectly balanced between BM subtypes, we fitted a multivariable linear regression including the three potential confounders as additional explanatory variables in the model (35). To extract the genes specifically associated to the BM subtypes, we selected the genes with a P-value inferior to \(5 \times 10^{-5}\) [corresponding to a Benjamini–Hochberg FDR (BH-FDR) of 0.002] for the effect of the BM subtype variable on gene expression, which led to the identification of 476 genes.

Molecular pathway and biological process analysis

Pathway analyses were performed by Gene Set Enrichment Analysis (GSEA) and are described in the Supplementary Methods.

Methylation, proteomic, survival analyses, and drug response prediction on cell lines

Analyses performed on external public data for DNA methylation, proteomics, and cell line gene expression are described in the Supplementary Methods, along with methods for survival analyses. The public cancer cell lines data were from refs. 36 and 37.

Results

Colorectal cancer BRAF V600E mutant population clusters into two distinct subtypes

We built a gene expression dataset of 218 BRAF V600E mutant (BM) colorectal cancer patients from different clinical trials (Fig. 1) to study gene expression profiles in this relatively rare subgroup. Although the profiles were obtained using different technologies and using fresh or FFPE specimen as source material, they did not show any evident cohort-bias clustering by PCA (Supplementary Fig. S1A) or hierarchical clustering (Supplementary Fig. S1B).

To assess if the tumor data presented a cluster structure, we applied a class-discovery approach based on NMF. The NMF
algorithm is stochastic; we computed 200 solutions for models with a factorization rank of two to six components. We judged the quality with two classic criteria: the cophenetic correlation coefficient (CCC, a measure of the overall stability of sample clustering over multiple solutions with a given rank) and the silhouette width (SW; a measure of the similarity of each sample to the samples in the same cluster relative to the similarity to the other samples; ref. 38). Very informative is also visual inspection of the consensus matrix (Fig. 2A), which suggests that the tumor set falls naturally into two separate groups. In agreement with this interpretation, the CCC and the average SW decrease significantly with increasing rank, and particularly from rank two to rank three. For rank two the CCC and SW were close to their maximum value of one, indicating, on an absolute scale, an appropriate clustering into two subtypes of tumors, whereas these criteria are not defined for rank one (the original data). A gene-randomized control dataset was not split at all (Fig. 2A).

We found that 476 genes were differentially expressed driven by differences between MSI and MSS tumors (Supplementary Fig. S4). We conducted gene set enrichment analyses (GSEA) to characterize the molecular pathways specific for the BRAF mutant subtypes. Using the 476 subtype-specific genes, we scored signatures of hallmark processes collected in the MSigDB molecular signature database (39) using the single sample GSEA (ssGSEA) method that indicates the degree to which the genes in a particular gene set are coordinately up- or downregulated within a sample (40). Prominent signatures scoring high in BM1 were EMT-related processes (EMT, apical junction, and myogenesis), KRAS signaling, and immune response (Fig. 3A; Supplementary Fig. S3). High scores in BM2 were achieved by cell-cycle and cycle checkpoints-related processes such as target genes of E2F transcription factors and genes involved in the G2 to M (G2–M) transition (Fig. 3A; Supplementary Fig. S3). Figure 3A indicates that several molecular signatures were characteristic for BM1, respectively for BM2, in a direct comparison between these two groups but not necessarily in comparison to other colorectal cancer tumors. To broaden the analysis to include all colorectal cancer, we compared ssGSEA signature scores between four groups: BRAF mutant subtypes, KRAS/BRAF double wild-type (WT2), and KRAS mutant colorectal cancer (see Materials and Methods). The KRAS signature was not only enriched in BM1 compared to BM2 but also compared with KRAS mutant colorectal cancer (Fig. 3B; Supplementary Fig. S4). BM2 displays similar levels of KRAS signaling than WT2 colorectal cancer. Cell cycle–related processes and glycolysis were not only overactivated in BM2 compared with WT2 and KRAS mutant colorectal cancer but also repressed in BM1 (Fig. 3B; Supplementary Fig. S4). Similarly, EMT-related processes were enriched in BM1 but also repressed in BM2 (Fig. 3B; Supplementary Fig. S4).

With the aim of characterizing the BM subtypes in their globality, we also conducted pathway analyses using the whole gene expression profile rather than by focusing on the 476 differentially expressed genes. In addition to the pathways found with the reduced list of genes, BM1 displays an overall stronger immune profile emphasized by activation of pathways such as IL2/STAT5, TNF signaling via NF-kB, IL6/JAK/STAT3, and allograft rejection (Supplementary Fig. S5). Angiogenesis and TGFβ processes were also enriched in BM1 compared to BM2. BM2 enrichment in metabolic process was more evident by looking at overall gene expression levels.
Colorectal cancer BRAF V600E mutant patients can be classified into two gene expression-based subtypes. **A,** Gene expression clustering of 218 CRC BRAF V600E mutant patients by non-NMF. Correlation matrix heatmaps appear on the left. The gene-randomized dataset was also analyzed (bottom heatmaps and dotted line). Cophenetic and silhouette scores (rank 2 to 6) are shown on the right. **B,** Association between clinicopathologic factors and the BRAF mutant subtypes. The NMF correlation heatmap for rank 2 is shown and the figure displays the annotation of the 218 BRAF mutant patients for the consensus molecular subtype (CMS), PI3K mutation, tumor site, gender, and MSI status. **C,** Mosaic plot displaying proportions of male:female (left), MSI:MSS (middle), and CMS subtypes (right) in the BM subtypes. Bar width is proportional to BM subtype proportions. **D,** Mosaic plot displaying the BM1:BM2 ratio across different cohorts. Bar width is proportional to BM subtype proportions. **E,** Venn diagram displaying the number of genes that were found specific to the indicated clinical factors in a multivariable linear regression (left). Heatmap displaying expression of genes specific to BM subtypes.
Figure 3.
GSEA analysis reveals functional differences between BM1 and BM2. A, ssGSEA was performed for BRAF mutant patients using the hallmarks gene signature collection from MSigDB. Signature enrichment scores were subjected to differential analysis (BM1 vs. BM2) and only enriched biological processes are displayed in the heatmap. Heatmap shows the z-score of ssGSEA enrichment scores. B, Heatmaps displaying the average ssGSEA hallmark signature scores for BM1, BM2, KRAS/WT2, and KRAS mutant using the 476 BM-specific genes. BH-FDR are shown on the right under the form of a heatmap. Heatmaps showing individual ssGSEA scores appear in Supplementary Fig. S4A. C, Heatmaps displaying ssGSEA signature scores of BM patients for selected signatures. MSigDB reference is displayed for each signature.
expression. This full transcriptomics analysis also revealed that both BM groups display less WNT activation than WT2 and KRAS mutant colorectal cancer, and that BM2 has less WNT activity than BM1 (using the MS5924 signature). Of note, this difference in WNT activation was not present by using the 476 BM specific genes (Supplementary Fig. S5).

To confirm the consistency of the signature enrichment, we performed ssGSEA with manually selected signatures covering the biological processes found to be enriched in Fig. 3A (Fig. 3C). The vast majority of KRAS and EMT-related signatures suggested activation of these processes in BM1. The same was true for cell-cycle–related signatures that were scoring high in BM2. Note-worthy, there was minimal gene overlap across this set of gene signatures (Supplementary Fig. S6).

The mTORC1 signature (MSigDB ref: MS5924) was scoring high in BM2. An mTORC1 complex activation can be the consequence of the activity of several other pathways including EGFR, MAPK, AKT, cytokines, hypoxia, and WNT (41). In turn, mTORC1 complex activation can lead to 4EBP1 and S6K pathways activation (41). Thus, to understand better the BM2 enrichment in mTORC1 signature, we have analyzed in greater details the scoring of some of these pathways in the BM subtypes. Surprisingly, we found that mTOR, 4EBP1, and two WNT signatures were enriched in BM1 instead of BM2 (Fig. 3C).

To elucidate this discrepancy, we checked the overlap of these signatures and found none (Supplementary Fig. S6). We therefore checked for KEGG biological process enrichment in these two signatures and found that M7561 (52 genes) was indeed highly representative of the mTOR pathway whereas MS5924 (200 genes) was not and was instead enriched for genes related to cell-cycle and metabolic processes (Supplementary Table S5). The pathway analyses, therefore, indicate that BM1 is characterized by mTOR and RAS signaling, whereas BM2 by cell-cycle and metabolic processes. The WNT signaling enrichment found in BM1 was not consistent across all signatures and should be interpreted cautiously.

To study in greater details the immune profile of BM subtypes, we used the recently reported tool called CIBERSORT that estimates the immune landscape based on gene expression profiles. This analysis revealed that BM1 tumors were significantly more strongly infiltrated by macrophages and monocytes and significantly less by resting dendritic cells whereas BM2 displayed more mast cell content (Supplementary Fig. S7; ref. 42).

Transcriptomics data do not account for posttranslational modifications. Proteomics reverse phase protein array (RPPA) and phosphoproteomics data are publicly available for some colorectal cancer of the TCGA, including 6 BM1 and 21 BM2 TCGA patients. The results of a differential expression analysis were consistent with the reported pathway analyses (Fig. 4A). In particular, AKT Serine 473 and 4EBP1 Threonine 70 were significantly more phosphorylated in BM1, whereas eukaryotic translation initiation factor 4E (eIF4E), another player of the mTOR pathway whereas MS5924 (200 genes) was not and was instead enriched for genes related to cell-cycle and metabolic processes (Supplementary Table S5). The pathway analyses, therefore, indicate that BM1 is characterized by mTOR and RAS signaling, whereas BM2 by cell-cycle and metabolic processes. The WNT signaling enrichment found in BM1 was not consistent across all signatures and should be interpreted cautiously.

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Drug–response prediction of BM subtypes
The Cancer Cell Line Encyclopedia (CCLE) and the Sanger institute and GlaxoSmithKline (GSK) have published gene expression profiles and drug screening results for a wide panel of cancer cell lines. We used these resources to investigate whether BM subtypes might differ in drug-response patterns. We built a penalized logistic model classifier for BM1/BM2, with a penalty coefficient that minimizes the misclassification error estimated by cross-validation. The classifier uses 44 genes (Supplementary Table S2) and has an estimated misclassification error of 11% (Supplementary Fig. S10). We applied this classifier to colorectal cancer cell lines: SW1417 and C2BBe1 were classified as BM1 whereas HCT115, LS1034, SW1116, RKO, SW48, and SW837 were classified as BM2, consistently in both datasets (supplementary Table S6). Twenty-five percent of these cell lines harbor the BRAF mutation and no evident enrichment toward BM subtypes was observed (one out of two BM1 cell lines and one out of six BM2 cell lines harbor the BRAF mutation; Supplementary Fig. S11).
**Figure 4.** Proteomics data analysis confirms observation done at the transcriptomic level. 

**A.** Analysis of proteomic and phosphoproteomic RPPA data for TCGA BRAF mutant patients. The heatmap displays the expression (in standard z-score) of proteins that were found differentially expressed between BM1 and BM2 ($P$-value < 0.05). Phosphorylation level of proteins are emphasized by a green circled P.

**B.** Scheme of pathways that are modulated in BRAF mutant subtypes. The left panel displays the PI3K/mTOR/AKT/4EBP1 pathway that is dysregulated in BM1 patients and the right panel displays the cell-cycle and checkpoints pathway that is dysregulated in BM2 patients.
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Next, we analyzed how these cell lines respond to selected drugs. According to Sanger data, a differential analysis revealed that BM1 cell lines are more sensitive to inhibition of BRAF (PLX4720; linear regression unadjusted \( P \)-value: 0.075), RCL2 (ABT-263; linear regression unadjusted \( P \)-value: 0.011), and MEK (CI-1040; linear regression unadjusted \( P \)-value: 0.101) compared to BM2 (Supplementary Table S7 and Supplementary Fig. S11). However, BM2 cell lines are more sensitive to CDK1 inhibition (by RO-3306) compared to BM1, although not statistically significantly. The analysis of CCLE data showed that an inhibitor of SRC (AZD0530), a protein that is upstream of RAS, RAF, PI3K, AKT, and MEK, is more efficient in killing BM1 cells compared to BM2 (linear regression unadjusted \( P \)-value: 0.076). The HSP90 chaperone 17-AAG displayed modest activity toward BM2 cells in both Sanger and CCLE data. These results support the notion that different pathways are preferentially activated in the two BM subtypes and allows prospective testing of novel drug combinations in stratified sets of models and patients.

Table 1. Survival analysis of BRAF mutant subtypes

<table>
<thead>
<tr>
<th>Strata</th>
<th>( N )</th>
<th>( P )</th>
<th>( HR )</th>
<th>95% CI</th>
<th>( N )</th>
<th>( P )</th>
<th>( HR )</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BM subtype + strata (cohort)</td>
<td>BM1/BM2</td>
<td>50 / 116</td>
<td>0.106</td>
<td>1.61</td>
<td>0.91 to 2.86</td>
<td>43 / 92</td>
<td>0.076</td>
<td>1.66</td>
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<tr>
<td>MSI status + strata (cohort)</td>
<td>MSS/MSI</td>
<td>52 / 88</td>
<td>0.053</td>
<td>1.93</td>
<td>0.99 to 3.76</td>
<td>47 / 62</td>
<td>0.053</td>
<td>1.88</td>
</tr>
<tr>
<td>Tumor site + strata (cohort)</td>
<td>Right/left</td>
<td>135 / 31</td>
<td>0.390</td>
<td>0.76</td>
<td>0.40 to 1.43</td>
<td>104 / 31</td>
<td>0.348</td>
<td>0.75</td>
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<td>Gender + strata (cohort)</td>
<td>Male/female</td>
<td>63 / 103</td>
<td>0.847</td>
<td>0.94</td>
<td>0.51 to 1.73</td>
<td>51 / 84</td>
<td>0.814</td>
<td>0.93</td>
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<tr>
<td>Cohort</td>
<td>PETACC5/GSE39582</td>
<td>50 / 43</td>
<td>0.157</td>
<td>1.73</td>
<td>0.81 to 3.71</td>
<td>50 / 43</td>
<td>0.264</td>
<td>1.5</td>
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<td></td>
<td>Agendia/GSE39582</td>
<td>42 / 43</td>
<td>0.466</td>
<td>1.34</td>
<td>0.61 to 2.93</td>
<td>42 / 43</td>
<td>0.518</td>
<td>1.27</td>
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<td></td>
<td>TCGA/GSE39582</td>
<td>31 / 43</td>
<td>0.542</td>
<td>0.52</td>
<td>0.07 to 4.16</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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<td>Stage III + strata (cohort)</td>
<td>Stage III/II and II</td>
<td>81 / 85</td>
<td>0.175</td>
<td>1.58</td>
<td>0.81 to 3.08</td>
<td>74 / 61</td>
<td>0.261</td>
<td>1.44</td>
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<td>BM subtype + MSI status + strata (cohort)</td>
<td>BM1/BM2</td>
<td>50 / 116</td>
<td>0.497</td>
<td>1.26</td>
<td>0.65 to 2.45</td>
<td>43 / 92</td>
<td>0.222</td>
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<td>MS5/MSI</td>
<td>52 / 88</td>
<td>0.096</td>
<td>1.81</td>
<td>0.90 to 3.62</td>
<td>47 / 62</td>
<td>0.159</td>
<td>1.71</td>
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<td>BM subtype + stage + strata (cohort)</td>
<td>BM1/BM2</td>
<td>50 / 116</td>
<td>0.155</td>
<td>1.53</td>
<td>0.85 to 2.75</td>
<td>43 / 92</td>
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<td>0.75 to 2.95</td>
<td>74 / 61</td>
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<td>0.099</td>
<td>1.63</td>
<td>0.91 to 2.90</td>
<td>43 / 92</td>
<td>0.070</td>
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<td>Male/female</td>
<td>63 / 103</td>
<td>0.728</td>
<td>0.90</td>
<td>0.49 to 1.66</td>
<td>51 / 84</td>
<td>0.664</td>
<td>0.88</td>
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<td>BM subtype + tumor site + strata (cohort)</td>
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<td>50 / 116</td>
<td>0.134</td>
<td>1.56</td>
<td>0.87 to 2.80</td>
<td>43 / 92</td>
<td>0.095</td>
<td>1.62</td>
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<td>Right/left</td>
<td>115 / 31</td>
<td>0.548</td>
<td>0.82</td>
<td>0.43 to 1.57</td>
<td>104 / 31</td>
<td>0.462</td>
<td>0.79</td>
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<td>BM subtype + MSI status + stage + tumor site + gender + strata (cohort)</td>
<td>BM1 / BM2</td>
<td>50 / 116</td>
<td>0.508</td>
<td>1.26</td>
<td>0.64 to 2.49</td>
<td>43 / 92</td>
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<td>MSS / MSI</td>
<td>52 / 88</td>
<td>0.175</td>
<td>1.72</td>
<td>0.79 to 3.74</td>
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<td>0.193</td>
<td>1.65</td>
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<td>Stage III/II and II</td>
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<td>1.03</td>
<td>0.47 to 2.27</td>
<td>74 / 61</td>
<td>0.399</td>
<td>0.72</td>
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<td>Right/left</td>
<td>115 / 31</td>
<td>0.610</td>
<td>0.81</td>
<td>0.37 to 1.79</td>
<td>104 / 31</td>
<td>0.406</td>
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<td>Male/female</td>
<td>63 / 103</td>
<td>0.641</td>
<td>0.86</td>
<td>0.45 to 1.64</td>
<td>51 / 84</td>
<td>0.526</td>
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<td>1.52</td>
<td>0.65 to 3.54</td>
<td>21 / 26</td>
<td>0.059</td>
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<td></td>
<td>BRAF mutant MSI + strata (cohort)</td>
<td>BM1/BM2</td>
<td>17 / 71</td>
<td>0.525</td>
<td>0.66</td>
<td>0.19 to 2.37</td>
<td>13 / 49</td>
<td>0.487</td>
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<td></td>
<td>BRAF mutant stage I and II + strata (cohort)</td>
<td>BM1 / BM2</td>
<td>19 / 66</td>
<td>0.220</td>
<td>1.78</td>
<td>0.71 to 4.48</td>
<td>14 / 47</td>
<td>0.052</td>
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<tr>
<td></td>
<td>BRAF mutant stage III + strata (cohort)</td>
<td>BM1/BM2</td>
<td>31 / 50</td>
<td>0.257</td>
<td>1.57</td>
<td>0.72 to 3.44</td>
<td>29 / 45</td>
<td>0.323</td>
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<td>BRAF mutant TCGA</td>
<td>BM1/BM2</td>
<td>7 / 24</td>
<td>0.682</td>
<td>1.24</td>
<td>0.44 to 3.46</td>
<td>10 / 32</td>
<td>0.301</td>
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<td>BM1/BM2</td>
<td>16 / 34</td>
<td>0.061</td>
<td>2.31</td>
<td>0.96 to 5.56</td>
<td>16 / 34</td>
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<td>BM1/BM2</td>
<td>10 / 32</td>
<td>0.682</td>
<td>1.24</td>
<td>0.44 to 3.46</td>
<td>10 / 32</td>
<td>0.301</td>
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<td></td>
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<td>BM1/BM2</td>
<td>17 / 26</td>
<td>0.588</td>
<td>1.41</td>
<td>0.41 to 4.91</td>
<td>17 / 26</td>
<td>0.967</td>
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Univariable and multivariable (using the indicated covariates) survival analyses using the Cox proportional hazard regression model. Single-test \( P \)-values (\( P \)) are shown in the table. None of the comparisons reached statistical significance with or without adjustment for multiple comparisons using the Bonferroni correction. The number of patients (\( N \)) used for the analyses is displayed for overall survival (OS) and relapse-free survival (RFS) analyses. Abbreviations: CI, confidence interval; HR, hazard ratio; P, \( P \)-value (Wald testing without adjustment).
Discussion

The CRCSC recently published a consensus classification system that unifies previously reported colorectal cancer subtypes based upon gene expression profiles (29). It consists of four subtypes called CMS1 to CMS4. In the largest yet reported colorectal cancer BRAF mutant patient cohort we describe here, we found that the vast majority classifies as CMS1 and only a few in any other groups. This does not explain the heterogeneity seen among BRAF mutant patients at the clinical level. The gene expression profiles segregate these carcinomas naturally into two subtypes, BM1 and BM2, which differ by the expression of a group of 476 genes, independently from factors such as tumor site, MSI, and gender. The two subtypes displayed difference in overall and relapse-free survival, although not significantly. MSI is well known to confer good prognosis independently of other clinical factors (45). One explanation for the survival difference between subtypes resides in the enrichment of BM2 with MSI patients as shown by a Cox multivariable analysis. However, although MSI status is a confounding factor for survival, our data indicate that it does not drive the difference in gene expression. Enrichment of BM1 in EMT signatures and MSS patients, which are associated with poor prognosis, correlates with poor survival of patients with a CMS4 consensus subtype colorectal cancer (29). Our survival analysis has some limitations: there are slight intercohort differences in the definition of events in relapse-free survival, which include secondary primary tumors in one cohort, in the computation of time to event, and in the distribution of censoring times (Supplementary Fig. S9). Moreover, due to the low number of patients and events in subset analyses, the statistical power of these analyses is relatively low.

Detailed pathway analysis of the 476 differentially expressed genes and of the overall transctriome as well revealed that the BM1 subtype is highly active in KRAS/mTOR/AKT/4EBP1 signaling and in genes associated with macrophage infiltration and EMT and the BM2 subtype in cell cycle and cycle checkpoint associated genes. Proteomics studies have revealed high levels of phosphorylation for 4EBP1 threonine 70 and AKT serine 473, which is in line with a high RAS signature. As a key effector of AKT-dependent oncogenic activation in tumors, 4EBP1 is released from its binding to eIF4E when phosphorylated by AKT, which subsequently initiates protein translation (46). Not surprisingly, phosphorylation of 4EBP1 at the threonine 70 residue is associated with poor survival in melanoma (47).

BRAF inhibition is known to initiate a feedback loop via reactivation of EGFR (48). As a result, single therapy with BRAF inhibitors has not been effective in colorectal cancer. An approach combining BRAF inhibition with EGFR blockade has been tested and showed promising results (48). Interestingly, BM1 subtype is enriched for an EGFR signature, derived from stable overexpression of ligand activated EGFR (M2634 in Fig. 3C). Nevertheless, we have found EGFR expression levels did not differ significantly between BM1 and BM2 (data not shown). Thus, this makes it unlikely that the pattern of resistance to BRAF inhibition differs between the two BM subtypes.

The CCLE and the Sanger institute have published gene expression profiles of a large panel of cancer cell lines. In addition, they screened these cell lines for drug sensitivity. We used these resources in an attempt to predict drug response according to BM subtype. Unfortunately, the very low number of cell lines classified as BM1 did not allow sufficient statistical power and therefore this analysis remains only exploratory. Nonetheless, our data suggest that BM1 and BM2 patients might benefit from different targeted approaches. A clinical trial assessing the combination of BRAF inhibitors with EGFR inhibitors and PI3K inhibitors (NCT01719380 in clinicaltrials.gov) is currently ongoing and its results are very much awaited. Our data suggest that BM1 patients may benefit from this combination (Fig. 4; Supplementary Fig. S11). An obvious link between BRAF activation and cell-cycle activation is the MAPK pathway (49). Another clinical trial evaluating the combination of BRAF inhibitors with EGFR inhibitors and MEK inhibitors (NCT01750918 in clinicaltrials.gov) is currently in progress with equally eagerly awaited results. Our data suggest that this combination might be effective in patients with a BM2 subtype colorectal cancer (Fig. 4; Supplementary Fig. S11).
Because BRAF mutant colorectal cancer cell lines and tumors do not respond efficiently to current anti-BRAF treatments such as Vemurafenib and PLX4720, strategies to combine with other drugs have recently been proposed (48, 50). Among them, combination of PLX4720 with the AZD6244, a MEK1/2 inhibitor and MK-2206, an AKT inhibitor, or with AZD7762, a CHK1/2 checkpoint inhibitors appeared highly effective in colorectal cancer cell lines (50). In this respect, the finding that BM1 is characterized by signatures related to RAS, a player of the MAP kinase signaling upstream of MEK, as well as related to AKT and 4EBP1, holds promise for treatment of patients with a BM1 tumor with combination of anti-BRAF, anti-MEK, and anti-AKT/mTOR/4EBP1 drugs. The combination of PLX4720 with the checkpoint kinase 1/2 (CHK1/2) inhibitor called AZD7762 strongly induced cell death in several BRAF mutant cell lines (50). This suggests that drugs targeting cell-cycle components, such as CHK1/2 and Aurora kinases, might be effective in BM2 patients. These hypotheses, however, need to be further explored.

Understanding what drives the difference between BM1 and BM2 is challenging. Because both subtypes harbor the BRAF mutation, it cannot itself be determining their differences. The analysis of the TCGA cohort did not reveal any differences in mutational events between BM1 and BM2 (data not shown). However, this analysis lacks statistical power due to low number of patients. Thus, it is difficult to say whether the nature of the “driving event” occurs at the mutational, epigenetic, or is a question of the state of the cell-of-origin of the cancer or of its interaction with the tumor stroma, for example.

We conclude that BM colorectal cancer can be segregated into two different subtypes based upon gene expression profiles. Further studies are needed to further elaborate the potential clinical relevance of these subtypes, notably in terms of different combinations of targeted therapies.

Disclosure of Potential Conflicts of Interest

M. Delorenzi reports receiving a commercial research grant from Bayer and has ownership interest (including patents) in Novartis and Roche. No potential conflicts of interest were disclosed by the other authors.

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# Clinical Cancer Research

**BRAF V600E Mutant Colorectal Cancer Subtypes Based on Gene Expression**

David Barras, Edoardo Missiaglia, Pratyaksha Wirapati, et al.


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