Neuroendocrine Differentiation, Microsatellite Instability, and Tumor-infiltrating Lymphocytes in Advanced Colorectal Cancer With *BRAF* Mutation

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Abstract

A clinicopathologic analysis of 59 cases of advanced colorectal cancer with *BRAF* mutation was performed to compare microsatellite unstable and stable cases, focusing on the inflammatory profiles and neuroendocrine differentiation of these tumors. Microsatellite stable tumors showed a high frequency of neuroendocrine differentiation. The combined presence of microsatellite instability and high CD8 T-cell content was associated with a 63% reduction in the risk of death.

Background: Approximately 10% of metastatic colorectal cancer (mCRC) cases will harbor the *BRAF* p.V600E mutation (BRAF-mCRC) and have been associated with a poor prognosis. Although they are usually considered a unique clinical entity, biologic heterogeneity has been described. We performed an extensive clinicopathologic study of a multicenter series of BRAF-mCRC to highlight differences between tumors with microsatellite instability (MSI) and microsatellite stable tumors, focusing on both inflammatory profiles and neuroendocrine differentiation. **Methods:** We included 59 BRAF-mCRC cases and collected the clinical data (ie, surgery, treatment, and follow-up). We evaluated MSI status, budding, lympho-angioinvasion, neuroinvasion, extent of active stroma, $CD3^+$ and $CD8^+$ intratumoral and peritumoral lymphocytes, programmed cell death ligand 1, p53, Ki-67, synaptophysin, and CDX2 expression. **Results:** The 22 MSI BRAF-mCRC cases were associated with the right side ($P < .0001$), an expansive grown pattern ($P < .01$), programmed cell death ligand 1 expression ($P < .0001$), high CD8 T-cell content ($P = .0001$), and lymph node metastases ($P < .029$). The 37 MSS BRAF-mCRC cases were characterized by a greater stromal component ($P = .0002$), pulmonary metastases ($P = .095$), and p53 and synaptophysin immunoreactivity ($P = .004$ and $P = .001$, respectively). Univariate analysis demonstrated that MSI and a high CD8 T-cell content were associated with a 34% (hazard ratio [HR], 0.66; 95% confidence interval [CI], 0.34-1.28; $P = .2$) and 33% (HR, 0.67; 95% CI, 0.45-0.99; $P = .04$) reduction in the risk of death, respectively. The combined presence of MSI and CD8 T-cell content decreased the hazard of mortality $\leq$ 63% (HR, 0.37; 95% CI, 0.14-0.97; $P = .2$), which was slightly reduced after multivariate analysis. **Conclusion:** A simultaneous evaluation of MSI, CD8 T-cell content, and neuroendocrine markers could allow for the identification of subsets of BRAF-mCRC with a different prognosis and potential eligibility for specific treatments.

Clinical Colorectal Cancer, Vol. 18, No. 2, e251-60

Keywords: Advanced colorectal cancer, BRAF mutation, CD8 T-cell content, MSI, Neuroendocrine differentiation

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Submitted: Oct 2, 2018; Revised: Dec 10, 2018; Accepted: Dec 14, 2018; Epub: Dec 20, 2018

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1533-0028/$ - see frontmatter
https://doi.org/10.1016/j.clcc.2018.12.003

Introduction

The overall prevalence of *BRAF* p.V600E mutation has been ~10% of advanced colorectal carcinoma (BRAF-metastatic CRC [mCRC]) and will be found in ~60% of CRC cases with microsatellite instability (MSI) and in only 5% to 10% of microsatellite stable (MSS) CRC.[1,2] Because the *BRAF* p.V600E mutation has been demonstrated to cause the epigenetic silencing of the *MLH1* promoter,[3] this mutation and/or *MLH1* methylation analysis have an established clinical utility to recognize sporadic CRC, excluding Lynch tumors.[4] MSI in CRC has been associated with a proximal location, poor histologic differentiation, mucinous or signet ring cell differentiation, and is common among stage II (20%) and III (12%) but rare in stage IV CRC cases (4%).[5] Moreover, MSI confers a favorable prognosis in patients with localized disease[6,7] but not in patients with metastatic disease. The host immune response has generally correlated with the survival benefit of MSI, because the presence of tumor-infiltrating lymphocytes (TILs) is more frequent in cases with MSI than in cases with MSS (21% vs. 3%).[8,9] Moreover, DNA mismatch repair (MMR) defects will lead to the introduction of immunogenic neoantigens.[10] Immunogenic neoantigens are potentially targetable by immune checkpoint inhibitors against the programmed cell death 1 (PD-1)/programmed cell death ligand 1 (PD-L1) axis, providing remarkable therapeutic promise for patients with MMR-deficient tumors.[11]

At present, the prognostic and predictive implications of TILs and inhibitory PD-1/PD-L1 cells in advanced CRC (mCRC) are poorly understood. In particular, in BRAF-mCRC, which has been associated with poor overall survival (OS) and progression-free survival,[12] the clinical relevance of a pronounced host immune reaction remains elusive, including both MSI and MSS tumors. In this setting, no extensive clinicopathologic studies have been reported to clarify whether MSI status and associated biologic and/or morphologic features will allow for the identification of clinically different tumor subsets. In a few recent 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 tumors.[13-15] Analogously, in 1 investigation, *TP53* was mutated more frequently in MSS/BRAF-mutant than in MSI/BRAF-mutant cancer.[16]

In the present study, we analyzed a series of 59 BRAF-mCRC cases to (1) compare the clinicopathologic characteristics of MSI versus MSS tumors; (2) analyze the extent of neuroendocrine differentiation and the types of neuroendocrine neoplasms between the 2 groups of BRAF-mCRCs; and (3) evaluate the density of intratumor $CD8^+$ T lymphocytes and the expression of PD-L1 by the tumor cells and their prognostic influence.

Methods

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 advanced CRC (mCRC). The inclusion criteria were as follows: (1) stage IV CRC, including metastatic disease (stage IV) at diagnosis and relapsed early-stage CRC (stage I-III); (2) the presence of *BRAF* p.V600E mutation; (3) the 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.

For each patient, we collected data on sex, age at the diagnosis, Eastern Cooperative Oncology Group performance status,[17] primary tumor 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−vascular endothelial growth factor or anti−epidermal growth factor receptor [EGFR] antibody). *BRAF* p.V600E mutation, MSI status, immunohistochemical expression of MMR proteins, and *MLH1* methylation were evaluated in accordance with previously reported protocols.[18] 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.[19] 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.

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.[20] The histopathologic revision evaluated the following features: histologic type (tubular, mucinous, medullary, signet ring cells, undifferentiated, and mixed adenoneuroendocrine histologic features [ie, MANEC]), grade (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).[21]

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[22] and graded as absent (no inflammatory cells at the tumor margin), weak (mild and patchy inflammatory cells at the tumor margin), moderate (evident band-like 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, ×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.[23] 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 Supplemental Table 1 (available in the online version).

Statistical Analysis

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 χ^2 test for continuous and discrete variables, respectively. We used box plots to represent the distribution of CD3, CD8, and PD-L1 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.

Results

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 1.

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%). Tubular and mucinous/signet ring cell cancer types were more frequent (49.2% and 23.7%, respectively), followed by medullary undifferentiated types (15.3%) and MANEC (12%). Most cases had an infiltrative pattern of growth (79.2%), and a proportion of stroma greater than average (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 the 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 Supplemental Figure 1 (available in the online version), 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.

Clinicopathologic Profiles of BRAF-mCRC According to MSI Status

The MSI mCRC tumors showed a positive correlation with right-sidedness ($P < .0001$), poor histologic differentiation ($P = .09$), an expansive pattern of growth ($P = .01$), and the presence of lymph node metastases ($P = .029$; Table 1). In contrast, the MSS mCRC tumors were characterized by a greater 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 tumors (16.2% vs. 4.5%; $P = .09$; Figures 1 and 2). 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 1; Supplemental Table 2; available in the online version).

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 2 and 3). 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 (Supplemental Table 3; available in the online version). No significant differences between the 2 groups were found for the Crohn-like lymphoid reaction.

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

Table 1 Clinicopathologic Profiles of MSI and MSS *BRAF*-mCRC

Variable	All Patients (n = 59)	MSI mCRC (n = 22)	MSS mCRC (n = 37)	*P* Value[a]
Age, y	66.9 ± 11.8	70 ± 9.6	65.1 ± 12.7	.1
Female sex	36 (61)	12 (54.5)	24 (64.9)	.4
Primary tumor side[b]				< .0001[c]
Right-sided	40 (69)	22 (100)	18 (50)	
Left-sided	18 (31)	0 (0)	18 (50)	
Histologic type				.09
Tubular	29 (49.2)	8 (36.4)	21 (56.8)	
Mucinous/signet ring cells	14 (23.7)	8 (36.4)	6 (16.2)	
Medullary/undifferentiated	9 (15.3)	5 (22.7)	4 (10.8)	
MANEC	7 (11.9)	1 (4.5)	6 (16.2)	
Grade				.09
1/2	30 (50.8)	8 (36.4)	22 (59.5)	
3	29 (49.2)	14 (63.6)	15 (40.5)	
Stage at diagnosis[b]				.6
I-III	21 (36.2)	9 (40.9)	12 (33.3)	
IV	37 (63.8)	13 (59.1)	24 (66.7)	
Distant metastasis[b]				.095
Liver	28 (55)	11 (55)	17 (56)	
Peritoneum	19 (38)	10 (50)	9 (30)	
Lung	14 (28)	3 (15)	11 (36)	
Lymph node metastasis[b]				.029[c]
Presence	14 (28)	9 (45)	5 (16.7)	
Absence	36 (72)	11 (55)	15 (83.3)	
Growth pattern[b]				
Expansive	11 (20.8)	8 (38.1)	3 (9.4)	
Infiltrative	42 (79.2)	13 (61.9)	29 (90.6)	.01[c]
Stroma ≥ 20%	34 (61.8)	6 (30)	28 (80)	.0002[c]
Synaptophysin ≥ 1	16 (29.1)	1 (4.5)	15 (45.5)	.001[c]
Ki-67	66.5 ± 20.5	70 ± 20	64.3 ± 20.7	.3
p53 ≥ 50	22 (37.3%)	3 (13.6)	19 (51.4)	.004[c]

Data presented as n (%) or mean ± standard deviation.
Abbreviations: MANEC = mixed adenoneuroendocrine carcinoma; mCRC = metastatic colorectal cancer; MSI = microsatellite instability; MSS = microsatellite stable.
[a]Student *t* test for continuous variables and χ^2 tests for categorical variables.
[b]Data were not available for all patients.
[c]Statistically significant.

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 ≤ 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).

Discussion

In the present study, 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. 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 the 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[24,25] and to define tailored treatments for these patients.[26,27] To date, neuroendocrine differentiation has been reported in BRAF-CRC at widely variable frequencies, ranging from 5% to 51.5%,[28-30] and is considered a negative prognostic marker. Although no association between the outcome and MANEC histologic type was observed in our series, likely owing to the limited

Figure 1 Histologic 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 (Original Magnification ×200). (B) Synaptophysin-stained Section (Original Magnification ×200). The Neuroendocrine Component Expressed General Neuroendocrine Markers Including Synaptophysin and the Exocrine Cells were Negative. (C) Acid Mucins of Non-neuroendocrine Cells Stained Strongly with Alcian Blue (Original Magnification ×400)

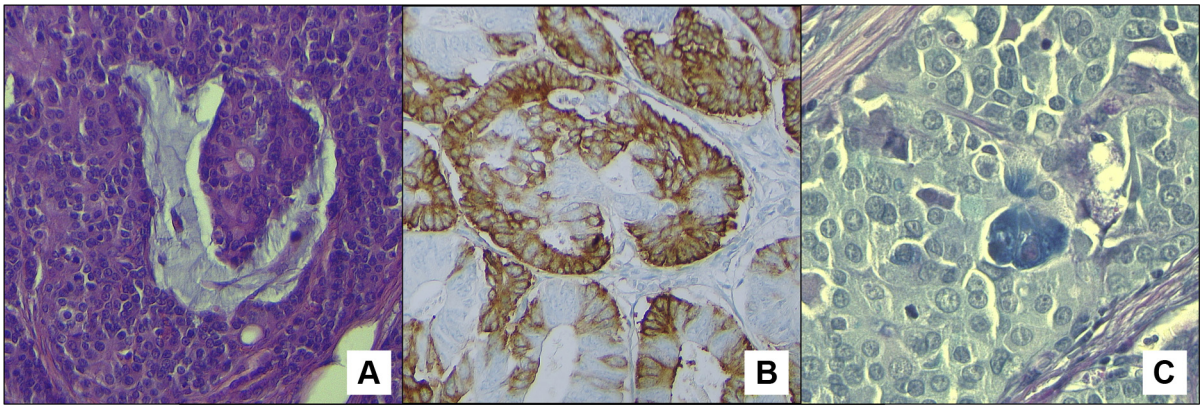


Figure 2 Comparison of Microsatellite Instability (MSI) Colorectal Cancer (CRC) and Microsatellite Stable (MSS) CRC. (A) MSI CRC Showing More Abundant Intratumor Lymphocytes and Lower Percentage of Stroma (Hematoxylin-eosin–stained Section; Original Magnification, ×200) Compared with (B) MSS CRC (Hematoxylin-eosin–stained Section; Original Magnification, ×100). (C) CD8 Immunostaining Highlighting the High Number of Immune Cells Infiltrating MSI CRC (Original Magnification, ×200) Compared with (D) MSS CRC Showing No Intratumor Lymphocytes (Original Magnification, ×100). (E) PD-L1 Expression on the Cell Membrane of Tumor Cells in MSI CRC (Original Magnification, ×400) and (F) intense p53 Nuclear Immunoreactivity in MSS CRC (Original Magnification, ×400)

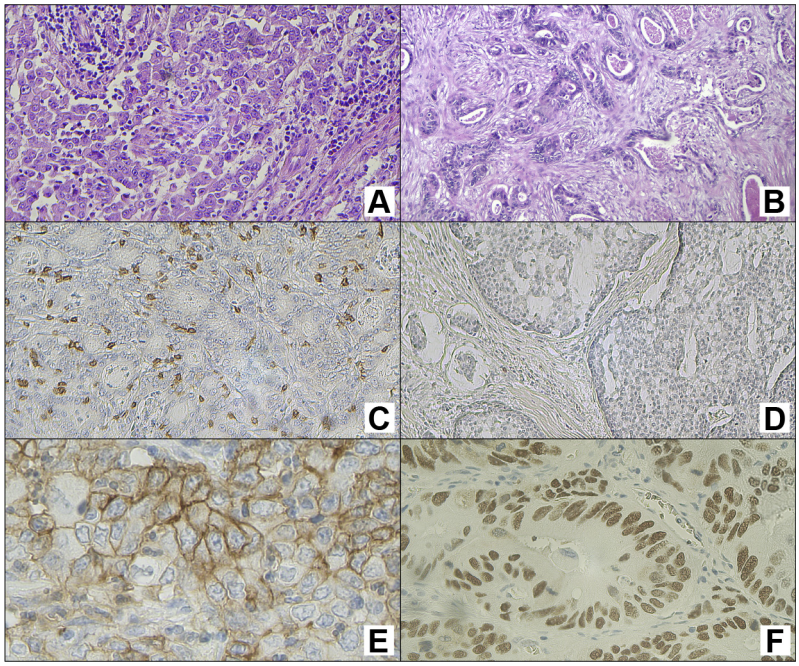
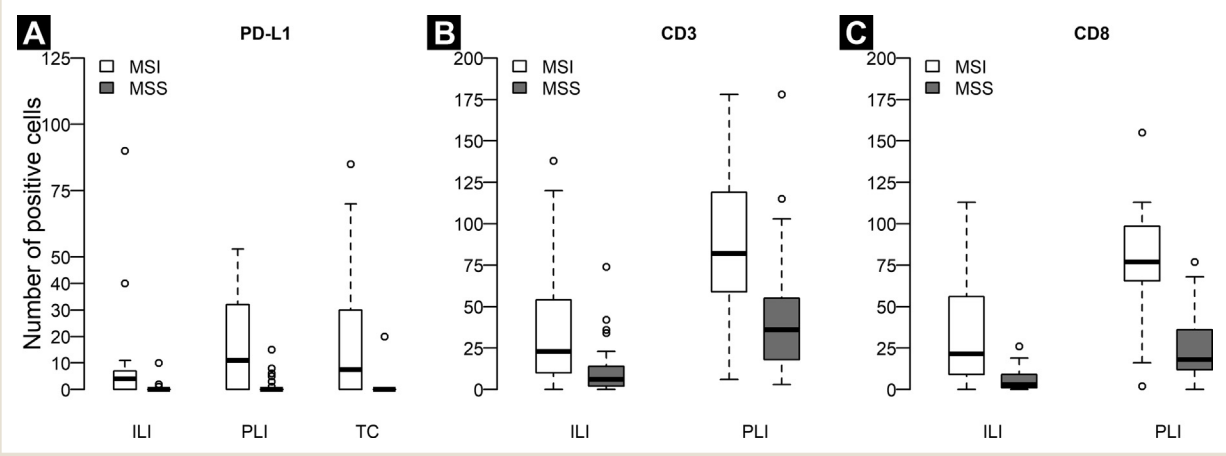


Figure 3 **Programmed Cell Death Ligand 1 (PD-L1) Expression and CD3 and CD8 T-cell Counts in microsatellite Instability (MSI) Compared with Microsatellite Stable (MSS) Tumors. The Expression of the PD-L1 Receptor, on Both Tumor Cells (TCs) and Intratumoral Lymphatic Invasion (ILI) and Peritumoral Lymphatic Invasion (PLI), were Associated Significantly More Often with MSI than with MSS (panel A, $P < .0001$). CD3 (panel B) and CD8 Lymphocytes (panel C) 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)**



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 neuroendocrine carcinoma of the gastrointestinal tract.[13]

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 presented with 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 $\leq$ 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, the present results have demonstrated that a pronounced host immune reaction is not unique to MSI BRAF-mCRC and suggests that the intratumoral CD8 T-cell content 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[31,32] 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,[33-35] but others have reported that PD-L1 expression is a negative prognostic marker.[36] Masugi et al[37] 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.[37] 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 the *BRAF* mutation and several histological features, including medullary histotype, a poor degree of differentiation, and the presence of a rich inflammatory infiltrate.[37]

The treatment of patients with BRAF-mCRC remains challenging because they have not responded efficiently to standard chemotherapy[38] and have shown heterogeneity in the drug response. Longer OS was observed in the TRIBE study (combination chemotherapy and bevacizumab as first-line therapy in treating patients with metastatic colorectal cancer) for patients with BRAF-mCRC.[39,40] The median OS was 19 months in the FOLFOXIRI (5-fluorouracil, leucovorin, oxaliplatin, irinotecan) plus bevacizumab group compared with 10.5 months in the FOLFIRI (leucovorin, 5-fluorouracil, irinotecan) plus bevacizumab group. Moreover, the median OS was 41 months in the *RAS* and *BRAF* wild-type subgroup compared with 13.4 months in the BRAF-mCRC subgroup.

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. Many of the patients included in the present study had selected during a long period of 7 years and had mainly received single- or multiagent chemotherapy; only a few patients had received a triplet regimen plus bevacizumab, which according to the TRIBE study results seems the best

treatment for these patients. Furthermore, many patients had received anti-EGFR therapy during the first or subsequent treatment lines, because only recently has the possibility of lower efficacy with this treatment for BRAF-mCRC been hypothesized.[41,42] In contrast, 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.

Conclusion

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. Although our results require validation against independent data, the present study has demonstrated a high frequency of MANECs among MSS BRAF-mCRC cases 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.

Clinical Practice Points

- *BRAF* mutation is a strong predictor of a poor prognosis in mCRC.
- Although these tumors are usually considered a unique clinicopathologic entity, they remain clinically challenging because they do not respond efficiently to standard chemotherapy, BRAF-targeted therapeutic approaches, or to recently reported combinations of MEK and EGFR inhibitors.
- To the best of our knowledge, our work has demonstrated for the first time that a simultaneous evaluation of MSI status and CD8 T-cell content in cases of BRAF-mCRC could be a useful strategy for identifying a subgroup of patients with a better prognosis and potential eligibility for cancer immunotherapy drugs.
- Our findings support previous data suggesting that the *BRAF* mutation could be an oncogenic driver of neuroendocrine carcinoma of the colon and rectum and shown that neuroendocrine differentiation is mainly observed in cases of MSS BRAF-mCRC.

Acknowledgments

The present study was supported by the Epigenomics Flagship Project—EPIGEN (project no. 08934412 to D.F.) and the University of Insubria. We thank Drs Chiara Albeni and Lucio Lettig for their helpful technical work. We thank Drs Barzaghi Sabrina, Barbarini Lucia, and Rossini Chiara for retrieving the clinical data.

Disclosure

The authors declare that they have no competing interests.

Supplemental Data

The supplemental figure and tables accompanying this article can be found in the online version at https://doi.org/10.1016/j.clcc.2018.12.003.

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Supplemental Table 1 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

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.

Supplemental Table 2 Histopathologic Features Evaluated in MSI and MSS Tumors

Variable	Total Patients (n = 59)	MSI (n = 22)	MSS (n = 37)	*P* Value
Crohn-like	12 (25)	5 (25)	7 (25)	1.0
Budding high grade	13 (25.5)	4 (20)	9 (29)	.5
Vascular space invasion	28 (54.9)	13 (65)	15 (48.4)	.2
Perineural invasion	29 (60.4)	9 (45)	20 (71.4)	.06
Necrosis				.1
Focal	13 (23.2)	4 (18.2)	9 (26.5)	
Geographic	24 (42.9)	13 (59.1)	11 (32.4)	

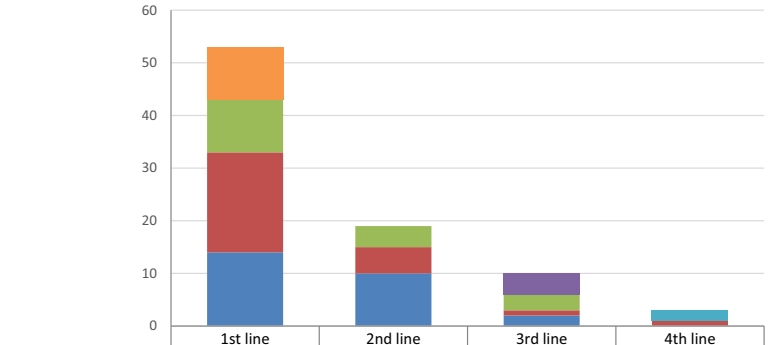
Abbreviations: MSI = microsatellite instability; MSS = microstatellite stable.

Supplemental Table 3 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[a]	0.632[a]	1.000	NA	NA	NA	NA	NA
CD3 PLI	0.504[a]	0.261	0.210	1.000	NA	NA	NA	NA
CD3 ILI	0.726[a]	0.370	0.546[a]	0.605[a]	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[a]	0.234	0.265	0.822[a]	0.727[a]	0.387	1.000	NA
CD8 ILI	0.813[a]	0.364	0.539[a]	0.591[a]	0.935[a]	0.265	0.740[a]	1.000

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

Supplemental Figure 1 Treatments Used in First and Subsequent Lines: 76% of Patients were Treated Upfront with Chemotherapy (CT) with or Without a Monoclonal Antibody (Anti–vascular Endothelial Growth Factor [VEGF] and/or Anti–epidermal Growth Factor Receptor [EGFR]), 33% of Patients Received a Second Line, 14%, a Third Line, and 5%, a Fourth Line of Treatment. Only 10 Patients (17%) were Treated with Best Supportive Care



	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