Molecular Therapy
Oncology
Original Article

Differential landscape of immune evasion in oncogenic RAS-driven primary and metastatic colorectal cancers

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Oncogenic drivers such as *KRAS* extensively modulate the tumor inflammatory microenvironment (TIME) of colorectal cancer (CRC). The influence of *KRAS* on modulating immune cell composition remains unclear. The objective of this study was to identify signatures of infiltrative immune cells and distinctive patterns that differ between *RAS* wild-type (WT) and oncogenic mutant (MT) CRC that explain immune evasion in MT tumors. A total of 7,801 CRC specimens were analyzed using next-generation DNA sequencing, whole-exome sequencing, and/or whole transcriptome sequencing. Deficiency of mismatch repair (dMMR)/microsatellite instability (MSI) and tumor mutation burden (TMB) were also assessed. *KRAS* mutations were present in 48% of CRC, similarly distributed in patients younger than vs. 50 years and older. In microsatellite stable (MSS) *KRAS* MT tumors, composition of the TIME included higher neutrophil infiltration and lower infiltration of B cells. MSI-H/dMMR was significantly more prevalent in *RAS* WT (9.1%) than in *KRAS* MT (2.9%) CRC. In MSS CRC, TMB-high cases were significantly higher in RAS MT (3.1%) than in RAS WT (2.1%) tumors. *KRAS* and *NRAS* mutations are associated with increased neutrophil infiltration, with codon-specific differences. These results demonstrate significant differences in the TIME of *RAS* mutant CRC that match previous reports of immunoevasive characteristics of such tumors.

INTRODUCTION

In the United States, over 152,000 people are diagnosed with colorectal cancer (CRC) and more than 50,000 die of the disease annually. The estimated 5-year survival rate for patients with CRC is 66%.[1] Improvements in screening have been successful in identifying polyps before they turn into cancer or detecting tumors earlier when CRC is easier to cure. Nonetheless, there remains a gap in knowledge regarding the biologic mechanisms of recurrence of both early- and late-stage tumors. For this reason, a major challenge is the emergence of chemoresistance to standard-of-care drug therapy for metastatic CRCs. Over 1 million deaths in the United States each year are due to cancers driven by mutant forms of the RAS oncogene (encoding guanosine triphosphatases that normally regulate cell proliferation). KRAS mutations are thought to be present in 33%–40% of CRC cases.[2,3] In CRC, mutated forms of KRAS are constitutively activated, resulting in the overstimulation of downstream signaling cascades and tumor initiation, progression, cell hyperproliferation, and malignant development and invasion.[4]

The tumor inflammatory microenvironment (TIME) in CRCs is in many ways modulated by oncogenic drivers such as *KRAS*.[5] The influence of oncogenic drivers on modulating immune cell composition, and the consequent effect on metastatic potential or response to therapy, remain unclear. With the rise of immuno-oncologic (IO) therapeutic agents spread across several drug classes, there is ever-increasing importance in identifying IO biomarkers that can be validated as predictive of efficacy in response to treatment. This issue is especially important in this tumor type, especially because

Received 28 December 2023; accepted 26 February 2024;
https://doi.org/10.1016/j.omton.2024.200786.

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Table 1. Patient demographics in relation to RAS mutation status of 7,801 cases of CRC

RAS category	N	Male	Female	Median age, y	Primary/local	Metastatic	Unclear
KRAS MT	3,727	1,758	1,969	61.0	2,008	1,672	47
HRAS MT	10	5	5	66.5	6	3	1
NRAS MT	285	115	170	60.0	153	130	2
Complex	43	17	26	59.0	27	15	1
WT	3,736	1,602	2,134	62.0	2,042	1,641	53
Total	7,801	3,497	4,304	62.0	4,236	3,461	104

the current standard marker of sensitivity to IO treatments in CRC, deficient mismatch repair (dMMR) with or without microsatellite instability (MSI), are altogether only present in up to ∼16% of cases of CRC.[6] Thus, there is strong impetus to discover predictive biomarkers of IO response in the remainder of cases that are marked as microsatellite stable (MSS).

The objective of this study was to confirm associations of mutant *RAS* with features of the TIME conducive to higher metastatic potential and chemoresistance, through genomic and transcriptomic analysis. We also sought to correlate characteristics of immune infiltration and presence of IO biomarkers, focusing on tumor mutation burden (TMB), programmed death ligand-1 (PD-L1), and MSI-high (MSI-H)/dMMR with mutant RAS and other aspects of the TIME.

RESULTS

We performed a retrospective examination of 7,801 CRC tumors identified within the Caris database. The cases were selected based on the identification of CRC tumors that had undergone genomic profiling using the NGS-592 or whole-exome sequencing panels and that had RAS results, with expression data available to analyze the TIME (Table 1).

The median age of all of the patients in this dataset was 62 years. The majority of analyzed tumor samples were from primary tumor locations or local recurrences (4,236 cases/54.3%); the others were from distant metastatic tumors (3,461 cases/44.4%) and 1.3% cases were from unknown sites. KRAS mutant cases comprised 47.7% of all of the cases; an additional 3.6% of cases harbored NRAS mutations, and mutations in HRAS were extremely rare (10 out of 7,801 cases = 0.13%) (Table 1). Of the 3,461 cases of distant metastasis, there was a wide distribution of anatomic site of metastatic spread analyzed for genomic profiling; the most prevalent metastatic sites were liver, lung, and peritoneum (Figure S1). The RAS mutation rate for primary CRC tumors was 51.5%, and 52.4% for distant metastatic tumors (excluding complex cases with >1 RAS mutation). The difference was not statistically significant. For all *RAS* mutations, there was no difference in the distribution between patients younger or older than 50 years (Figure 1).

TMB has been US Food and Drug Administration (FDA) approved as a companion diagnostic for programmed cell death 1 (PD-1) inhibitors in tumor-agnostic fashion.[7] The FDA-approved cutoff for use of IO inhibitors is 10 mutations (mt)/megabase (Mb). We examined TMB in our dataset in relation to the *RAS* status of CRC tumors. TMB-high (TMB-H) (defined as $\geq$ 10 mt/Mb) was higher in wild-type (WT) RAS in the overall cohort. However, when looking at MSS tumors, TMB-H appeared higher in RAS MT compared to the WT (Figure 2). The presence of PD-L1 and of MSI-H was also higher in the setting of WT *KRAS* (Figure 2). Overall, these results indicated that IO markers were consistently more prevalent in tumors harboring WT *KRAS*.

We further examined the overall distribution of TMB across *RAS* subtypes. As seen in the accompanying boxplot (Figure 3), looking at TMB as a continuous variable, the median was higher in KRAS MT compared to WT ($p < 0.001$) (Figure 3). We next examined TMB levels more specifically in populations of the MSS CRC cases. The relation of TMB and *RAS* mutations in MSS tumors is shown in Figure 3. ** indicates $p < 0.0001$.

RAS WT tumors harbored an especially wide distribution of range of TMB. The median TMB was lower in the *RAS* WT population (4 mt/Mb) than in *KRAS* MT tumors (5 mt/Mb) ($p < 0.001$). This finding also held for the few detected cases of *HRAS* MT, as well as cases of combined *KRAS/NRAS* MT and *KRAS/HRAS* MT, but not for *NRAS* MT alone. The median distribution of TMB across *RAS* subtypes was relatively uniform in MSS CRC samples (Figure 3). The differences in TMB levels were significant between *KRAS* MT and *KRAS* WT ($p < 0.001$), and between *NRAS* MT and *NRAS* WT ($p < 0.001$). Using the standard and FDA-approved TMB cutoff of 10 mt/Mb to define the TMB-H population, we found that aside from *HRAS*, the prevalence of TMB-H with *KRAS* or *NRAS* mutations was 5%–18%; these results were independent of the microsatellite status (Figures 3A and 3B). The percentage of TMB-H was highest in *HRAS* MT tumors, although the total number of *HRAS* mutants was very low. HRAS is not a classical mutation in CRC, and this high level is maintained in the MSS cohort; the overall numbers are too low to determine the underlying driver of this finding. Aside from *HRAS*, the prevalence of TMB-H was only slightly higher in *KRAS* MT (3.1%) and *NRAS* MT (3.3%) as compared to all-*RAS* WT tumors (2.1%) (Figure 3).

The TIME of CRC comprises a constantly evolving landscape affected significantly by the extent and composition of immune infiltration. We used QuanTIseq to quantify the extent of immune cell

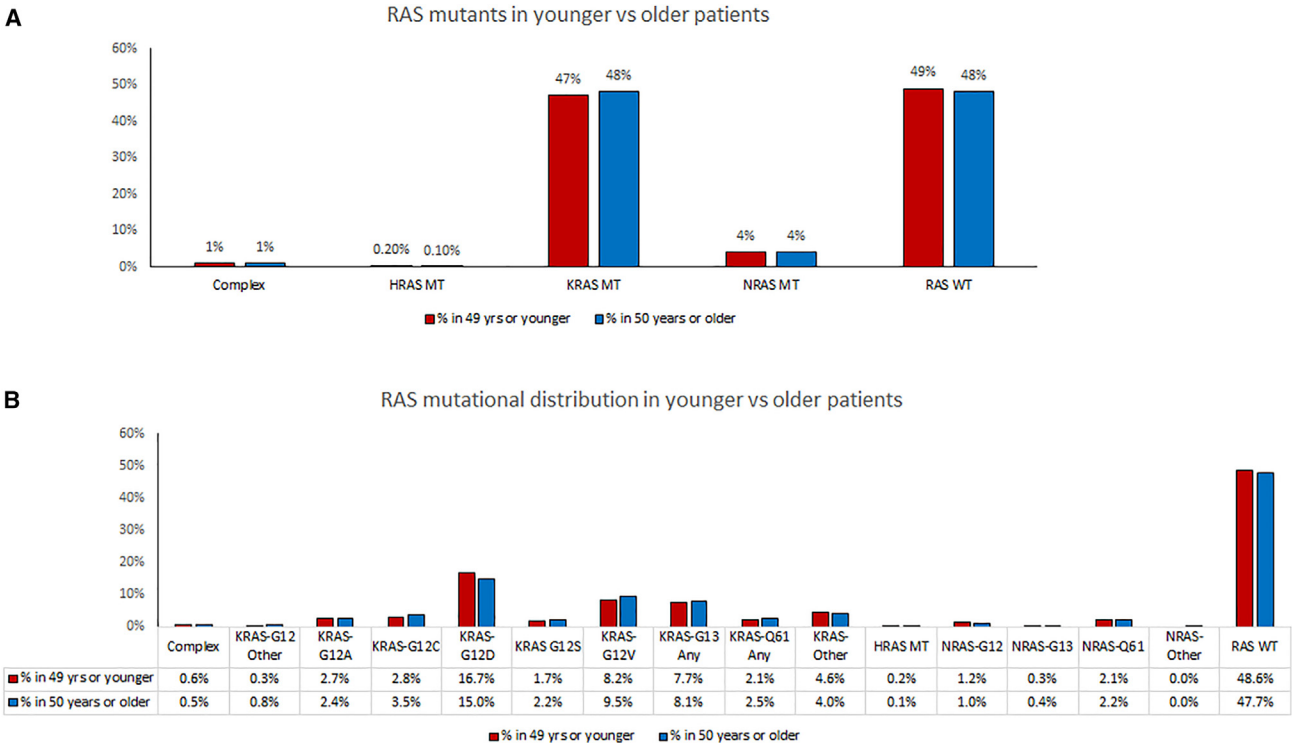


Figure 1. Distribution of RAS mutations across our cohort of 7,801 cases of CRC identified in the Caris database

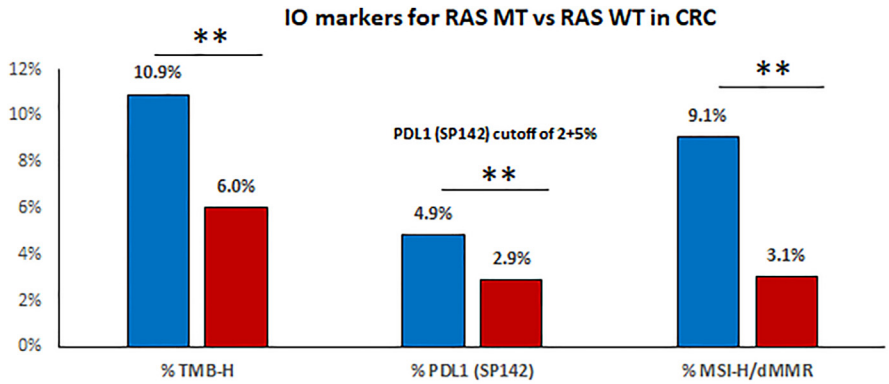
(A) Distribution of RAS mutants in patients <50 years vs. >50 years of age.

(B) Distribution of specific isoforms of RAS in patients <50 years vs >50 years of age.

infiltration in our specimens, and analyzed the association of these infiltration landscapes with status of *RAS* in MSS CRC tumors. We investigated potential differences in immune cell composition and quantity among primary CRC tumors and then separately in a cohort of distant metastatic tumors. The TIME of primary CRC differed based on *RAS* mutation status, in that *KRAS* and *NRAS* MT tumors displayed less infiltration of M1 macrophages, $CD8^+$ T cells, and B cells as compared to their *RAS* WT counterparts (Figure 3A). Neutrophilic infiltration was higher in *KRAS* MT primary tumors as well. By contrast, in metastatic tumors, there were no discernible differences in M1 macrophage infiltration in *KRAS* MT vs. WT. The metastatic tumors retained the decreased infiltration of B cells and $CD8^+$ T cells in *KRAS* and *NRAS* MT cases and increased neutrophils in *KRAS* MT (Figure 3B). However, there was less infiltration of natural killer (NK) cells in *KRAS* MT metastatic tumors as compared to *KRAS* WT, a difference not seen in primary tumor samples. Furthermore, the fraction of regulatory T cells (Tregs) differed significantly between tumors from these anatomic sites, with increased Tregs seen in metastatic CRC and decreased Tregs seen in primary tumors.

When focusing on the MSS population, we found that neutrophil infiltration in the TIME of MSS tumors was significantly higher in tumors that harbored *KRAS* MT compared to *KRAS* WT (Figure 4). When we examined the full landscape of immune cell types comprising B cells, M1 and M2 macrophages, $CD8^+$ and $CD4^+$ T cell subtypes, NK cells, and monocytes in addition to neutrophils, the MSS tumors and TIME-profiled B cells, M2 macrophages, $CD8^+$ T cells, dendritic cells, and fibroblasts all were lower in number in *KRAS* MT CRCs. In *NRAS* MT tumors, B cells and M1 macrophages in particular were lower in prevalence. In contrast, neutrophils were higher in both *KRAS* and *NRAS* MT tumors. We summarize our findings from MSS tumors in Figure S2. Overall, there was a significantly higher extent of neutrophil infiltration in *KRAS* MT (median cell fraction 6.6% vs. 5.9%). This finding was also seen when individual codons were studied. Similarly, *NRAS* MT (6.9%) CRCs showed higher neutrophil infiltration than WT tumors. B cells, M2 macrophages, $CD8^+$ T cells, dendritic cells, and fibroblasts were lower in *KRAS* MT tumors; B cells and M1 macrophages were lower in *NRAS* MT samples ($q < 0.05$).

MSI-H/dMMR is of extreme interest in the CRC oncology community due to the association of this trait with susceptibility to checkpoint immune inhibition. Thus, we delved into this small but clinically significant subpopulation further by identifying the immune landscape in this context. We found that MSI-H/dMMR was significantly more prevalent in *RAS* WT (9.1%) than in *KRAS* MT (2.9%) or *NRAS* MT (1.8%) tumors, and was the highest in *HRAS* MT tumors (60%, $q < 0.05$), keeping in mind that *HRAS* was seen in very few tumors (10 of 7,801 total CRC cases; Table 1). Accordingly, TMB-H ($\geq$10 mt/Mb) was more prevalent in *RAS* WT (10.9%) than *KRAS*



RAS mutation status	RAS WT	KRAS/NRAS/HRAS MT
Positive	175	114
Negative	3432	3820
Total	3607	3934
% PDL1 (SP142)	***4.9%***	***2.9%***
Positive	395	233
Negative	3222	3629
Total	3617	3862
% TMB-H	***10.9%***	***6.0%***
Positive	339	124
Negative	3385	3927
Total	3724	4051
% MSI-H/dMMR	***9.1%***	***3.1%***
Fisher's exact p value	p<0.0001	

MSS

	RAS WT	RAS MT
Positive	69	117
Negative	3218	3625
Total	3287	3742
% TMB-H	2.1%	3.1%
Fisher's exact p value	p=0.0073	

Figure 2. Comparison of TMB, PD-L1, and MSI-H in the WT vs. mutant RAS populations

(5.8%) or *NRAS* (5.1%) MT, and highest in *HRAS* MT tumors (70%, all $q < 0.05$) (Figure 3). However, once MSI-H cases were removed and only the MSS were analyzed (Figure S2), there were more MSS^{+}*KRAS* MT tumors with a TMB-H profile than MSS^{+}*KRAS* WT (3.1% vs. 2.1%, $q < 0.05$) (Figures 2 and 3), especially in *KRAS* non-12/13/61 codon mutations (5.5%, vs. 2.1%, $q < 0.05$), and G12C (4.4%, $p < 0.05$). We also analyzed PD-L1 expression; in MSS tumors, *KRAS* G12D (10.4%) and G13 MT (11.8%) showed higher expression than *RAS* WT tumors ($q < 0.05$).

Using QuanTISeq, we detected significantly increased infiltration of NK cells in *KRAS* MT MSI-H/dMMR CRC, with concurrent decreased $CD4^{+}$ and $CD8^{+}$ T cells (Figure 5). No differences were seen in the Treg or other relevant immune populations, including M1 or M2 macrophages, monocytes, myeloid dendritic cells, neutrophils, or B cells, in this context.

Finally, we sought to investigate gene sets in the MSI-H/dMMR subgroup, with special focus on pathways associated with resistance vs. response to immune checkpoint immunotherapy (e.g., interferon γ [IFN-γ] signaling, WNT, RIG-I, PD-L1, TMB, CXCL9, TRAF2, STK11), comparing *RAS* MT vs. WT cases. We performed gene set enrichment analysis to identify molecular and immune pathways that were differentially expressed between MSI-H tumors that were *RAS* MT vs. WT. Tumors with more than one *RAS* MT were excluded from this analysis. Using this approach, we detected enrichment in immune profile subsets representing IFN-α and -γresponses, as well as those associated with allograft rejection (Figure S3). The Transducer of ERBB2 antiproliferative pathway is implicated in T cell regulation and activation, with expression being the lowest in activated T cells. We found that this pathway was downregulated in the *RAS* WT MSI-H group, as compared to *RAS* MT MSI-H. When we performed similar analyses focusing on the MSS populations that were *RAS* MT vs. *RAS* WT, there were no significantly enriched pathways detected in the Hallmark, BioCarta, and Kyoto Encyclopedia of Genes and Genomes gene sets (Figure S3).

We analyzed the dataset in context of consensus molecular subtyping (CMS) for CRC. The CMS molecular classification system was established and adapted following publication in 2015 as a way to distinguish subtypes based on gene expression.[8] We examined the results of our entire cohort in the context of the CMS categories and determined that there were significantly more KRAS MT than KRAS WT in CMS3 subtype cases ($p < 0.001$, 23% vs. 10%), whereas RAS WT was significantly higher than KRAS MT in CMS2 ($p < 0.001$, 37% vs. 27%) (Figure S4). CMS1 subtype cases demonstrated

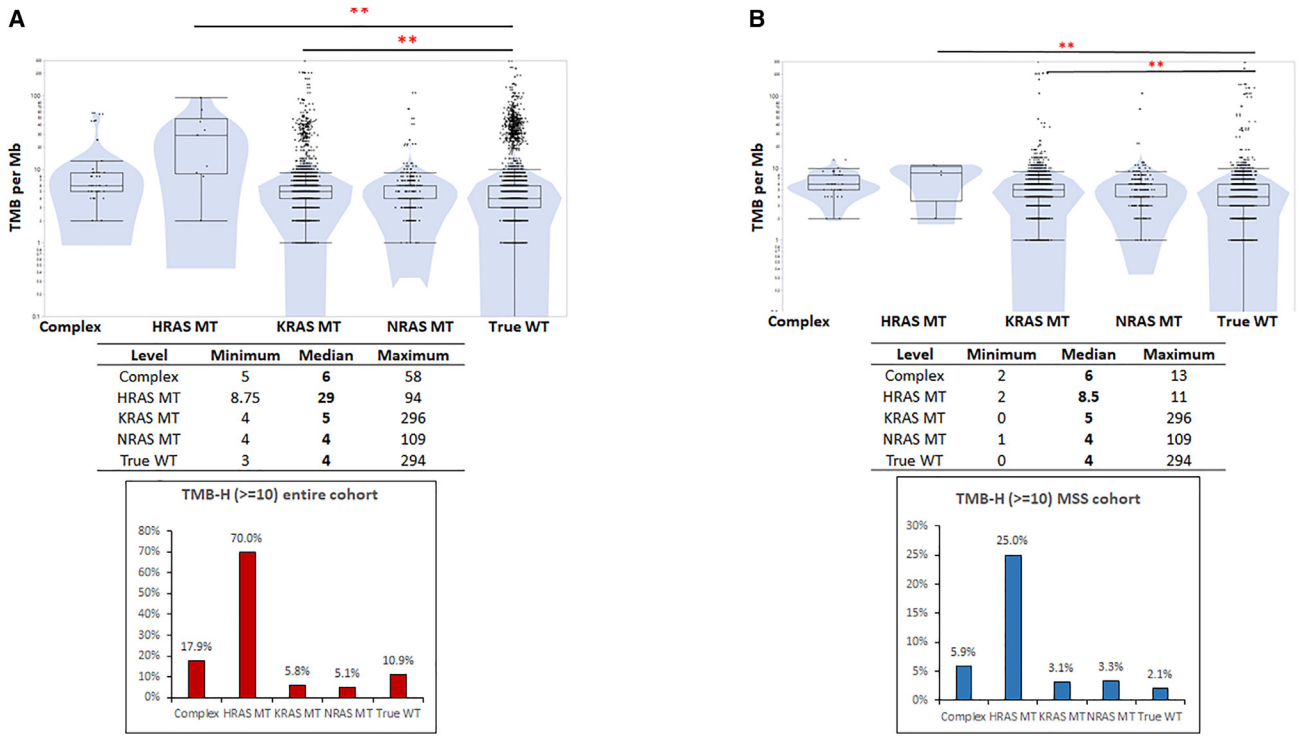


Figure 3. Analysis of TMB in context of RAS status

(A) TMB and RAS mutations in entire cohort and (B) in MSS. **p < 0.001.

significantly higher RAS WT than KRAS MT ($p < 0.01$, 17% vs. 15%). In NRAS MT cases, WT tumors were significantly higher in CMS1 (17% vs. 8%, $p < 0.001$); however, CMS2 was significantly more enriched in NRAS MT vs. WT (44% vs. 37%, $p = 0.02$), as was CMS 3 (16% vs. 10%, $p < 0.01$). The rare HRAS MT cases were significantly higher in CMS 1 than RAS WT ($p < 0.01$, 60% vs. 17%). When we isolated the MSS-specific cohort, the same significant differences were seen, with the exception of the finding that in the CMS1 subtype cases, KRAS MT was significantly higher than KRAS WT. For the NRAS MT cases, similarly significant differences were seen except for CMS1 (no significant differences).

DISCUSSION

In this study, we characterized the prevalence of IO biomarkers (e.g., TMB, PD-L1, MSI-H/dMMR), the composition of the immune microenvironment, and their relationship with mutations in the *RAS* oncogenes in metastatic CRC. We performed genomic and transcriptomic analyses, including RNA deconvolution analysis, to infer the cellular composition of the microenvironment. We found that *KRAS* and *NRAS* mutations in CRC tumors were associated with increased neutrophil abundance compared to WT counterparts, with codon-specific differences, and lower prevalence of MSI-H/dMMR status (see overall summary in Figure S5). *HRAS* mutations were confirmed to be extremely rare alterations in CRC, showed no difference in neutrophil abundance compared to *HRAS* WT, and were associated with a higher prevalence of MSI-H/dMMR. Overall, $CD8^+$ T cells and B cells were less abundant in *KRAS* and *NRAS* mutants, although substantial variability was seen among different protein changes. As a whole, *RAS* mutations were more prevalent in our analysis than has been generally reported in other studies, but this incidence did not vary by age.

The issue of microsatellite stability vs. instability has predominated discussions of efficacy of IO therapeutic drugs, even more so than PD-L1. PD-L1 has not been validated yet as a predictive biomarker in the setting of metastatic CRC, although expression on the higher end of the spectrum, as assessed using the Combined Positive Score system, has revealed this surface marker to be associated with higher responsiveness to immune checkpoint inhibitors in various other cancer types, including upper gastrointestinal cancers. Whether MSS tumors, which make up the vast majority of all of the cases of metastatic CRC, harbor other features that will allow this susceptibility is a popular line of investigation. The extent of immune infiltration is emerging as a potential new biomarker of treatment response, often with the designation of "hot," indicating tumors with heavy amounts of immune infiltration, and "cold" for tumors lacking notable amounts. These designations remain vague and not well defined to this point. Here, we found that in MSS tumors, *KRAS* mutations were associated with a higher TMB almost unanimously, whereas PD-L1 was elevated in CRC samples harboring either the G12D or G13 isoforms of *KRAS*. There was a large group of TMB-H in WT (likely due to MSI-H; MSI-H tumors are enriched for *BRAF*

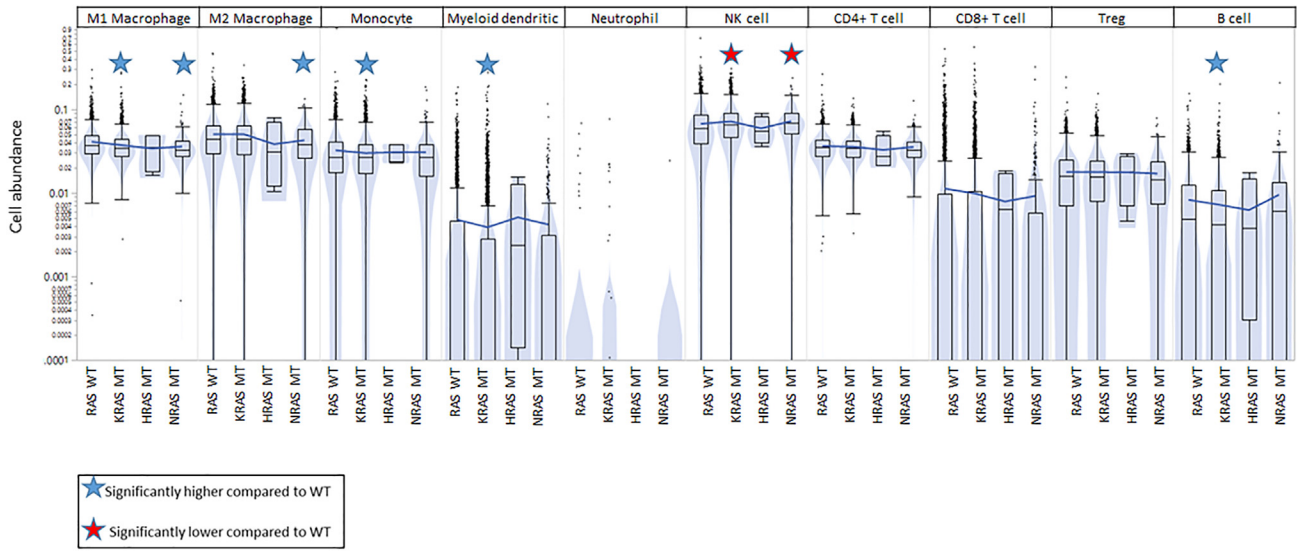


Figure 4. Immune infiltration and the TME in RAS buckets in MSS tumors

mutations for the CpG island methylator phenotype subtypes, which are mutually exclusive of *KRAS* mutations). Therefore, the proportion of TMB ≥ 10 was higher in WT tumors, but the median of *KRAS* is actually higher, due to different distribution patterns. We speculate that the increased prevalence of TMB-H in the MT RAS population may be due to coexpression of altered DNA polymerase epsilon (encoded by the POLE gene) or other drivers that increase immunogenicity and that merit future exploration.

The anti-PD-1 immune checkpoint inhibitor pembrolizumab has been approved for MSI-H/dMMR metastatic CRC in the first-line setting.[9] Compared to standard therapy, pembrolizumab improved progression-free survival and overall response rate in this subset of patients. However, despite better prognosis of MSI-H/dMMR CRC, only ~50% of patients have shown response upon pembrolizumab therapy. Notably, patients harboring an *RAS* mutant tumor seem to not benefit from pembrolizumab therapy.[10] *KRAS* mutation has been increasingly recognized for its role in engaging TIME to favor tumor progression and elicit resistance to targeted and IO therapy.[9] In our study, both *KRAS* and *NRAS* mutations were associated with increased neutrophil prevalence and infiltration within the TIME, with codon-specific differences, whereas *HRAS* showed no difference. This is in line with a recently published study that examined chemokines in *KRAS* mutant CRCs.[11] Using a murine model, the authors could show that interleukin-8 production increases intratumoral neutrophil enrichment. Another study revealed that neutrophils are increased in the serum of metastatic CRC patients and are believed to play an important role in the metastatic spread of CRCs.[12] In terms of the exact players from the immune system, overall $CD8^+$ T cells and B cells were less abundant in *KRAS* and *NRAS* mutants, and substantial variability was seen among different molecule changes; *HRAS* mutation was associated with the highest $CD8^+$ T cell and B cell abundance.

We detected some noticeable differences in immune infiltration, assessed using QuanTISeq, between primary CRC and distant metastatic tumor specimens. Specifically, there were fewer NK cells in KRAS MT distant metastatic samples, whereas there were no differences in NK volume in KRAS MT vs. WT primary tumor samples; also, Tregs were enriched in the metastatic tumors but not in primaries (Figure 3). How these differences may play a role in the efficacy of immune checkpoint inhibition and other forms of immunotherapies including NK and T cell-based cell therapies is a fertile field for further investigation in this era of burgeoning clinical trial investigation of these strategies in CRC. The clinical relevance of our findings is highlighted by subgroup analyses of the KEYNOTE-177 trial, which reported that the location of metastatic spread is associated with pembrolizumab efficacy (overall survival: pulmonary metastasis hazard ratio [HR] 1.99 vs. hepatic metastasis HR 0.68).[9] Immune infiltration patterns that differ between primary and metastatic sites may provide a window of opportunity for analysis that could be used to tailor the use of immune checkpoint inhibition based on anatomic sites of spread and other factors unique to each individual patient, to further maximize efficacy beyond genetic biomarkers.

Overall, the KRAS MT subpopulation in our cohort displayed increased infiltration of M2 macrophages along with decreased abundance of M1 macrophages. The extent of M2 macrophage infiltration has been reported to be relatively higher in more advanced stages of CRC, localizing most prominently at the invasive front of tumors and within lymph node metastases; thus, this finding is also associated with worse prognosis, independent of MSI status.[13,14] Conversely, M1 infiltration is associated with better patient prognosis because low counts of this tumor-associated macrophage (TAM) are associated with lymph node metastases,[14] even as infiltration by both forms of these TAMs occur together rather than in a mutually exclusive manner.[13] Activated M2 macrophages secrete factors that induce

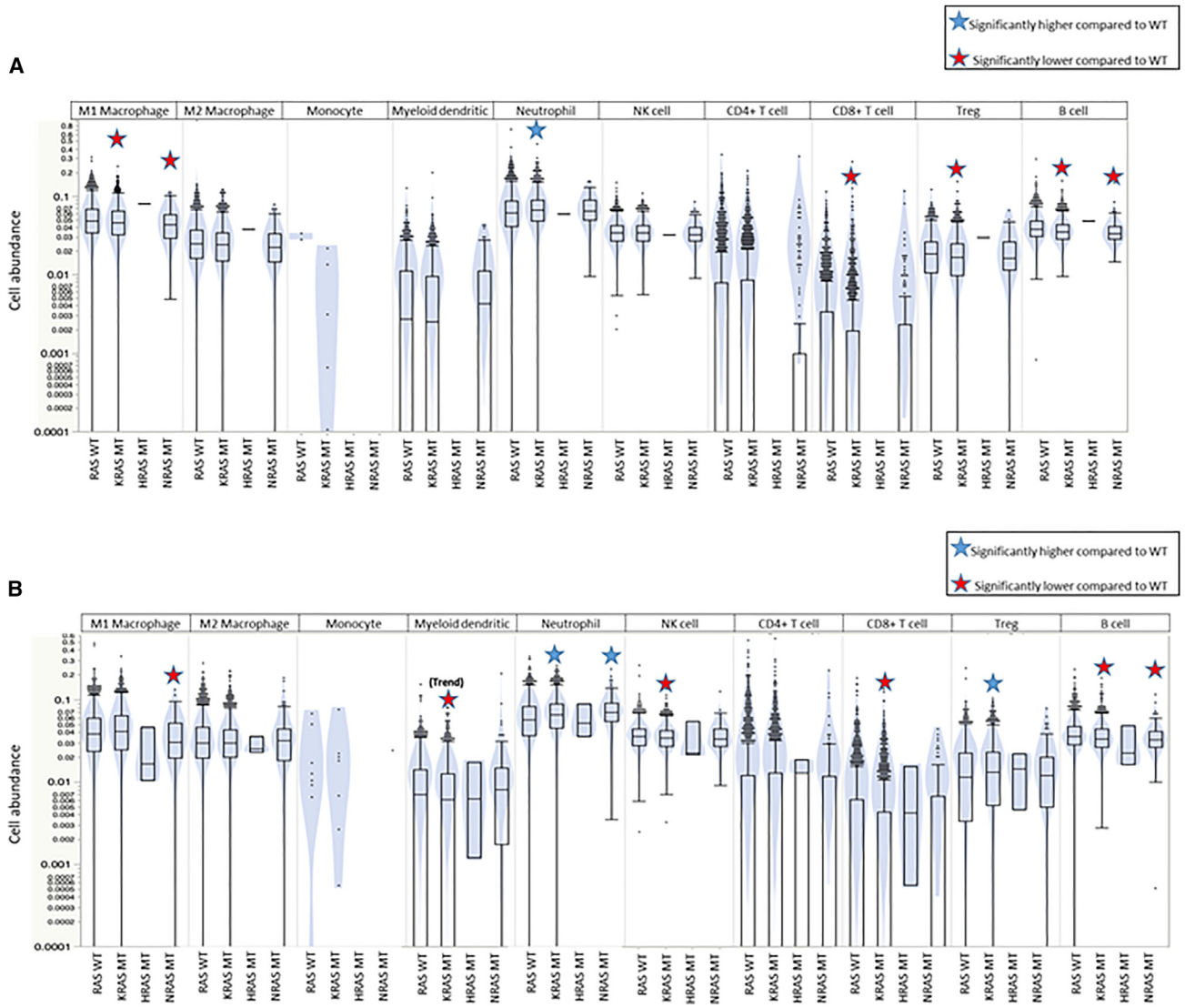


Figure 5. Immune infiltration patterns

TME in (A) primary tumors (MSS) and in (B) metastatic tumors (MSS).

tumor growth and progression while simultaneously producing immunosuppression of the surrounding microenvironment.[13] Notably, although a difference in M1 infiltration was seen in KRAS MT vs. WT primary CRC cases, we did not detect any significant differences in metastatic tumors. This finding provides a basis for a more pivotal role of M1 macrophages in modulating the TIME at the earliest stages of CRC, and less so in clones that spread via hematogenous routes and require variable microenvironmental factors that permit growth of those clones in distant organs. Likewise, a role of M1 TAMs more focused on locoregional spread to lymph nodes would be more consistent with the current understanding of its role at the cellular level. We also found a higher extent of neutrophil infiltration in KRAS MT tumors. In a fashion similar to that of TAMs, tumor-associated neutrophils (TANs) have been categorized as N1 and N2 subtypes associated with antitumorigenic and protumorigenic properties, respectively.[15,16] Although our data did not distinguish between these particular subtypes, the overall increased abundance of neutrophils in tandem with increased M2 TAMs suggests an additive, and possibly synergistic, inflammatory composition that induces immunosuppression in KRAS MT cases. The reported role of TANs in angiogenesis and overall promotion of metastasis is believed to be orchestrated in part by mesenchymal stromal cells.[16] Overall, defining differences in the CRC TIME within KRAS MT primary and metastatic tumors provides another layer of understanding that will inform targeted therapeutic strategies in the era of direct targeting of specific isoforms of KRAS, with or without incorporation of immune checkpoint inhibitors under investigation.[17] Examples have emerged over the past several years that inhibition of KRAS MT

(e.g., G12C) induces changes in the surrounding TIME that sensitize tumors to checkpoint inhibitor therapy.[18] Such work has profound implications for expanding the sphere of patient populations that are likely to benefit from combination immunotherapy regimens, beyond the current requirement of dMMR/MSI-H.

The vast amount of attention garnered by dMMR/MSI-H CRC cases, in relation to eligibility for immunotherapy, is being addressed by research aimed at the majority of CRC cases, which are MSS. The theory that the TIME can be modulated to induce a higher level of immunogenicity in MSS tumors is an area of active exploration. The use of multikinase inhibitors such as regorafenib administered in concert with immune checkpoint inhibitors is one relatively recent example leading to significant response rates, with findings that this success may be limited to some anatomic sites over others.[19–21] How and to what extent immune infiltration patterns and composition vary among tumors driven by oncogenic KRAS is an area that requires deeper exploration in clinical trial design, in the context of emerging small-molecule inhibitors of specific isoforms of KRAS of varying clinical efficacy.[5] Patterns of immune infiltration stratified by CMS subtypes have been reported by Becht et al..[22] and others. Our results showing the increased prevalence of MT KRAS and NRAS in CMS3, the metabolic CRC subtype associated with low degrees of inflammation, support a growing body of data indicating the ability of RAS to orchestrate immune evasion. Likewise, lower rates of MT RAS in CMS1, associated with a relative more immune-rich TIME, particularly in cytotoxic lymphocyte penetration, also provides support to this notion.[22] The fact that 4%–5% of tumors with now-targetable KRAS G12C alterations in MSS^{+}*KRAS* MT tumors were also TMB-H points toward a small but nonetheless significant subset of patients who may benefit from covalent RAS-targeted small-molecule inhibitors in combination with immunotherapeutic agents. With more such RAS-targeted inhibitors in development and in clinical trial stages, these subsets are going to increase in proportion over the next decade.[5]

With increasing attention to and interest in young adult/early-onset forms of CRC, fueled by recent reports of the rise in incidence and prevalence of this subpopulation,[23] we sought to examine whether there were any differences in *RAS* mutations in CRC in this subpopulation vs. the population of CRC patients 50 years and older. If the prevalence of RAS mutations is significantly enriched in patients with young adult/early-onset forms of CRC, defined as patients diagnosed with CRC at younger than 50 years of age,[24] then this disparity would have critical implications for the notion that early-onset CRC has a different biologic imprint and thus clinical behavior than CRC that occurs in older adults. This is especially important in young-onset CRC because of the role of mutated RAS in the early stages of CRC carcinogenesis in young adults (30–50) proposed by Vogelstein and Fearon 3 decades ago.[25,26] Our large dataset afforded us the opportunity to confirm the reported prevalence of *RAS* mutations in CRC in general and in the young adult/early-onset CRC subpopulation. Here, we did not detect any differences in the distribution of RAS expression between patients younger or older than 50 years.

An additional finding of this study was that the percentage of *KRAS* MT cases is slightly higher than expected (48.1%) but mirrors other recent studies confirming KRAS alterations in nearly half of all cases of CRC.[27]

Although the large numbers in our dataset provide advantages to examining subset, there are limitations of this study that include the retrospective nature of the study and analysis, as well as limited availability of some clinical outcomes and related data. We analyzed metastatic tumors collectively for comparison to profiles of primary CRC tumors; future focus on anatomic site-specific analyses may yield additional insights into differences between metastatic lesions that may be attributable to varying extent of immune infiltration and content in the surrounding microenvironments.

In summary, these results demonstrate significant differences in the TIME of *RAS* mutant CRC that identify variable susceptibilities to IO agents and provide further detailed characterization of heterogeneity between *RAS* variants at the molecular and immunogenic levels.

MATERIALS AND METHODS

Samples

A total of 7,801 CRC tumors were submitted to Caris Life Sciences (Phoenix, AZ). This study was conducted in accordance with guidelines of the Declaration of Helsinki, Belmont report, and U.S. Common rule. In keeping with 45 CFR 46.101(b) (4), this study was performed using retrospective, deidentified clinical data. Therefore, this study is considered institutional review board exempt and patient consent was not required.

Next-generation sequencing (NGS)

NGS was performed on genomic DNA isolated from formalin-fixed paraffin-embedded (FFPE) tumor samples using the NextSeq platform (Illumina, San Diego, CA). Matched normal tissue was not sequenced. A custom-designed SureSelect XT assay was used to enrich 592 whole-gene targets (Agilent Technologies, Santa Clara, CA). All of the variants were detected with >99% confidence based on allele frequency and amplicon coverage, with an average sequencing depth of coverage of >500 and an analytic sensitivity of 5%. Before the molecular testing, tumor enrichment was achieved by harvesting targeted tissue using manual microdissection techniques.

TMB

TMB was measured by counting all nonsynonymous missense, nonsense, in-frame insertion/deletion, and frameshift mutations found per tumor that had not been previously described as germline alterations in dbSNP151, Genome Aggregation Database (gnomAD) databases, or benign variants identified by Caris geneticists. A cutoff point of $\geq$10 mt/Mb was used based on the KEYNOTE-158 trial,[28] which showed that patients with a TMB of $\geq$10 mt/Mb across several tumor types had higher response rates than patients with a TMB of <10 mt/Mb. Caris Life Sciences is a participant in the Friends of Cancer Research TMB Harmonization Project.

MSI

A combination of multiple test platforms was used to determine the MSI-H/dMMR status of the tumors profiled, including fragment analysis (FA; Promega, Madison, WI), immunohistochemistry (IHC; MLH1, M1 antibody; MSH2, G2191129 antibody; MSH6, 44 antibody; and PMS2, EPR3947 antibody [Ventana Medical Systems, Tucson, AZ]), and NGS (for tumors tested with NextSeq platform, 7,000 target microsatellite loci were examined and compared to the reference genome hg19 from the University of California). The three platforms generated highly concordant results, as previously reported, and in the rare cases of discordant results, the MSI or MMR status of the tumor was determined in the order of IHC, FA, and NGS.

mRNA expression (whole-transcriptome sequencing)

Expression data were evaluated on mRNA isolated from a FFPE tumor sample using the Illumina NovaSeq platform and the Agilent SureSelect Human All Exon V7 bait panel; transcripts per million were reported. Gene fusions were detected using the Illumina NovaSeq platform. In addition, immune cell fraction was calculated by QuanTIseq using these transcriptomic data.

Data and statistical analysis

Cohorts were defined by having a pathogenic/presumed pathogenic mutation in *KRAS*, *NRAS*, or *HRAS* or being *RAS* WT (identified by the NGS platform previously described). Comparative analysis of molecular alterations in the cohorts were analyzed using chi-square or Fisher exact tests. TMB distribution as well as tumor microenvironment cell fractions were analyzed among cohorts using nonparametric Kruskal-Wallis testing. A p value of <0.05 was considered a trending difference; p values were further corrected for multiple comparison using the Benjamini-Hochberg method to avoid type I error, and an adjusted p value (q value) of <0.05 was considered a significant difference.

DATA AND CODE AVAILABILITY

The study protocol and statistical analysis plan are available in the paper. Other data (including the summary of genomic data) will be made available upon reasonable request from jxiu@carisls.com.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omton.2024.200786.

ACKNOWLEDGMENTS

The authors thank all of the participating centers of the Caris Precision Oncology Alliance and their support staff for contributing to this research project. This work was presented in poster format at the 2021 Annual Meeting of the American Society of Clinical Oncology.

AUTHOR CONTRIBUTIONS

Conceptualization: E.L. Methodology: E.L., J.X., Y.B., and M.O. Writing – original draft: E.L., J.X., and Y.B. Software, formal analysis, data curation: J.X., Y.B., and M.O.. Investigation, writing – review & editing: E.L., J.X., Y.B., A. Saeed, A.P., S.G., S.S., T.S., E.F., R.P., H.J.L., A.F.S., C.N., M.O., A. Seeber, and W.E. Supervision, project administration: E.L.

DECLARATION OF INTERESTS

J.X., Y.B., C.N., and M.O. are employed by Caris Life Sciences. E.L. reports support from the University of Minnesota Clinical Center for the Study of Pancreatic Disease, part of The Chronic Pancreatitis Diabetes Pancreatic Cancer Research consortium funded by the National Institute of Diabetes and Digestive and Kidney Diseases (5U01DK126300-03). He reports support from research grants from the American Cancer Society (RSG-22-022-01-CDP) 2022–2026; the Minnesota Ovarian Cancer Alliance in 2019, 2021, and 2022; the American Association for Cancer Research (2019 AACR-Novocure Tumor-Treating Fields Research Grant 1-60-62-LOU); the Randy Shaver Cancer Research and Community Fund; honoraria and travel expenses for lab-based research talks 2018–2021 and equipment for laboratory-based research 2018–present from Novocure; honorarium for panel discussion organized by Antidote Education for a CME module on diagnostics and treatment of $HER2^{+}$ gastric and CRCs, funded by Daiichi-Sankyo, 2021 (honorarium donated to lab); compensation for scientific review of proposed printed content from Elsevier Publishing and The Johns Hopkins University Press; institutional principal investigator for clinical trials sponsored by Celgene, Novocure, Intima Bioscience, and the National Cancer Institute; and University of Minnesota membership in the Caris Life Sciences Precision Oncology Alliance (no financial compensation). He acknowledges and thanks the following groups for donations in support of cancer research: friends and family of Gayle Huntington; the Mu Sigma Chapter of the Phi Gamma Delta Fraternity, University of Minnesota; the Litman Family Fund for Cancer Research; Dick and Lynnae Koats; Ms. Patricia Johnson; and the Love Like Laurie Legacy.

A. Saeed reports research grants (to institution) from AstraZeneca, Bristol Myers Squibb, Merck, Clovis, Exelixis, Actuate Therapeutics, Incyte Corporation, Daiichi Sankyo, Five Prime Therapeutics, Amgen, Innovent Biologics, Dragonfly Therapeutics, KAHR Medical, Oxford Biotherapeutics, BioNTech, and Data Safety Monitoring Committee (DSMC); consulting fees from Arcus Therapeutics; and advisory board fees from AstraZeneca, Bristol Myers Squibb, Exelixis, Pfizer, and Daiichi Sankyo.

E.F. reports the following disclosures: employee of Hospital Corporation of America (HCA) International; personal financial interest (conference attendance): Repair Therapeutics, CARIS Life Science, Seagen, and Sapience Pharma; funding to institution: Repair Therapeutics, Bicycle Therapeutics, Artios Pharma, Seagen, Amgen, Nurix Therapeutics, BioNTech SE, Relay Therapeutics, Tahio Pharmaceutical, Pfizer, Roche, Daiichi Sankyo, Gilead Science, Basilea Pharmaceutica, Jiangsu Hengrui Medicine, Mereo Biopharma, HUTCHMED, Merus, Crescendo Biologics, GSK plc, BeiGene, Turning Point Therapeutics, Sapience Pharma; and leadership role: European Organisation for Research and Treatment of Cancer.

A.F.S. reports funding from Caris Life Sciences for research, travel, and the speaker's bureau.

H.J.L. reports advisory board/honoraria from Bayer, BMS, Merck, 3TBioscience, Fulgent, Boehringer-Ingelheim, UCB, Replimune, and Adagene, and is supported by SWOG Cancer Research Network, the National Cancer Institute, and the NIH.
A. Seeber is on the speaker's panel for Caris Life Sciences.

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