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Tumor mutational burden modulates the prognostic effect of *RAS* mutations in metastatic colon cancer: mechanistic insights and genotype-phenotype correlations

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Abstract

Background *RAS* mutations, present in 40–50% of metastatic colorectal cancer (mCRC) cases, drive oncogenic signaling and confer resistance to anti-EGFR therapies. Tumor mutational burden (TMB), a marker of genomic instability, has recently emerged as a predictive biomarker of response to immunotherapy. However, the prognostic interaction between *RAS* status and TMB in mCRC remains poorly defined.

Patients and methods We analyzed 108 patients with microsatellite-stable metastatic colon cancer (mCC). Tumor samples were profiled using the TruSight Oncology® platform. Eligible patients had an ECOG Performance Status < 2, a cachexia risk score < 1, and no peritoneal carcinomatosis. TMB and *RAS* mutation status were assessed, and the prognostic significance of the different *RAS*/TMB combinations was evaluated for overall survival (OS) using Kaplan–Meier and Cox proportional hazards models. Biological differences across selected subgroups were explored using Gene Ontology (GO) enrichment and Phenolyzer network analyses.

Results *RAS* mutations were associated with reduced OS (46.4 vs. 67.9 months for mutant vs. wild-type; HR 1.76; $P = 0.0495$). Stratified analysis showed that the adverse effect of *RAS* mutations was restricted to patients with low TMB (< 10 mutations/Mb). The subgroup with both *RAS* mutations and low TMB had the poorest OS (28.0 months; HR 2.34; $P = 0.0058$), whereas patients with either *RAS* wild-type or high TMB showed comparable survival. GO analysis revealed

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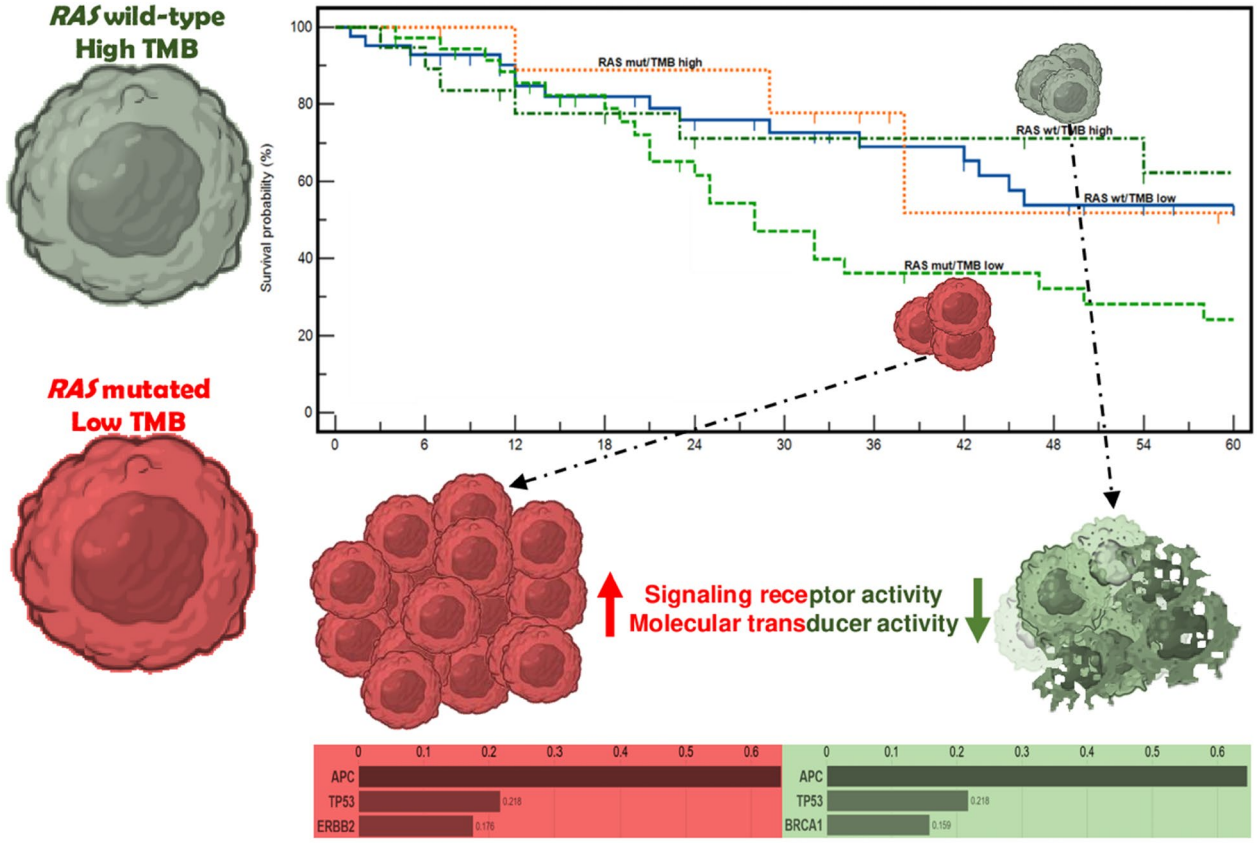
enrichment of receptor-mediated signaling pathways in *RAS*-mutant/TMB-low tumors. Phenolyzer highlighted distinct molecular networks, with *APC*, *TP53*, and *ERBB2* as central hubs in *RAS*-mutant/TMB-low tumors, and *APC*, *TP53*, and *BRCA1* in *RAS*-wild-type/TMB-high tumors.

Conclusions This study demonstrates a prognostic interaction between *RAS* mutations and TMB in mCC, identifying the *RAS*-mutant/TMB-low subgroup as having the poorest outcomes. Integrative bioinformatic analyses suggest distinct biological mechanisms underlying these differences. These findings support the development of tailored therapeutic and monitoring strategies for specific molecular subgroups.

Keywords *RAS* mutations, Tumor mutational burden, Prognosis, Colorectal cancer, Colon cancer

Graphical Abstract



Patients with *RAS*-wild-type/high-TMB metastatic colon cancers show the most favorable prognosis, likely due to weaker proliferative signaling and enhanced immune recognition. Conversely, *RAS*-mutant/low-TMB tumors exhibit the worst survival, driven by hyperactivated oncogenic pathways and reduced immunogenic visibility.

Background

Colorectal cancer (CRC) is the third most commonly diagnosed cancer worldwide and the second leading cause of cancer-related mortality, with an estimated 1.9 million new cases and approximately 935,000 deaths reported annually. This global burden is expected to rise due to population aging and lifestyle factors [1]. A substantial proportion of CRC cases (20–25%) are diagnosed at an advanced stage as metastatic colorectal cancer (mCRC), which carries a poor prognosis [2]. Despite significant progress in systemic treatments, including targeted therapies such as anti-EGFR (epidermal growth factor receptor) and anti-VEGF (vascular endothelial growth factor) antibodies, immunotherapies, and oral agents, the median overall survival for patients with mCRC remains approximately three years, with long-term survival achieved by only a minority of patients [3].

Understanding the genetic and molecular underpinnings of mCRC is essential for improving patient outcomes and optimizing treatment strategies. Mutations

in the *RAS* family of oncogenes are among the most extensively studied genetic alterations in mCRC [4]. *RAS* genes encode small GTPases that regulate key cellular signaling pathways involved in proliferation, differentiation, and survival. Mutations, particularly in codons 12, 13, and 61, lead to constitutive activation of RAS proteins, driving persistent stimulation of downstream signaling pathways such as MAPK and PI3K/AKT [5]. This aberrant signaling promotes oncogenesis by facilitating uncontrolled cell growth, apoptosis evasion, and metastasis. *RAS* mutations, present in approximately 40–50% of mCRC cases, are established negative prognostic and predictive biomarkers, particularly in the context of anti-EGFR therapy [6, 7]. Patients with *RAS*-mutant tumors derive no benefit from anti-EGFR monoclonal antibodies like cetuximab and panitumumab and often exhibit more aggressive tumor phenotypes and poorer outcomes.

At the same time, tumor mutational burden (TMB) has emerged as a pivotal biomarker for predicting responses to immunotherapies, particularly immune checkpoint inhibitors (ICIs) [8]. TMB quantifies the total number of somatic mutations per coding area of the tumor genome, reflecting the tumor's genetic instability. High TMB is associated with increased tumor immunogenicity due to the generation of neoantigens, which are novel peptides resulting from tumor-specific mutations that can elicit immune responses [9, 10]. Pembrolizumab, an anti-PD-1 therapy, has been approved for the treatment of solid tumors, including mCRC, with high TMB (> 10 mut/Mb –mutations/megabase–) [11, 12].

The interplay between *RAS* mutations and TMB is less well understood. While *RAS* mutations are well-documented drivers of oncogenic signaling and poor prognosis, their relationship with TMB remains unclear. This study aims to investigate the prognostic significance of *RAS* mutations across low- and high-TMB subgroups, offering a deeper understanding of the molecular heterogeneity of mCRC and its implications for patient management.

Methods

Patients' selection and clinical management

Consecutive patients who underwent genetic sequencing at the AMES center and were managed according to the European Society for Medical Oncology (ESMO) guidelines [13] were included in this study. They were diagnosed between January 2016 and January 2024. All patients had an Eastern Cooperative Oncology Group (ECOG) Performance Status < 2 and a cachexia risk score < 1 [14]. Furthermore, none had peritoneal carcinomatosis or an anticipated survival of less than three months, as assessed by their treating clinicians. Eligible patients were required to be 18 years of age or older with microsatellite stable (MSS) mCC. Furthermore, only individuals with colon cancer were included to ensure homogeneity, excluding cases of rectal cancer due to the established differences in molecular biology, clinical behaviour, and prognosis between these tumor types. In accordance with European Medicines Agency (EMA) guidelines, none of the patients in this cohort received ICIs, nor did they participate in any clinical trials involving ICIs. In fact, current EMA guidelines do not recommend ICIs for MSS colorectal cancer; their use is indicated only in cases of microsatellite instability (MSI) or mismatch repair-deficient (dMMR) mCRC [15]. In selected cases, following multidisciplinary team evaluation, loco-regional ablative procedures were performed with the intent of metastatic disease control. These included surgical metastasectomies, primarily of hepatic or pulmonary lesions, and stereotactic body radiotherapy (SBRT), which was selectively applied to pulmonary metastases using variable dose and fractionation schedules, based on lesion characteristics and anatomical considerations.

Patients were monitored through routine follow-up, including total body computed tomography (tbCT) or magnetic resonance imaging (MRI) scans and tumor response was evaluated using the Response Evaluation Criteria in Solid Tumors (RECIST v1.1) [16]. Disease control (DC) was defined as achieving complete response, partial response, or stable disease, while progressive disease referred to failure in attaining disease control.

The study was conducted in accordance with the ethical principles of the Declaration of Helsinki. The patients signed an informed consent at the time of clinical genetic testing, which authorized the future use of their anonymized data and samples for research purposes. The study received approval from the Institutional Review Board (IRB) of the AMES Center (approval number CA01/2024).

Tumor specimens and sequencing

Formalin-fixed, paraffin-embedded (FFPE) tissue samples of primary colon cancer were selectively microdissected under morphological guidance. DNA extraction was carried out using the MGF03-Genomic DNA FFPE One-Step Kit in accordance with the protocol provided by the manufacturer (MagCoreDiatech). The extracted DNA underwent quality assessment in triplicate using the FFPE QC Kit (Illumina, San Diego, USA), following the manufacturer's recommendations. Library preparation was completed with the TruSight Oncology® 500 kit (TSO500), designed to target 523 genes implicated in cancer (details available in Supplementary File 1). This platform identifies various genomic alterations, including single nucleotide variants (SNVs), insertions and deletions (indels), splice variants, copy number changes, gene fusions, and biomarkers related to immunotherapy, such as TMB and microsatellite status. Sequencing was

performed on an Illumina NovaSeq 6000 instrument (San Diego, USA).

TMB was calculated as outlined by Chalmers et al. [17]. The analysis incorporated all coding somatic substitutions and indels within the targeted regions, including synonymous mutations. To ensure accuracy in identifying coding variants, independent algorithms were applied for variant calling and TMB computation (refer to Manufacturer Instructions at https://emea.support.illumina.com/). The target genomic region spanned 1.9 Mb. For MSI classification, a statistical model based on somatic mutation profiles was employed, accurately distinguishing MSI from MSS tumors [18]. This model was developed using mutation data from 999 exome-sequenced TCGA samples with known MSI status (determined by mononucleotide markers) and achieved a positive predictive value of 98.9% and a negative predictive value of 98.8% in an independent validation set of 427 samples (refer to Manufacturer Instructions at https://emea.support.illumina.com/).

Bioinformatics analysis and data presentation

The bioinformatics pipeline of Illumina TSO500 was used for the analysis of sequencing data. A median of 117 million reads was generated per sample, with coverage in the target region exceeding the manufacturer's suggested threshold of 150X. Sequence data were aligned to the human reference genome GRCh37 using the Burrows–Wheeler Aligner with default parameters [19]. Population- and cancer-specific variants were cross-referenced with several databases, including GENCODE, dbNSFP, ICGC-PCAWG, COSMIC, 1000Genomes, ClinVar, CancerMine, OncoScore, CIViC, and CBMDB, to evaluate clinical significance. Variants were filtered using unmatched normal datasets and excluded if the global minor allele frequency was < 1%. Prioritization of variants followed a four-tiered structure (Tier 1–4), in line with AMP (Association for Molecular Pathology)/ACMG (American College of Medical Genetics and Genomics)/ASCO (America Society of Clinical Oncology)/CAP (College of American Pathologists) joint consensus recommendations [20, 21]. Variants with strong clinical significance in cancer were identified based on evidence levels from databases such as CIViC and Cancer Biomarkers.

The prognostic relevance of clinicopathological factors on overall survival (OS) was evaluated in this study. OS was defined as the time from the diagnosis of metastatic disease to death due to colorectal cancer (cancer-specific survival). Progression-free survival (PFS) was not analyzed because treatment regimens and radiological assessments varied, rendering vital status a more consistent and reliable endpoint. Covariates were dichotomized for both univariate and multivariate analyses: age (≤ 70 vs. >70 years), sex (male vs. female), extent of metastatic spread (single site vs. multiple sites), response to first-line therapy (disease control vs. no disease control), *RAS* mutation status (wild-type vs. mutated), and tumor mutational burden (TMB high, >10 mut/Mb vs. TMB low, < 10 mut/Mb). The classification of TMB categories was guided by the FDA's approval of pembrolizumab for metastatic tumors with TMB >10 mut/Mb, supported by real-world evidence demonstrating the predictive value of this threshold for immunotherapy response [22]. The decision to adopt the 10 mut/Mb cut-off was further solidified by consensus among the authors. Two sensitivity analyses were conducted using alternative thresholds (8 and 12 mut/Mb) of TMB, in combination with *RAS* mutational status. Kaplan–Meier survival analysis was used to estimate OS, with the log-rank test employed for univariate comparisons. Multivariate analysis was conducted using the Cox proportional-hazards model to examine interactions between OS and the defined covariates. Hazard ratios (HRs) were calculated alongside their corresponding 95% confidence intervals (CIs). A Forest plot was generated to display the HRs along with their 95% CIs, providing a clear visual comparison of the relative risks associated with each factor. Comparisons of continuous variables, such as mean age between TMB subgroups, were performed using the t-test. Relationships between categorical clinicopathological factors and classification groups were analyzed through contingency tables and chi-square tests.

Statistical significance was established at a p-value threshold of <0.05. Analyses were carried out using Microsoft Excel and MedCalc® software, version 20.112.

Gene ontology and phenolyzer analyses

To systematically annotate and interpret the functional implications of genes exhibiting differential alterations between the two most prognostically dichotomized subgroups of mCC patients (*RAS*-mutant/TMB-low and *RAS*-wild type/TMB-high) a comprehensive Gene Ontology (GO) enrichment analysis was performed. GO analysis facilitates the functional annotation of genes by categorizing them into three primary ontological domains: molecular function (MF), biological process (BP), and cellular component (CC). This classification provides a structured representation of gene functions and their relationships, enabling the identification of overrepresented biological themes within gene sets. The analysis employed the Gene Ontology (GO) database, a structured and standardized framework that captures biological knowledge by describing gene product attributes across all species. Each term in the ontology is hierarchically connected to others through defined relationships, enabling a comprehensive interpretation of gene functions and their interactions. To account for

Table 1 Clinico-pathological characteristics of analysed patients

Variable	No. (%)	RAS wt	RAS mut	*P*
Age				
Median (range)	63.5 (27–82)	66	63	0.6251
Gender				
Female	38 (35.2)	21	17	
Male	70 (64.8)	40	30	0.8514
Side				
Left	72 (66.7)	42	30	
Right	36 (33.3)	19	17	0.5848
Grading				
G1	7 (6.5)	3	4	
G2/3	101 (93,5)	58	43	0.4542
pT				
1/2	13 (12.03)	7	6	
3	57 (52.7)	34	23	
4	14 (12.9)	6	8	0.5158
Unknown	24 (22.2)	14	10	
pN				
0	30 (27.7)	20	10	
1	33 (30.5)	14	19	
2	21 (19.4)	13	8	0.1256
Unknown	24 (22.2)	14	10	
No. of metastatic sites				
1	28 (25.9)	17	11	
≥ 2	80 (74.07)	44	36	0.6013
Liver involvement				
Yes	78 (72.2)	46	32	
No	30 (27.7)	15	15	0.4017
Type of first-line therapy				
Chemotherapy plus anti-EGFR	58 (53.7)	58	0	
Chemotherapy plus anti-VEGF	47 (43.5)	3	44	
Only chemotherapy	3 (2.8)	0	3	< 0.0001
Surgery of metastases				
No	89 (82.4)	51	38	
Yes	19 (17.6)	10	9	0.7105
Liver	16	8	8	
Lung	3	2	1	
Stereotactic radiotherapy on lung				
No	103 (95.4)	58	45	
Yes	5 (4.6)	3	2	0.8715
Response to first-line therapy				
CR	4 (3.7)	3	1	
PR	50 (46.3)	28	22	
SD	31 (28.7)	18	13	
PD	23 (21.3)	12	11	0.8571
TMB (mut/mb)				
≥ 10	29 (26.8)	19	10	
< 10	79 (73.1)	42	37	0.2534

CR: Complete Response; EGFR: Epidermal Growth Factor Receptor; PD: Progressive disease; PR: Partial response; SD: Stable disease; TMB: Tumor mutational burden; VEGF: Vascular Endothelial Growth Factor

unequal subgroup sample sizes, a bootstrap resampling procedure with 50 iterations was applied. The analyses were performed in R with the clusterProfiler package, version 4.2.2. Multiple testing correction was performed, and only GO terms meeting a significance threshold of adjusted $P < 0.05$ were retained, ensuring the robustness and biological relevance of the enriched pathways. Furthermore, to investigate potential gene hierarchies and interaction networks, differentially altered genes between the two subgroups were further analyzed using Phenolyzer. Phenolyzer is a computational tool that integrates diverse sources of genomic and phenotypic data, including gene–disease associations, protein–protein interactions, shared biological pathways, gene families, and transcriptional regulation. It incorporates information from curated databases such as OMIM, Orphanet, ClinVar, GeneReviews, and the GWAS Catalog [23]. The tool prioritizes genes and variants according to their likely relevance to a specific phenotype or disease and provides both a scoring system and network visualizations depicting gene–gene and gene–disease relationships. Legends for the network visualizations, detailing specific interactional contexts, are provided in a supplementary file (Supplementary File 2). A mutation-aware approach was employed to select candidate genes for Phenolyzer analysis. Genes were ranked based on the number of mutations per gene within each subgroup, and only those with a prevalence ≥ 0.2 were included in the primary analysis. A supplementary table lists a broader set of genes with prevalence ≥ 0.1 (Supplementary File 3). Phenolyzer outputs were used to identify the most closely connected genes within each subgroup, highlighting potential functional hubs that may contribute to clinical behavior.

Results

Clinical and pathological characteristics of the cohort

The study analyzed a cohort of 108 patients with mCC. Clinical and pathological characteristics of these patients are detailed in Table 1, stratified by *RAS* mutation status (wild-type vs. mutant). Median age was consistent across both groups, with a median of 63.5 years (range 27–82). The cohort comprised 64.8% males and 35.2% females. Most tumors were left-sided (66.7%), with a smaller portion located on the right side (33.3%). Tumor grade was predominantly G2/3 (93.5%), with only 6.5% classified as G1.The majority of patients had pT3 tumors (52.7%), while pT1/2 and pT4 accounted for 12.03% and 12.9% of cases, respectively. Regarding nodal status, 27.7% were pN0, 30.5% pN1, and 19.4% pN2, with 22.2% of cases having unknown nodal status. Most patients (74.07%) had more than two metastatic sites, with liver involvement in 72.2% of cases.

The majority of patients received combination therapy with chemotherapy plus anti-EGFR agents (53.7%), all of

whom belonged to the *RAS* wild-type group. In contrast, 43.5% received chemotherapy plus anti-VEGF, predominantly in the *RAS*-mutant group, while only a small fraction of patients (2.8%) received chemotherapy alone. The distribution of first-line treatment significantly differed between groups ($P < 0.0001$). Surgical resection of metastases was performed in 17.6% of patients, with a similar frequency between the two groups (wild-type: $n = 10$; mutant: $n = 9$; $P = 0.7105$). The majority of surgical interventions involved the liver ($n = 16$), followed by the lung ($n = 3$). Stereotactic body radiotherapy (SBRT) to the lung was administered in 4.6% of patients, with comparable distribution across the groups.

In terms of response to first-line therapy, 3.7% of patients achieved a complete response (CR), 46.3% a partial response (PR), 28.7% had stable disease (SD), and 21.3% showed progressive disease (PD). Additionally, TMB was below 10 mut/Mb in 73.1% of patients. No statistically significant associations were observed between *RAS* mutation status and any of the clinical or pathological variables described.

Overview of *RAS* mutations

Table 2 provides a detailed overview of the *RAS* mutation landscape in this cohort, highlighting the distribution frequencies of both common and less frequent variants within *KRAS* and *NRAS*. Among the *KRAS* variants, the most prevalent mutation was *KRAS* p.G12D, occurring in 23.4% of cases, followed by p.G13D (17.0%) and p.G12V (14.9%). These mutations represent the most common alterations observed, consistent with established patterns in mCRC. Less frequent *KRAS* mutations included p.G12C (6.4%), and p.Q61H, p.G12A, and p.A146T, each occurring in 4.2% of cases. Rare mutations within the *KRAS* gene, such as p.Q61L, p.K117N, and p.G13C, were each identified in only 2.1% of cases. In *NRAS*, two mutations (p.Q61K and p.G12D) were each observed in 4.2% of cases. Other rare variants, such as p.Q61L, p.G13D, and p.Q61R, were each present in 2.1% of the cohort, indicating a lower frequency of *NRAS* mutations relative to *KRAS*. Additionally, two instances of co-mutations involving both *KRAS* and *NRAS* genes were identified. These rare co-occurrences (*KRAS* p.Q61H/*NRAS* p.Q61K and *KRAS* p.G13D/*NRAS* p.Q61L) each represented 2.1% of cases and highlight the potential complexity of the mutational landscape in a subset of patients.

Table 2 Distribution frequency of *RAS* variants

Gene variant	No	%
KRAS		
p.G12D	11	23.4
p.G13D	8	17.0
p.G12V	7	14.9
p.G12C	3	6.4
p.Q61H	2	4.2
p.G12A	2	4.2
p.A146T	2	4.2
p.Q61L	1	2.1
p.K117N	1	2.1
p.G13C	1	2.1
NRAS		
p.Q61K	2	4.2
p.G12D	2	4.2
p.Q61L	1	2.1
p.G13D	1	2.1
p.Q61R	1	2.1
KRAS/NRAS co-mutations		
p.Q61H/p.Q61K	1	2.1
p.G13D/p.Q61L	1	2.1

Prognostic role of *RAS* mutations and subgroup analysis

After a median follow-up of 50.7 months, 53 events (deaths) occurred among the 108 patients. As shown in Fig. 1, the presence of a *RAS* mutation significantly impacts patient prognosis (median OS for *RAS* wild-type vs. *RAS* mutant: 67.9 months vs. 46.4 months; HR: 1.76; 95% CI: 1.00–3.11; Log-Rank test $P = 0.0495$). Figure 2 illustrates the HRs with corresponding 95% CIs for selected variables, stratified by the presence or absence of *RAS* mutations. Response to first-line therapy was excluded from this analysis, as therapeutic choice is closely determined by *RAS* mutation status. Point estimates for each variable are detailed in Supplementary File 4. Among all subgroups, the only variables for which *RAS* mutations did not universally adversely impact prognosis were tumor side and TMB.

Prognostic role of *RAS* mutations in relation to TMB subgroups

Although the prognostic impact of *RAS* mutations according to tumor location (e.g., a stronger effect in left-sided colon cancers) is well established, their relationship with TMB introduces a novel point of investigation. The scientific literature strongly supports the prognostic role of *RAS* mutations, but the lack of prognostic impact in high-TMB tumors is particularly striking, as highlighted in Fig. 3. These molecular insights are now routinely collected for most patients to guide therapeutic decisions. Interestingly, in multivariate analysis, both the number of metastatic sites (1 vs. >1) and combined TMB/*RAS* status emerged as independent prognostic variables (Table 3). Specifically, patients with low TMB and *RAS* mutations showed a markedly reduced median survival (28.0 months) compared to other combinations (87.0 months), with this combination significantly associated with poorer survival in both univariate ($P = 0.0071$) and multivariate analyses (HR = 2.34; 95% CI, 1.27–4.28; $P = 0.0058$). Therefore, patients with low TMB and *RAS*

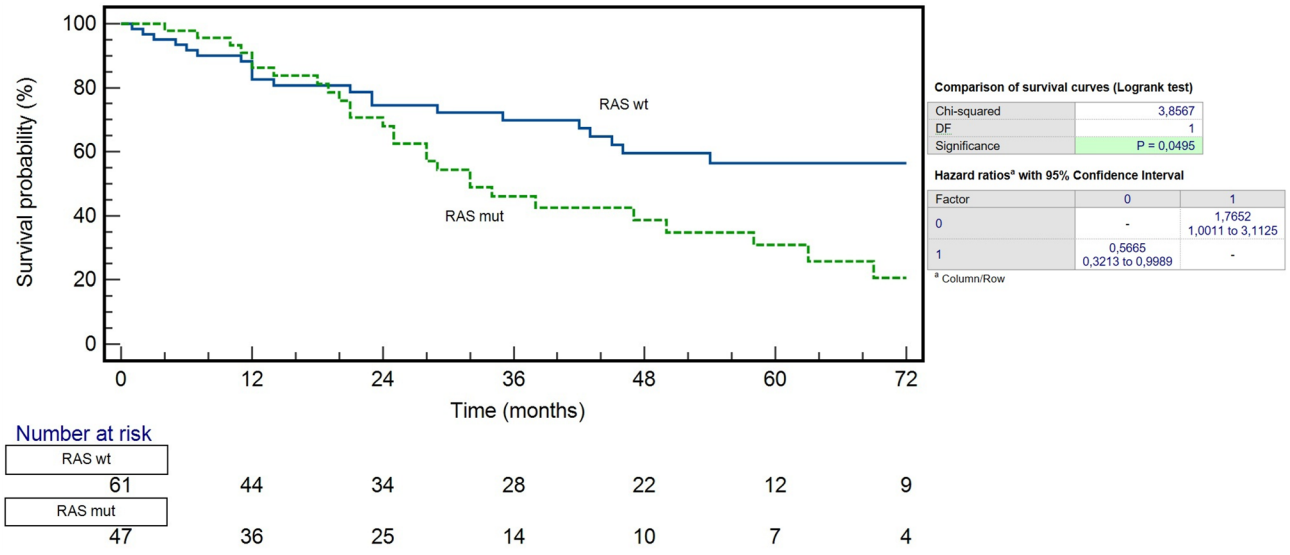


Fig. 1 Kaplan–Meier curves illustrating the prognostic impact of *RAS* mutation status is presented. The output of the log-rank test, including the corresponding HRs (Hazard Ratios) and 95% CIs (Confidence Intervals), is provided in the tables adjacent to the survival curves

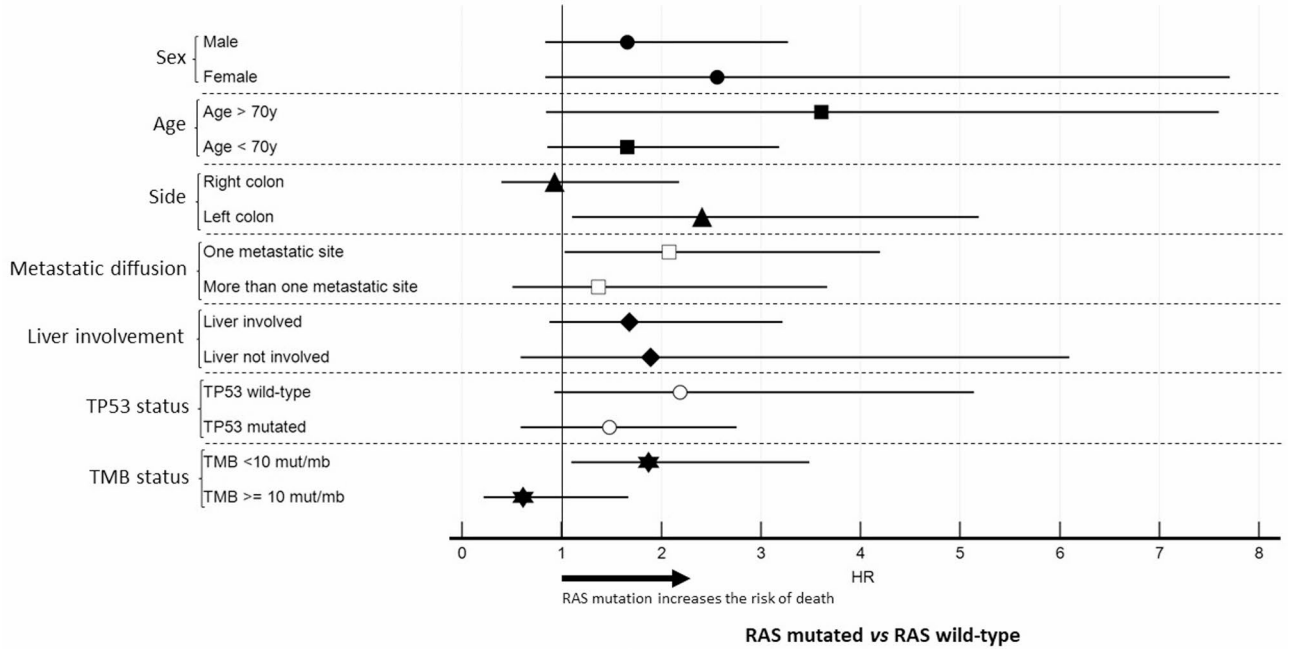


Fig. 2 A Forest plot displaying HRs (Hazard Ratios) and 95% CIs (Confidence Intervals) was generated to illustrate the effect of *RAS* mutation status on survival for the subgroups listed on the left side of the graph. An HR greater than 1 indicates that the presence of *RAS* mutations is associated with an increased risk of mortality, whereas an HR less than 1 suggests a reduced risk

mutations constitute a distinct subgroup with a significantly worse prognosis compared to all other TMB/*RAS* combinations (i.e., low TMB with *RAS* wild-type, high TMB with *RAS* mutations, or high TMB with *RAS* wild-type). In contrast, patients with tumors characterized by high TMB and *RAS* mutations, low TMB and *RAS* wild-type, or high TMB and *RAS* wild-type exhibited broadly similar survival patterns, although in longer follow-up the subgroup with the most favorable outcome (highest proportion of surviving patients, > 60%) was represented by those with high TMB and *RAS* wild-type tumors (Supplementary File 5). The distribution of treatments according to different combinations of *RAS* mutation and TMB status is shown in Supplementary File 6. The prognostic impact of different TMB/*RAS* interactions, using alternative TMB thresholds (8 and 12 mut/Mb), is represented in Supplementary File 7.

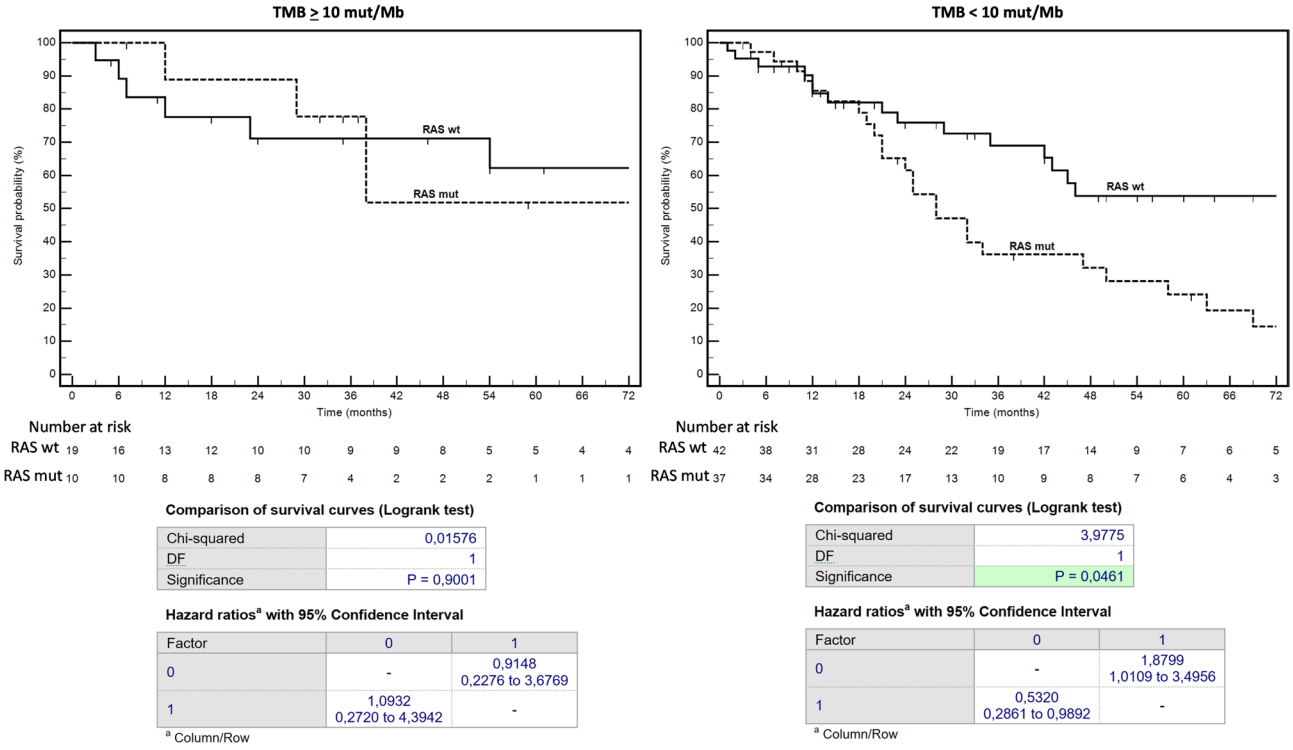


Fig. 3 Kaplan–Meier curves illustrating the prognostic impact of *RAS* mutation status on survival are presented for patients with TMB > 10 mut/Mb (on the left side of the figure) and TMB < 10 mut/Mb (on the right side of the figure). The output of the log-rank test is provided below each Kaplan–Meier curve

Table 3 Uni- and multi-variate analyses of the prognostic interactions including clinic-pathological variables and *RAS*/TMB combined status

Co-variate	Dicothomization	Median survivals (months)	No. of events/patients	*P* at univariate	HR	95% CI	*P* at multivariate
Age	≥ 70y vs. < 70y	54.0 vs. 47.0	16/31 vs. 37/77	0.8201	1.62	0.85–3.08	0.1387
Gender	M vs. F	42.0 vs. 50.0	36/70 vs. 17/38	0.6422	1.02	0.56–1.85	0.9474
No. of metastatic sites	1 site vs. > 1 site	80.0 vs. 32.0	18/28 vs. 35/80	0.0057	0.48	0.26–0.88	0.0189
Response to first-line CT	DC vs. no DC	63.0 vs. 29.0	37/85 vs. 16/23	0.0211	1.40	0.70–2.80	0.3385
Liver involvement	Yes vs. No	45.0 vs. 80.0	41/76 vs. 12/32	0.1897	0.69	0.38–1.23	0.2160
TMB/*RAS* mutational status	TMB low/*RAS* mut vs. other combinations	28.0 vs. 87.0	24/37 vs. 29/71	0.0071	2.34	1.27–4.28	0.0058

CI: Confidence Interval; CT: chemotherapy; DC: Disease Control; F: Female; HR: Hazard Ratio; M: Male; y: years; TMB: Tumor mutational burden

Integrative gene ontology and phenolyzer analyses in prognostically divergent subgroups

To investigate the biological mechanisms underlying the most divergent clinical outcomes, a Gene Ontology (GO) enrichment analysis was performed, focusing on the two most prognostically dichotomized subgroups: *RAS*-mutant/TMB-low patients *versus* *RAS*-wildtype/TMB-high patients. Among the three dimensions explored [Molecular Function (MF), Biological Process (BP), and Cellular Component (CC)] only MF showed significant differences between groups (Fig. 4). Specifically, the RAS-mutant/TMB-low subgroup exhibited a significant enrichment in signaling receptor activity (enrichment score > 7.5), as well as in molecular transducer activity and transmembrane signaling receptor activity. To explore potential hierarchies and functional connections among genes and to identify whether distinct genetic interaction patterns exist between these two subgroups, the differentially altered genes were further analyzed using Phenolyzer. Phenolyzer is a computational tool that integrates known gene-disease associations, functional annotations, and interaction networks to prioritize genes based on their relevance to a phenotype or disease. Genes were ranked according to their frequency of alteration (see Methods). As shown in Fig. 5, Phenolyzer network analysis (panel A) and the corresponding

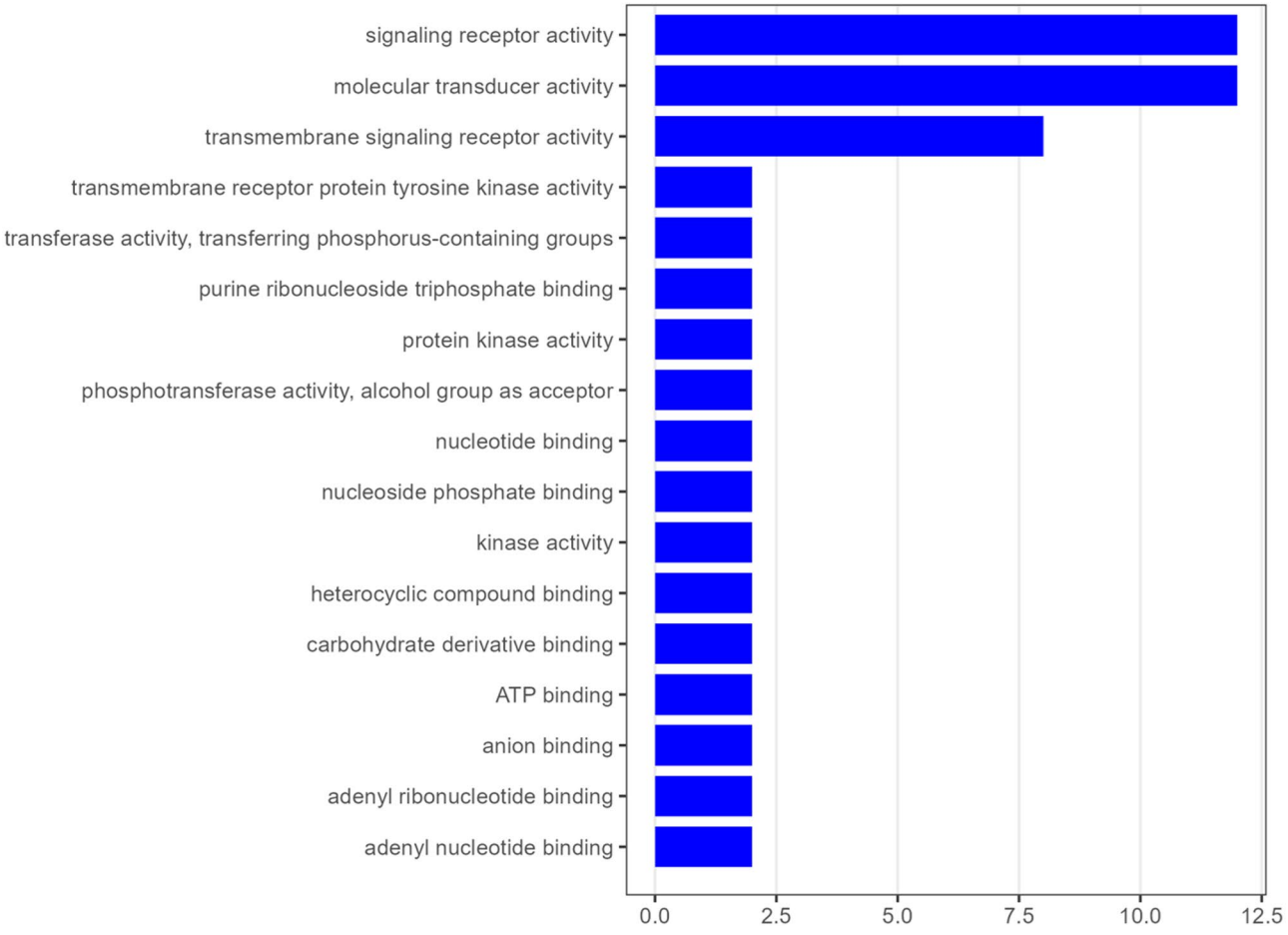


Fig. 4 The figure displays statistically significant enriched pathways on the Y-axis, with the enrichment score on the X-axis, reflecting the strength of overrepresentation in *RAS*-mutant/TMB-low versus *RAS*-wild-type/TMB-high tumors

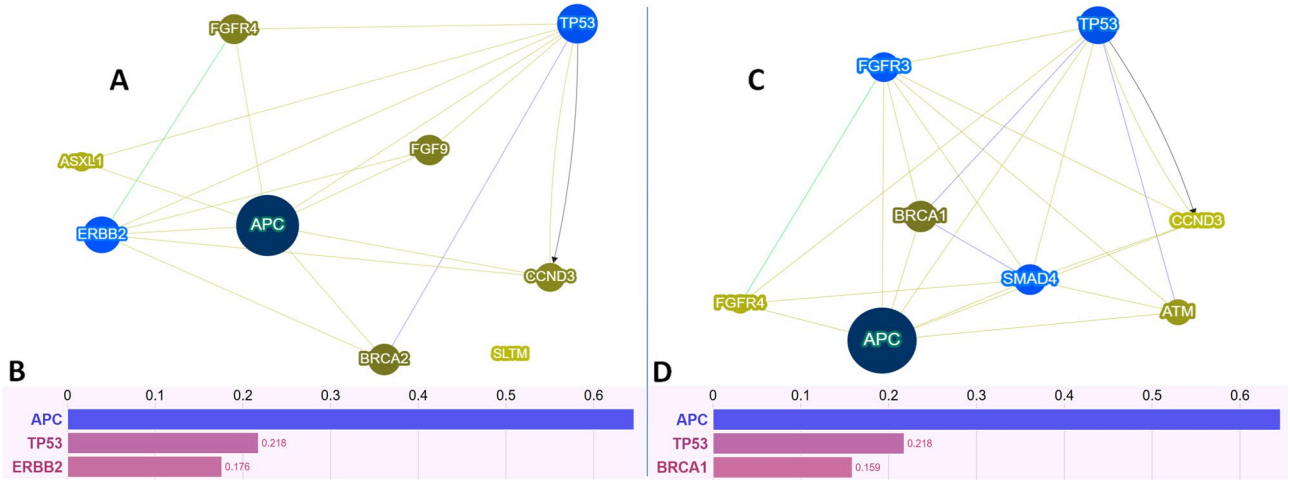


Fig. 5 The upper panels depict the interactional contexts of the selected genes, while the lower panels show bar plots of Phenolyzer-based gene prioritization. *RAS* mutant/TMB-low tumors are shown in Panels **A** and **B**, and *RAS* wild-type/TMB-high tumors in Panels **C** and **D**. Bar height corresponds to the Phenolyzer score, reflecting both the predicted relevance of each gene to the disease and its network interactions (see Methods for details)

Phenolyzer score ranking (panel B) highlight that, in the *RAS*-mutant/TMB-low group, the three most closely interrelated genes are *APC*, *TP53*, and *ERBB2*, whereas in the *RAS*-wild type/TMB-high group (panels C and D), they are *APC*, *TP53*, and *BRCA1*.

Discussion

In our study performed in 108 mCC patients and analyzed with the same NGS test, we found that *RAS* mutations, emerged as a significant prognostic factors. This effect was confined to patients with low TMB. Furthermore, the combination of low TMB and *RAS* mutations was associated with markedly reduced survival, highlighting a subgroup with significantly worse outcome if compared to other TMB/*RAS* combinations. In our study, TMB was quantified using the TSO500 assay, a comprehensive genomic profiling panel that demonstrates robust performance in clinical samples. The TSO500 panel offers superior accuracy in defining TMB cut-offs [24, 25], thereby enhancing the reliability of our results.

RAS mutations are established as key diagnostic and prognostic biomarkers in mCRC, influencing therapeutic decisions and predicting clinical outcomes. In fact, mutations in *RAS* identify patients unlikely to benefit from anti-EGFR therapies, thereby guiding treatment stratification [26]. Beyond treatment implications, *RAS* mutations have prognostic significance, correlating with reduced OS [4].

Prognostic prediction in mCRC is inherently multifaceted and cannot rely solely on individual biomarkers. Incorporating molecular perspectives, such as TMB and *RAS* mutation status, may enable a more nuanced stratification of patient subgroups. TMB, which measures the total number of mutations per megabase of DNA, is increasingly recognized as an independent prognostic and predictive biomarker across multiple cancers, including mCRC [27]. Together with MSI, a high TMB is often associated with an increased neoantigen load, which facilitates immune recognition and enhances responsiveness to ICIs [28]. However, in mCRC, the distribution of TMB indicates that up to 20% of patients exhibit high TMB (≥ 10 mut/Mb), a threshold commonly employed in clinical studies to assess immunogenic potential [29]. This proportion is considerably higher than the < 5% of cases characterized by MSI, underscoring how this threshold captures a broader subset of patients and contextualizing its relevance within the overall TMB landscape of CRC. In contrast, the majority of mCRC cases are microsatellite stable (MSS) and typically exhibit low TMB, characterized by a reduced neoantigen load and diminished immunological visibility of the tumor. Therefore, integrating TMB assessment with *RAS* mutation status offers a more nuanced stratification, identifying high-risk subgroups such as TMB-low/*RAS*-mutated tumors, which may benefit from targeted therapies rather than immunotherapy.

In fact, our findings emphasize the prognostic relevance of integrating TMB with *RAS* mutation status in mCC highlighting that TMB may attenuate or mitigate the negative prognostic impact commonly associated with the presence of *RAS* mutations. This hypothesis is supported by recent research showing a better prognosis in CRC patients with *KRAS* mutations when TMB levels are elevated [30]. Analysis of data from the Memorial Sloan Kettering Cancer Center (MSKCC) and The Cancer Genome Atlas (TCGA) cohorts revealed that, while TMB alone did not significantly influence OS across all CRC patients, high TMB was linked to improved OS in those harbouring *RAS* mutations. These observations highlight TMB potential as an independent prognostic factor and its promise in enhancing clinical prognostic models [30]. However, the reliance on public datasets introduces inherent limitations, including heterogeneity in populations, different cancer sites (colon and rectum), differing disease stages, and variable treatment histories, all of which may confound results. In contrast, our analysis validates, for the first time, these findings in a more uniform patient cohort derived from real-world clinical practice.

Although we have already provided a rationale for the use of the 10 mut/Mb cut-off, it is important to acknowledge that there is still no universally accepted standard for TMB thresholds. In fact, the threshold of 10 mut/Mb, initially introduced following FDA guidance for the use of pembrolizumab in MSI tumors [22], has been widely adopted across various tumor types, including MSS mCRC, as a practical value for defining TMB-high status in studies exploring immune responsiveness and prognostic stratification. In addition, analysis of data from 117 clinical trials encompassing 12,450 patients receiving immunotherapy revealed that a TMB ≥ 10 mut/Mb correlates with significantly higher objective response rates to ICIs across multiple tumor types, including MSS mCRC [31]. However, we fully acknowledge that this cut-off is somewhat arbitrary, and we concur that future prospective studies are needed to refine and validate optimal TMB thresholds, particularly in light of emerging immunogenomic insights that may better evaluate the complexity of tumor–immune interactions in these patients [32]. To address this, we performed an exploratory and sensitivity analysis using alternative TMB cut-offs of 8 and 12 mut/Mb. Although these thresholds have been variably proposed in prior studies, our results indicate that neither the 8 nor the 12 mut/Mb cut-off achieved a prognostic stratification as consistent or clinically meaningful as the originally applied 10 mut/Mb threshold. A possible explanation is that the 8 mut/Mb threshold may be too-much permissive, including a broader and more

heterogeneous group of tumors as TMB-high, considering also those with limited immunogenic potential or inherently indolent biology. In contrast, the 12 mut/Mb threshold may be too-much restrictive, potentially excluding tumors that, despite falling below this higher cut-off, still harbor significant neo-antigen production and release and aggressive behavior. Within our cohort, the 10 mut/Mb cut-off provided an optimal balance between sensitivity and specificity for identifying the prognostically adverse subgroup characterized by low TMB and *RAS* mutation. These findings support the use of TMB as a continuous biomarker with context-dependent interpretability, particularly when integrated with key oncogenic alterations such as *RAS* mutations.

From a biological and therapeutic perspective, the TMB-low/*RAS*-mutated subgroup likely represents a particularly unfavorable molecular profile. Multiple lines of evidence support this view. *RAS* mutations are well-established drivers of resistance to anti-EGFR therapies such as cetuximab and panitumumab, and are consistently associated with poorer clinical outcomes [4]. At the same time, low TMB is typically associated with a limited neoantigen repertoire, which, in turn, leads to reduced immunogenicity and poor infiltration by cytotoxic T lymphocytes [33]. In this immunologically "cold" context, tumors may evade immune recognition more effectively. Furthermore, *RAS*-mutant CRC often exhibits a profoundly immunosuppressive tumor microenvironment, characterized by decreased densities of $CD8^+$ T cells and antigen-presenting cells, along with increased infiltration of neutrophils and other myeloid subpopulations [34]. On these bases, RAS-driven signaling through the MAPK and PI3K pathways has been shown to downregulate the expression of MHC molecules and immune checkpoint ligands, further diminishing tumor visibility to the host immune system [35]. Taken together, these data suggest that TMB-low/*RAS*-mutated tumors not only resist targeted therapies but also fail to trigger effective immune responses, creating a biologically aggressive and clinically high-risk phenotype. In contrast, tumors with either wild-type *RAS* or high TMB, alone or in combination, may retain greater therapeutic vulnerability and exhibit a more immunologically active microenvironment, which could translate into improved patient outcomes.

From a practical point of view, patients within the TMB-low/*RAS*-mut subgroup, likely to be immunologically "cold", may derive greater benefit from oncogene-targeted strategies. Recent pharmacological advances have enabled direct inhibition of *RAS* oncogenes previously considered undruggable [36]. Sotorasib, an irreversible *KRAS* p.G12C inhibitor, has demonstrated clinical efficacy in refractory mCRC [37], with enhanced activity observed when combined with EGFR blockade [38]. Similarly, adagrasib in combination with cetuximab inducedresponse rates of up to 34% and a median PFS of 6.9 months in *KRAS* p.G12C-refractory mCRC [39]. These findings suggest that TMB-low/*RAS*-mut tumors, though resistant to immunotherapy, may be amenable to targeted *RAS*-directed treatments. Moreover, emerging compounds such as MRTX1133, which targets *KRAS* p.G12D (currently in preclinical development), underscore the expanding therapeutic landscape [40]. Collectively, these developments highlight a compelling rationale for shifting from broad immunogenic strategies toward precision-targeted interventions in TMB-low subgroups.

This interpretation is supported by the results of GO and Phenolyzer analyses. GO enrichment analysis indicates that tumors from *RAS*-mutant/TMB-low patients exhibit a functional landscape dominated by cell surface receptors and signal transduction pathways. As with most GO analyses, these results should be interpreted as hypothesis-generating. Nevertheless, they suggest that *RAS*-mutant/TMB-low tumors rely predominantly on intracellular signaling for the maintenance of proliferation and survival, thereby sustaining constitutive activation of *RAS*-mediated pathways. In this context, tumor growth appears to be driven mainly by intracellular signaling. Additionally, in TMB-low tumors, relatively conservative DNA repair mechanisms may limit neoantigen formation, potentially reducing immunological recognition by the host immune system. These features likely contribute to the poorer prognosis observed in this patient subgroup and indicate potential benefits from targeted inhibitors or innovative combination strategies. In contrast, the *RAS* wild-type/TMB-high subgroup, which does not show receptor-mediated pathway enrichment, may benefit from enhanced immune recognition and more effective antitumor immune surveillance, consistent with its more favorable prognosis. Furthermore, Phenolyzer analysis in the *RAS*-mutant/TMB-high group identified, in addition to canonical cancer-driving genes *APC* and *TP53*, somatic alterations in *BRCA1*. This suggests that even within a microsatellite-stable cohort, these patients harbor genetic changes that could facilitate DNA damage and subsequent immune recognition, potentially contributing to improved survival outcomes despite not receiving immune checkpoint inhibitors. Collectively, these findings raise the possibility that *RAS* wild-type/TMB-high tumors may engage alternative, yet uncharacterized, immunologic mechanisms in the immune evasion and antitumor activation balance, underscoring the need for future studies to investigate their immunogenomic landscape and clarify the biological basis of these prognostic differences.

Finally, the poorer prognosis observed in patients with *RAS*-mutant/low-TMB tumors cannot be attributed to a

higher incidence of *BRAF* mutations. In fact, among the eight patients in this cohort harboring a *BRAF* p.V600E mutation, only one belonged to the RAS-mutant/low-TMB subgroup (Suppelmentary File 8).

Although ICIs are not currently standard of care in MSS mCRC, integrating TMB with *RAS* mutation status may provide several translational opportunities. Our data indicate that patients harboring *RAS* mutations with low TMB constitute a high-risk subgroup with markedly reduced OS. This stratification could inform clinical decision-making, enabling more precise risk assessment and tailored monitoring strategies. Furthermore, in the context of clinical trial design, identifying TMB-low/*RAS*-mutated patients may offer a rationale for cohort enrichment in studies evaluating novel targeted therapies. For example, early-phase trials could prioritize this high-risk subgroup to assess the efficacy of RAS-directed inhibitors, potentially in combination with agents modulating the tumor microenvironment to overcome immunological resistance. Conversely, patients with high TMB and wild-type *RAS* may represent a subgroup more likely to benefit from immune-based interventions despite MSS status, supporting rational patient stratification and adaptive trial designs.

By focusing on a well-defined patient population, our study provides clinically relevant insights into the interplay between TMB and *RAS* mutations. However, several limitations should be acknowledged. In particular, the retrospective design introduces potential biases, including those related to data collection, patient selection, and unmeasured confounding factors. Furthermore, the relatively small sample size (total $n = 108$, including $n = 10$ in the TMB-low/*RAS*-mutated subgroup) underscores the need for larger, prospective, multi-institutional cohorts to confirm and extend our findings, particularly within increased molecular subgroups. In this context, the identification of patients with *HER2* amplification, which was observed in only four cases in our dataset, deserves particular attention, as none of these patients received HER2-targeted therapy due to the absence of EMA-approved anti-HER2 regimens for mCRC at the time of data collection, limiting access to such treatments outside of clinical trials.

A notable feature of this cohort deserving to be discussed is its higher median OS of both *RAS* mutated and wild-type patients, which significantly exceeds the approximately 30 months commonly reported for mCC. This outcome reflects the stringent patient selection criteria (see Methods), including excellent performance status, absence of peritoneal metastases, and a cachexia risk below 1. These criteria ensure the inclusion of patients with uniformly favorable clinical profiles. This methodological approach aims to isolate the prognostic influence of biological factors, minimizing the confounding effects of variables related to overall clinical condition.

Furthermore, we acknowledge that progression-free survival data were not available in our cohort, which represents a limitation of the study. Although OS may be influenced by subsequent lines of therapy and post-progression treatments, the prognostic effect observed in the TMB-low/RAS-mutated subgroup likely reflects a biologically high-risk profile rather than treatment-related confounding alone. The stringent selection criteria, including excellent performance status and absence of peritoneal metastases, likely further reduce the impact of post-progression variability.

Conclusions

The identification of distinct subgroups characterized by low TMB and *RAS* mutations highlights the importance of comprehensive molecular profiling, paving the way for personalized monitoring and tailored therapeutic strategies in specific molecular subsets. Future studies should aim to clarify the mechanistic interplay between *RAS* mutations and TMB, particularly in relation to tumor immunogenicity and resistance to ICIs.

Abbreviations

CRC	Colorectal cancer
mCRC	Metastatic colorectal cancer
mCC	Metastatic colon cancer
EGFR	Epidermal growth factor receptor
VEGF	Vascular endothelial growth factor
RAS	Rat sarcoma virus oncogene family
MAPK	Mitogen-activated protein kinase
PI3K	Phosphatidylinositol-3-kinase
AKT	Protein kinase B
TMB	Tumor mutational burden
ICIs	Immune checkpoint inhibitors
PD-1	Programmed cell death protein 1
mut/Mb	Mutations per megabase
AMES	Advanced Molecular and Experimental Sciences (center)
ESMO	European Society for Medical Oncology
ECOG	Eastern Cooperative Oncology Group
MSS	Microsatellite stable
EMA	European Medicines Agency
MSI	Microsatellite instability
dMMR	Mismatch repair-deficient
SBRT	Stereotactic body radiotherapy
tbCT	Total body computed tomography
MRI	Magnetic resonance imaging
RECIST	Response Evaluation Criteria in Solid Tumors
DC	Disease control
IRB	Institutional Review Board
FFPE	Formalin-fixed, paraffin-embedded
QC	Quality control
TSO500	TruSight Oncology 500
SNVs	Single nucleotide variants
indels	Insertions and deletions
Mb	Megabase
TCGA	The Cancer Genome Atlas
GO	Gene Ontology
MF	Molecular function
BP	Biological process
CC	Cellular component
OMIM	Online Mendelian Inheritance in Man
GWAS	Genome-Wide Association Studies

OS	Overall survival
PFS	Progression-free survival
HRs	Hazard ratios
CIs	Confidence intervals
AMP	Association for Molecular Pathology
ACMG	American College of Medical Genetics and Genomics
ASCO	American Society of Clinical Oncology
CAP	College of American Pathologists
CR	Complete response
PR	Partial response
SD	Stable disease
PD	Progressive disease
MSKCC	Memorial Sloan Kettering Cancer Center
MHC	Major histocompatibility complex
APC	Adenomatous polyposis coli
TP53	Tumor protein p53
BRAF	v-Raf murine sarcoma viral oncogene homolog B
ERBB2	Erb-B2 receptor tyrosine kinase 2
BRCA1	Breast cancer type 1 susceptibility protein
HER2	Human epidermal growth factor receptor 2

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12967-025-07273-w.

Supplementary Material 1: Genes analyzed using the TruSight Oncology® 500 panel

Supplementary Material 2: Legends for Phenolyzer network visualizations, detailing specific interactional contexts

Supplementary Material 3: Gene ranking by mutation prevalence across subgroups.

Supplementary Material 4: Hazard ratios and 95% confidence intervals for prognostic variables, stratified by RAS status

Supplementary Material 5: Kaplan–Meier survival curves illustrating prognostic trajectories according to RAS status (WT: wild-type; MUT: mutated) and tumor mutational burden (TMB) levels (TMB-high: >10 mut/Mb; TMB-low: <10 mut/Mb). Event counts are reported below each graph. Analyses are descriptive; log-rank test results, along with hazard ratios (HRs) and 95% confidence intervals (CIs), are displayed within the plot area

Supplementary Material 6: Distribution of treatment regimens according to RAS mutation and TMB status in metastatic colorectal cancer patients

Supplementary Material 7: Kaplan–Meier survival curves showing prognostic trajectories stratified by RAS status (WT: wild-type; MUT: mutated) and TMB levels. The upper panel uses a TMB threshold of 8 mut/Mb (TMB-high: >8; TMB-low: ≤8), and the lower panel uses a threshold of 12 mut/Mb (TMB-high: >12; TMB-low: ≤12). Event counts are reported below each graph. Analyses are exploratory and descriptive; log-rank test results, hazard ratios (HRs), and 95% confidence intervals (CIs) are displayed within each plot

Supplementary Material 8: Distribution of molecular subgroups by ***BRAF*** p.V600E mutation status

Supplementary Material 9: Accession numbers for raw sequencing data of the clinical cohort submitted to ENA

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Data availability
Raw sequencing data have been deposited in the European Nucleotide Archive (ENA) under the accession numbers provided in Supplementary File 9.

Declarations

Ethics approval and consent to participate
The study was approved by the Institutional Review Board of Centro AMES under protocol no. CA01/2024. It was conducted in accordance with the principles of the Declaration of Helsinki and its subsequent amendments.

Consent for publication
Not applicable.

Competing interests
Monica Ianniello, Raffaella Ruggiero, Roberto Sirica, Nadia Petrillo, Antonio Fico, Luisa Circelli, Antonio Barone, and Giovanni Savarese are employed by AMES, Centro Polidiagnostico Strumentale srl, 80013 Naples, Italy. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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