# RNA-Based Diagnostic Panel Matches TME Phenotype to Therapeutic Mechanism of Action in Colorectal Cancer


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## 1 Background

Colorectal Cancer (CRC) is the third most common type of cancer, and is deadly in its advanced stages. While curative surgery is appropriate for early-stage disease, up to 30% of those patients experience recurrence within 2-5 years[1,2]. Certain targeted therapies are available for late-stage CRC, such as anti-EGFR depending on mutation status, anti-angiogenics, or checkpoint inhibitors in cases where patients are shown to be MSI-H/dMMR (**Figure 1**). Unfortunately, few diagnostic tools exist to match an individual with recurrent metastatic disease to the optimal therapy regime, and for the majority of patients with metastatic disease, the clinician must choose therapies without the benefit of precision tools that would predict the best treatment.

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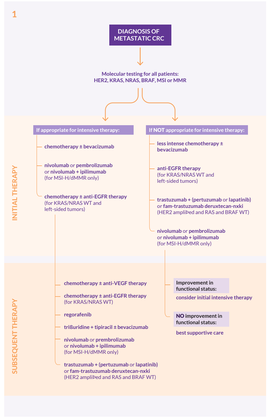


▲ *Figure 1. Flow Chart of the Treatment Algorithm For Colorectal Cancer Based on NCCN Guidelines*

*A simplified representation of the National Comprehensive Cancer Network (NCCN) Guidelines for Colon Cancer and Rectal Cancer, including systemic and targeted therapies.*

*Abbreviations:*

- *CRC, colorectal cancer*
- *dMMR, mismatch repair deficient*
- *EGFR, endothelial growth factor receptor*
- *MMR, mismatch repair*
- *MSI, microsatellite instability*
- *MSI-H, microsatellite instability-high*
- *VEGF, vascular endothelial growth factor*
- *WT, wild type*

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In 2015, a collaboration across six research groups produced the Consensus Molecular Subtypes (CMS) model for typing CRC patients[3]. The CMS model represents the synthesis of six different classification schemes, and based on gene expression data returns four distinct subtypes: CMS groups 1 - 4 (with a fraction of patients indeterminant, herein "IND"). These four groups have been further annotated based on analysis of additional molecular features: briefly, CMS1 is immunogenic and includes MSI-H; CMS2 is WNT & MYC active; CMS3 includes KRAS mutations and metabolic dysregulation; and CMS4 is stromal or angiogenic in nature. CMS subgroups by-and-large accord with known pathological features of the disease[4], and are prognostic for overall survival (OS) and progression free survival (PFS)[3]. Nevertheless, CMS has not proved to be predictive for targeted therapies such as bevacizumab, and has yielded some confounding results between different trials (e.g. CALBG/SWOG 80405 vs FIRE-3)[5,6]. Thus patients and clinicians are still in need of predictive diagnostic tools to guide precision treatment of CRC.

OncXerna Therapeutics, in collaboration with Genialis, has developed TME Panel-1, a novel diagnostic panel that uses RNA-based gene expression data to classify patients based on the dominant biologies of the tumor microenvironment (**Figure 2**)[7]. The input gene signature represents angiogenic and immunogenic properties of stromal biology, and the neural network that comprises the TME Panel-1 algorithm has learned interactions between these critical processes. The algorithm classifies patients into one of four TME phenotypes—Angiogenic (A), Immune Suppressed (IS), Immune Active (IA) and Immune Desert (ID). We are exploring the predictive value for each class or combination of classes, to determine which patients are more likely to respond to various targeted therapies, including anti-angiogenics and checkpoint inhibitors, as well as other TME-based targets[7].

TME Panel-1 was originally trained and validated on gastric cancer, but with care taken in the design of the algorithm workflow to enable diagnostic application in other solid tumors. Analysis of over 2,000 biobank patient samples suggested the model may be applicable in ovarian and colorectal cancers as well. Retrospective analysis of the phase Ib trial of navicixizumab, a bispecific monoclonal antibody against VEGF and DLL4, supports this hypothesis in ovarian cancer[7]. In this study, we begin to address whether TME Panel-1 might be applicable in colorectal cancer, explore how it might complement the use of the CMS system, and propose an explanation for how TME Panel-1 may succeed, where others have not, as a predictive diagnostic.

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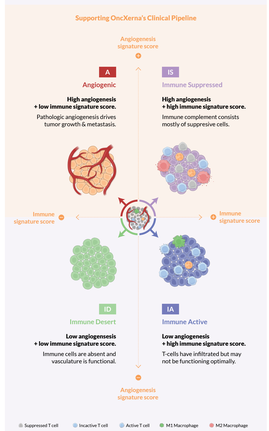



▲ *Figure 2. TME Panel-1 Predicts Four Phenotype Classes based on Interactions between Angiogenesis and Immune Biologies*

*TME Panel-1 employs a machine learning model that has learned gene signatures representing the angiogenic and immune biologies that dominate the stroma. The combinations of these biologies result in four different tumor microenvironment (TME) phenotypes: Angiogenic (A), Immune Suppressed (IS), Immune Active (IA) and Immune Desert (ID). The model classifies patients into one of these four phenotype classes based on gene expression from patient tumor samples. Previous work reported at SITC 2019 showed these phenotypes are independent of disease stage or demographics, and that the TME phenotypes confer distinct prognostic risk. More recent work presented at SITC 2020 showed TME Panel-1 is predictive of outcome for anti-angiogenic and checkpoint inhibitor therapies, including approved and investigational drugs in gastric cancer.*

## 2 Methods

### Generation of Wood Hudson Dataset

Transcriptome data were generated from formalin fixed paraffin embedded (FFPE) surgical specimens of human colorectal carcinomas (CRC) that were donated by St. Elizabeth Healthcare to the Wood Hudson Cancer Research Laboratory. Location of the primary tumor and metastasis for each patient was determined from inspection of the anonymized surgical pathology reports. The study had the approval of the St. Elizabeth Healthcare Institutional Review Board. Sections were cut and stained (hematoxylin and eosin) and confirmation of the cancer diagnosis was performed by a board-certified pathologist. Unstained slides were sent to Almac for RNA-sequencing using the Roche Kapa Total RNA kit, and the resulting data analyzed by Genialis to quantify and normalize expressions. Ninety-two samples had sufficiently high-quality expression data for further analysis.

### Processing of CIT Dataset

A publicly available dataset of colon cancer gene expression, collected for the Cartes d'Identité des Tumeurs (CIT) program from the French Ligue Nationale Contre le Cancer, was downloaded from Synapse. These data were previously analyzed for mRNA expression profiles using Affymetrix U133plus2 chip[8], and were among the various datasets used in the synthesis of the Consensus Molecular Subtypes[3]. The 566 tumoral samples were included in further analysis. In order to analyze the CIT data using the OncXerna TME Panel-1 algorithm, the Affymetrix probes were mapped to Biomart (Ensembl v103). Probes that mapped to multiple genes were removed, and in cases where multiple probes map to the same gene, the probe with the highest mean over all samples was retained. Lastly, we manually curated mappings for five TME Panel-1 signature genes missing from the Affy<>Biomart conversion. CIT gene expression quantifications were normalized in accordance with the TME Panel-1 workflow, and annotated with the associated metadata for tumor stage, side and CMS group.

### TME Panel-1 and CMS Biomarker Analysis

All Wood Hudson (WH) and CIT samples were classified using the TME Panel-1 algorithm into one of four TME phenotype classes (**Figure 2**)[7]. This enabled tabulation of the prevalence of each phenotype by disease stage and Left (distal) or Right (proximal) tumor side. Survival analysis was performed on the CIT patients to evaluate the prognostic potential of TME Panel-1. Recurrence free survival (RFS) was evaluated as months from surgery to recurrence, and overall survival (OS) on late stage (3-4) patents as time from recurrence to death. The relationship between CMS and TME Panel-1 was explored by mapping CIT patients onto the latent space created by two hidden nodes of the TME Panel-1 artificial neural network. In this manner, we could compare TME and CMS assignments.

**Gene Expression Datasets Used In Study**

The following datasets were used to explore the application of the TME Panel-1 diagnostic biomarker in colorectal cancer.

**Cartes d'Identite des Tumeurs (CIT)**

- GEO: GSE39582
- Ref: Marisa *et al.*, 2013
- Public dataset containing 566 primary tumor samples from patients in stage 1-4 CRC who had curative surgery between 1988 and 2007 in France. RNA expression data were generated by microarray, and the metadata include: CMS classification; mutational status of KRAS, TP53, BRAF; MMR and CIN status; stage of disease at diagnosis; site of primary tumor; disease-free interval; and overall survival status.

**Wood-Hudson Biobank Specimens (WH)**

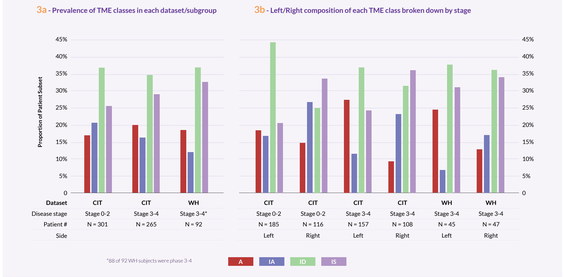
- Proprietary collection of 92 samples from the Wood Hudson Cancer Research Laboratory of patients with metastatic CRC that were treated with various regimen including targeted therapies following surgery. Gene expression was measured by RNA-seq and each sample was evaluated histologically. Metadata included stage of disease at diagnosis, stage at surgery and site of primary tumor.

## 3 Results

### TME Panel-1 Accords with Known Biological Characteristics of CRC

All patients from the CIT and WH datasets were classified according to the TME Panel-1 into one of four phenotype classes: Angiogenic (A), Immune Suppressed (IS), Immune Active (IA) or Immune Desert (ID). The prevalence of patients in each class was tabulated based on disease stage (**Figure 3a**) and tumor side (**Figure 3b**).

We observed a majority of patients classified in the IS and ID groups, which is consistent with the notion that CRC has a "cold" tumor microenvironment. The prevalence in IS suggests that many patients have tumors with high scores on both the angiogenesis and immune axes. Furthermore, the prevalence of the CIT stage 3-4 phenotypes were more similar to WH, for which 88 of the 92 patients were also stage 3-4, than it was to CIT stage 0-2 (**Figure 3a**). Considering Left and Right-sidedness (**Figure 3b**), the Left was found to be more angiogenic (A), while the Right was more immune active (IA). Taken together, the consistencies between TME class and previously reported attributes of stage and sidedness support the potential use of TME Panel-1 to characterize dominant biology in CRC.



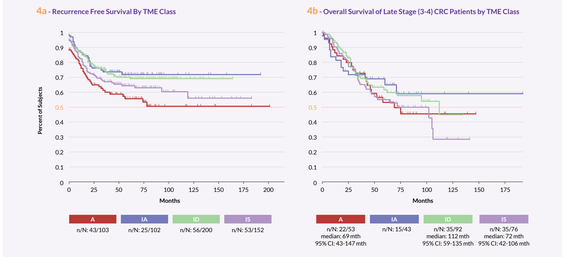

◀ *Figure 3. Prevalence of TME Phenotype Classes In the CIT and WH Datasets by Stage and Side*

***(a)** The CIT dataset was split into early- (0-2) and late-stage (3-4) disease, and compared to Wood Hudson for which 88 of 92 patients were stage 3-4. For each data subset, the proportion of patients classified as Angiogenic (A), Immune Active (IA), Immune Desert (ID) and Immune Suppressed (IS) was tabulated. TME classes are color coded according to the figure legend.*

***(b)** The data subsets were further split based on the side of tumor, Left (distal) or Right (proximal), and the TME class proportions retabulated.*

### TME Classes Appear Prognostic For RFS and OS

Since TME Panel-1 recapitulated fundamental aspects of CRC biology, we asked whether the TME classes were prognostic of the disease. Patients from the CIT dataset were analyzed for survival probability. Consistent with previous reports, the high angiogenic phenotypes (A) and (IS) showed worse recurrence-free survival, and worse overall survival among late-stage patients. Note, in the RFS analysis, none of the subgroups crossed the 50% threshold to compute median statistics, but the trend was evident. In both analyses, immune active (IA) patients had the best prognosis, consistent with observations in the field. We acknowledge heterogeneity of treatment in the Stage 3-4 cohort may impact assessment of disease prognosis in this analysis.




◀ *Figure 4. Kaplan Meier Survival Curves of Patients in the CIT Dataset Stratified by TME Phenotype Class*

*Recurrence free survival (RFS) of all available patients (N = 557, **4a**) and overall survival (OS) in late stage metastatic (stage 3-4) CRC patients (N=264, **4b**) was analyzed from the CIT dataset. Each survival curve represents a TME Panel-1 phenotype class, as indicated in the legend. For the RFS analysis (**4a**), none of the four patient classes had yet crossed the 50% threshold, and therefore median statistics have not been computed. Nevertheless, a clear trend may be observed in which the immune active phenotype has the best outcome, and the angiogenic class the worst prognosis. Despite the heterogeneous treatment histories of the patients in the OS analysis (**4b**), a similar trend is observed with the (A) and (IS) phenotypes showing lowest median survival.*

### TME Provides Potentially Actionable Phenotypic Details to Complement CMS Approach

To explore how TME Panel-1 relates to CMS, patients from the CIT dataset were classified according to the TME Panel-1 algorithm, and projected on the latent space of the TME model defined by an immune X-axis and angiogenic Y-axis (**Figure 5a**). Patients in each CMS group could then be evaluated based on these biological axes, and more specifically, based on the TME phenotypes defined by the latent space quadrants.

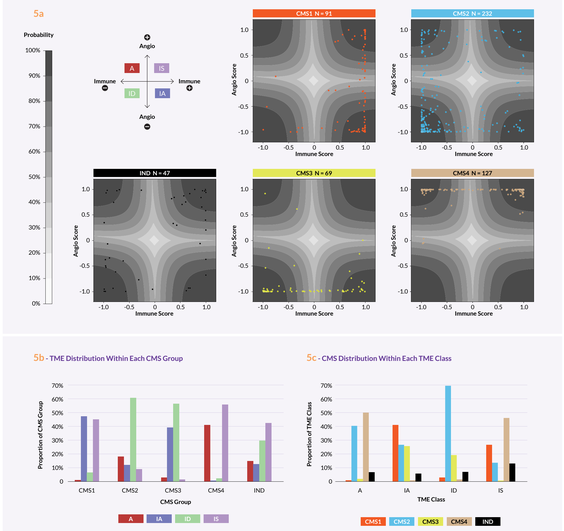
Consistent with Guinney *et al* 2015, CMS1 subjects were mostly high immune (positive on X-axis, top middle panel, orange), and CMS4 were mostly angiogenic (positive on Y-axis, bottom right panel, tan). CMS2 (top right, blue) were distributed in all four quadrants, though enriched for ID, while CMS3 was low angiogenic (bottom middle, yellow).

Despite the consistencies with the immune CMS1 and angiogenic CMS4, TME Panel-1 provides more granularity in terms of the molecular biological characteristics of the patients. For example, we observed a considerable number of CMS1 patients that had high TME angiogenic scores, and many of the CMS4 patients having high TME immune scores. We quantified the distribution of patients between CMS groups and TME classes to better appreciate how these classification approaches may lead to different conclusions about patient biology.

### TME Panel-1 Captures the Interactions Between Immune and Angiogenic Processes

While nearly all (92%) of CMS1 patients scored on the TME Immune axis, and 77% were dMMR (**Figure 6a**), almost half of those (45%) were in the (IS) TME class and therefore also angiogenic (**Figure 5b**). Conversely, 97% of CMS4 subjects were angiogenic, but the majority of those (56%) were in the (IS) TME class and therefore also scored positively on the TME immune axis. A plurality of indeterminant CMS patients were found to be (IS) as well, suggesting that the interactions between different biological processes in the stroma may confound models that do not explicitly account for these biologies.

Over half of all TME immune active (IA) patients were found in groups other than CMS1, including nearly 40% of patients previously classified as CMS3 (**Figures 5b, 5c**). Likewise, nearly 50% of TME (A) patients were observed in CMS groups other than CMS4. These results highlight differences in the very conception of the TME Panel-1 and CMS systems, with the former focused on detecting dominant biological processes of the tumor microenvironment and their interactions, while the CMS system derives from models including both tumor cell and stroma. Consequently, the CMS system is CRC-focused while TME Panel-1 aims to be tumor agnostic.




◀ *Figure 5a. Mapping CIT Patients and Their CMS Groupings Onto the TME Panel-1 Phenotype Landscape*

*Each patient was plotted on the TME Panel-1 landscape as defined by the Immune (x-axis) and Angio (y-axis) scores. The TME phenotypes corresponding to different regions of the landscape are shown in the diagram in the upper left panel. CMS subgroups were plotted separately to aid in visualization, and are identified by color legend, with IND (black) = CMS Indeterminate (per Guinney et al. 2015). The grayscale contours are probability bands that represent the probability of a particular TME classification by the TME Panel-1 algorithm.*

◀ *Figure 5b,c. Quantification of the Mapping of CMS Groups onto TME Classes*

***b)** For each CMS group, the proportion of patients of each TME class is shown, colored according to the legend.*

***(c)** For each TME class, the proportion of patients of each CMS group is shown, colored according to the legend. These plots represent the same but converse tabulation analysis.*

## 4 Discussion

Unlike the meta-model synthesis of CMS, the TME Panel-1 was built to abstract the biology of the tumor microenvironment for all solid tumors, not just CRC. The Panel was designed to be predictive—classification based on those biologies allows matching TME phenotypes with appropriate therapies. This has turned out to be the case when examined in other tumor types, such as Gastric and Ovarian[7,9] (publications in preparation). The goal of this analysis was to understand how TME Panel-1 accords with known CRC biology such as as differences in Left and Right sided cancers, and correlates with prior subtyping efforts in CRC, such as CMS. We have observed consistencies with prior analysis of immune and angiogenic biologies such as:

- Consistent enrichment for TME Angiogenesis (A) class and CMS4, particularly in Left sided tumors
- Consistent enrichment for TME Immune Active (IA) class and CMS1
- Similar prognostic relationships for TME (A) and CMS4 and TME (IA) and CMS1

Observed differences largely reflect the composition and distribution of TME phenotypes across CMS groups, where the TME panel identifies immune and angiogenic signal in patients outside of the canonical CMS definitions. For example, in our analysis of the CIT dataset, patients assigned to (A) and (IA) classes were dispersed more broadly than just CMS4 and CMS1. In fact, only half of the (A) patients are CMS4, and half of (IA) are CMS1 (see **Figure 5**). The data on the predictive nature of CMS groups is limited, but in several large studies the hypothesis that CMS4 could be a strong predictor for response to Avastin has not held up. We wonder whether the model-synthesis approach used to derive CMS resulted in a compromise between tumor cell and tumor microenvironment signals, such that the dilution of the angiogenesis signal has led to a less effective predictor of response for drugs that target processes of the TME. Might the core focus of TME Panel-1 on the interactions between these biologies perhaps improve predictive power for drugs that target the microenvironment such as Avastin and Checkpoint Inhibitors? In CRC this hypothesis awaits further testing.

Even less is known about the predictive nature of CMS1 to immune targeted therapies such as checkpoint inhibitors. We do know that dMMR/MSI-H are mostly captured by CMS1 and this is the most validated group of CRC patients to CPI (30-50% response)[10]. However, recent studies of the MSS population through HLA mutation and immune cell infiltration analyses has suggested there is another 20% of MSS CRC that may be appropriate for CPI treatment[11]. Similar to the relationship between TME (A) and CMS4, the TME (IA) class is made up of 41% CMS1 and then significant contributions from CMS2 and CMS3. An interesting future question will be whether the TME IA subgroup can capture the 20% of possible MSS responders to CPI.

In addition, TME Panel-1 defines an Immune Suppressed (IS) class. CRC is characterized as 'cold,' but this could be for either a lack of immune activity or immune suppression blocking CPI activity. Of note, almost half of the dMMR patients in the CIT dataset are classified as TME (IS) (**Figure 6**). Understanding the relationship between IS and response to CPI in this disease setting is an interesting future question. Emerging therapies focused on immunosuppressive cells and cytokines, such as myeloid targeting agents or next-generation immune modulators such as anti-TIM3 or LAG3, may be able to 'warm up' the IS group and further enhance immune therapy opportunities in CRC.

OncXerna is developing novel therapeutics that target the tumor microenvironment: navicixizumab, an anti-angiogenic bispecific antibody; and bavituximab, a phosphatidylserine-targeting monoclonal antibody that may help activate cold microenvironments. Future clinical studies may afford us the opportunity to test these hypotheses on CMS and TME subtyping for predicting response to therapies in CRC.

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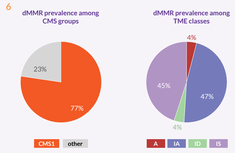


◀ *Figure 6. Proportion of dMMR patients in the CIT dataset represented in CMS groups and TME classes*

*About three quarters of dMMR patients were captured by CMS1 (77%) (left), whereas 92% of dMMR patients were classified as high immune TME phenotypes (IA) and (IS) (right). Groups and classes are colored according to the legends.*

## 5 Conclusions

- The TME Panel-1 RNA-based gene signature captures the interplay between angiogenic and immunogenic properties using machine learning to classify patients into one of four TME phenotypes.
- Prevalence of TME Panel-1 classes identified in CRC was similar in other solid tumors (e.g. gastric cancer). In addition, TME Panel-1 was prognostic for recurrence-free and overall survival in CRC, further supporting its use as a novel, pan-tumor biomarker.
- TME Panel-1 phenotypes reflected the expected biology based on known molecular characterization of CRC by stage of disease, distal/proximal sidedness, and CMS subtype. Yet, the Panel's focus on the tumor microenvironment may make it more predictive of response to TME-targeted agents such as immunotherapy and anti-angiogenics, and rational combinations.
- Testing whether TME Panel-1 can prospectively predict outcome of targeted therapy in CRC is planned, with trials for bavituxmab and navicixizumab, as well as broader exploration of additional clinical datasets.


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### References


1. *Fatemi, S. R. et al. Recurrence and Five Year Survival in Colorectal Cancer Patients After Surgery. Iran. J. Cancer Prev. 8, (2015).*
2. *Duineveld, L. A. M. et al. Symptomatic and Asymptomatic Colon Cancer Recurrence: A Multicenter Cohort Study. Ann. Fam. Med. 14, 215–220 (2016).*
3. *Guinney, J. et al. The Consensus Molecular Subtypes of Colorectal Cancer. Nat. Med. 21, 1350–1356 (2015).*
4. *Baran, B. et al. Difference Between Left-Sided and Right-Sided Colorectal Cancer: A Focused Review of Literature. Gastroenterol. Res. 11, 264–273 (2018).*
5. *Lenz, H.-J. et al. Impact of Consensus Molecular Subtype on Survival in Patients With Metastatic Colorectal Cancer: Results From CALGB/SWOG 80405 (Alliance). J. Clin. Oncol. 37, 1876–1885 (2019).*
6. *Stintzing, S. et al. Consensus Molecular Subgroups (CMS) of Colorectal Cancer (Crc) and First-Line Efficacy of Folfiri Plus Cetuximab or Bevacizumab in the fire3 (Aio Krk-0306) Trial. Ann. Oncol. 30, 1796–1803 (2019).*
7. *Strand-Tibbitts, K. et al. Development of an RNA-based Diagnostic Platform Based on the Tumor Microenvironment Dominant Biology. SITC (2020).*
8. *Marisa, L. et al. Gene Expression Classification of Colon Cancer Into Molecular Subtypes: Characterization, Validation, and Prognostic Value. PLoS Med. 10, e1001453 (2013).*
9. *Strand-Tibbitts, K. et al. Working Towards Precision Medicine for the Tumor Microenvironment. in SITC (2019).*
10. *André, T. et al. Pembrolizumab in Microsatellite-Instability-High Advanced Colorectal Cancer. N. Engl. J. Med. 383, 2207–2218 (2020).*
11. *Giannakis, M. et al. Genomic Correlates of Immune-Cell Infiltrates in Colorectal Carcinoma. Cell Rep. 15, 857–865 (2016).*