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Title Immunologic Mechanisms of Colorectal Liver Metastases: Potential for Novel Therapies

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Immunologic Mechanisms of Colorectal Liver Metastases: Potential for Novel Therapies

By

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Doctor of Medicine

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An abstract of

A thesis submitted to the Faculty of the

James T. Laney School of Graduate Studies of Emory University

In partial fulfillment of the requirements for the degree of

Master of Science

In Clinical Research

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Abstract

Immunologic Mechanisms of Colorectal Liver Metastases: Potential for Novel Therapies

By

Caroline R. Goel, MD

Colorectal cancer is the third most common cancer worldwide. Over half of patients with colorectal cancer (CRC) develop liver metastases. While immunotherapy is an emerging treatment for patients with solid tumors, its use among patients with CRC is limited due to poor efficacy. Furthermore, gene expression patterns of liver-specific CRC metastases remain unclear. The purpose of this study was to identify and validate a high-risk gene expression profile for patients with colorectal liver metastasis (CRCLM) to better inform prognosis and development of novel targeted therapies.

CRCLM samples from patients who underwent complete metastatectomy from 2009-2017 at Emory University were examined. Expression profiling of extracted RNA was performed using the NanoString Immuno-Oncology (IO360) 770-gene panel. Statistical analysis using cutoffs of absolute log 2-fold change ≥ 1.5 and p-value ≤ 0.05 were performed. Patients were analyzed by extremes of outcomes: survival time in the lowest quartile, compared to those still alive at last follow-up. Four genes had higher expression in tumors from patients with worse overall survival compared to patients still alive at last follow-up: CSF1, MGMT, IL6R, and LILRB4.

CSF1 signaling leads to M2-macrophage polarization, a well-studied anti-inflammatory and tumor-tolerant phenotype. MGMT encodes a DNA repair enzyme that repairs alkylating chemotherapy damage and is implicated in carcinogenesis and chemotherapy response. IL6R signaling affects tumor proliferation through tumor-associated macrophages, myeloid derived suppressor cells, and T cells. LILRB4 signaling from myeloid-derived suppressor cells leads to T cell anergy and tumor tolerance. On Kaplan-Meier survival analysis, increased expression relative to the median of CSF1, MGMT, and IL6R was significantly associated with poor survival (all $p<0.05$). LILRB4 expression trended towards significance in its association with poor survival ($p=0.124$). On multivariable cox regression adjusted for age, gender, and tumor sidedness, increased expression of CSF1, MGMT, and IL6R was associated with increased hazard of death (all $p<0.05$). LILRB4 expression did not reach statistical significance ($p=0.162$).

Immunohistochemical analysis was performed to validate these gene expression findings at the protein level. Consistent with our gene expression data for CSF1, we saw increased M2-macrophage polarization, a known downstream effect of its signaling, among tumors of patients with poor survival. Direct antibody staining confirmed protein expression of MGMT and LILRB4. Although analysis did not reach statistical significance for any genes of interest, immunohistochemistry did demonstrate expected phenotypic and spatial distribution of cells of interest.

These findings suggest a myeloid-predominant tumor immune microenvironment in which overexpression of genes of interest promote a pro-tumorigenic environment.

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Patients and families

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Introduction

Colorectal cancer is the third most common cancer worldwide. Over half of patients develop metastatic disease, most commonly to the liver.[1,2] Large-scale population-based studies have shown that up to 80% of colorectal liver metastases (CRCLM) develop within 2 years of primary colectomy.[3] CRCLM are among the few Stage IV cancers routinely managed surgically. However, despite optimal systemic and surgical treatment, median survival is 48 months following liver resection or approximately 40% at 5 years.[4,5] Furthermore, only 20% of patients are eligible for surgical resection, and 5-year survival among unresectable patients is 10-15%.[6] In addition to developments in surgical techniques to improve safety and feasibility of metastatectomy, chemotherapy and immunotherapy, in select cases, remain an important focus in the management of CRCLM. Despite significant advances in the use of immunotherapy for treatment of many solid tumors, its use among patients with colorectal cancer remains low due to its poor efficacy in the majority of patients. Although currently available immune checkpoint inhibitor therapies have shown promising results among colorectal cancer patients with gene mutation repair defects (microsatellite instability), this feature of tumors is present in only 5% of all colorectal cancer patients.[7] In order to broaden the population of patients who may benefit from immunotherapy, a deeper understanding of the colorectal cancer-specific tumor immune microenvironment is needed.

The mechanisms by which metastatic tumor cells survive and ultimately proliferate in the body are poorly understood. Variability in the development of metastatic disease is thought to be due to tumor escape from dormancy. Cancer dormancy is the propensity for tumor cells to exist as asymptomatic, microscopic disease within an individual.[2] These dormant cells are thought to evade detection by the immune system while possessing the ability to form clinically-relevant

metastatic disease at a later time. The immunologic mediation of tumor dormancy is an emerging focus of cancer research, although often difficult to study due to the limited role for surgery in most cases of metastatic disease, and thus limited tissue to study. As a disease where surgery plays an important role, CRCLM represent a prime opportunity to study the host immune response to dormant tumor cells.

The development of clinically significant metastatic disease is related to both biologic features of the tumor and the host immune response. Previous studies have demonstrated concordant morphological and cytogenetic changes between primary colorectal tumors and metastasis.[8] The similarity in genomic profiles between primary and metastatic tumor cells and the wide range of timing to development of metastatic disease underscores the role of host factors in the activation and proliferation of dormant microscopic disease.[9] There is a growing body of literature to support the role of immune cells in regulating the progression to metastatic disease and the need for further characterization of the tumor immune microenvironment. For example, Galon *et al.* found a positive correlation between presence of markers for Th1 activation and cytotoxic and memory T cell infiltration and CRC recurrence.[10] Similarly, by quantifying density of CD3+ and CD8+ T cells within colorectal tumors, Pagès *et al.* were able to more accurately predict prognosis than current AJCC TNM classification.[11] Other cell populations including tumor-associated macrophages (TAMs) play a major role in cancer-related inflammation and tumor progression. Through complex intercellular signaling pathways these TAMs can have both inhibitory and supportive roles in cancer, depending on their polarization and function.[12] The association between intratumoral immune cell infiltration and patient outcomes underscores the importance of further characterization of the tumor immune microenvironment. Identifying effective targets to limit metastatic spread or the viability of

metastatic cells within the tumor immune microenvironment is a highly important and innovative area of research with both prognostic and therapeutic potential.

Our laboratory examines how innate host factors including the tumor immune microenvironment impact clinical outcomes in patients with colorectal liver metastases. We hypothesize that resected colorectal liver metastases will have unique immune signatures that will accurately prognosticate clinical outcomes for patients and potentially lead to novel therapeutic strategies.

Therefore, the aim of this study was to identify differentially expressed genes associated with survival in patients with CRCLM who underwent surgical resection in order to better inform prognosis and future therapeutic targets.

Methods

Patient Selection

This study included patients with CRCLM who underwent completion hepatic metastatectomy at Emory University from 2009-2017. IRB approval was obtained prior to study commencement. Specimens were obtained as formalin-fixed paraffin-embedded (FFPE) tissues. Tissue processing was performed by the Emory Winship Cancer Institute Cancer Tissue and Pathology Shared Resource. Serial sections were cut at 4-5 µM thickness from the same tissue blocks for immunohistochemical analysis.

For each patient, relevant demographic, operative, perioperative, histopathologic, genetic, and long-term survival data were collected. Histopathologic data included tumor grade and primary tumor side (left or right colon). Genetic data included presence of commonly identified and clinically-relevant gene mutations; these included v-raf murine sarcoma viral oncogene homolog B (BRAF), microsatellite instability (MSI), and Kirsten rat sarcoma viral oncogene homolog (KRAS).

NanoString Analysis

Gene expression analysis focused on the immune features present in histologic specimens of CRCLM. RNA was extracted from FFPE tissues obtained from the Emory Cancer Tissue and Pathology Shared Resource. Analysis of gene expression was performed using the NanoString nCounter PanCancer Immune Oncology 360 Panel (IO360), a 770-gene expression panel. Samples were de-identified with respect to patient data during experimental and analytical process to avoid potential observer bias. Transcript counts were determined using the nCounter

Analyzer. Expression counts were normalized using geometric mean of positive control, and genes were selected using the geNorm algorithm and log2 transformation. Patients were dichotomized as "high-risk" or "low-risk" using survival quartiles, with those still alive at last follow-up considered "low-risk" and those in the lowest survival quartile considered "high-risk." Overall survival (OS) was defined as time from surgery to death by any cause (Figure 1). High gene expression was defined as log 2-fold change ≥ 1.5 relative to the median, and significance was set at $p \leq 0.05$.

Survival Analysis

Survival analysis was performed by converting aggregate NanoString gene expression data to dichotomous outcome variables denoting gene expression relative to the median for survival groups. Kaplan-Meier and log-rank survival analysis was performed to identify univariate association between gene expression and overall survival. Multivariable cox regression was used to identify association between gene expression and overall survival adjusting for pertinent covariables. All analysis was done with the assistance of the Winship Cancer Institute Biostatistics and Bioinformatics Shared Resource.

Immunohistochemistry Antibody Protocol Development and Staining

Each antibody was optimized by testing it with appropriate positive control tissue at a minimum of 3 antibody dilutions and additional staining conditioning as needed to determine the optimum staining conditions for each antibody prior to sectioning and staining the study samples. Formalin-fixed and paraffin-embedded tissue sections from each subject were cut to a 5-μm thickness and air-dried. Four sets of immunohistochemical stains were performed on individual slides from each tissue sample. Staining was performed using Ventana DISCOVERY Ultra

Automated Immunohistochemistry Stainer (Ventana Medical Systems, Tuscon, AZ). Slides were deparaffinized with EZ-Prep (# 05279771001, Ventana) and then were antigen retrieved for 40 minutes with CC1 reagent (#950-500, Ventana). Antibody staining was performed using the below stains at listed dilutions and incubated for 40 minutes. DISCOVERY OmniMap anti-Rabbit HRP was applied and incubated for 12 min and the detection was completed in combination with DISCOVERY ChromoMap DAB kit, as per manufacturer recommendations. Slides were counterstained with hematoxylin for 12 min. Slides were then dehydrated, cover-slipped, and evaluated by light microscopy.

Immunohistochemical Staining

Assessment of CSF1 expression was performed using dual antibody co-staining for macrophages, using CD68 (Agilent, Santa Clara, CA) at 1:200 and for M2-polarized macrophages using CD163 (Abcam, Cambridge, MA) at 1:2000 dilution. Total counts of M2-differentiated macrophages were determined in the peritumoral space, at the tumor-liver interface, and within the tumors. The percentage of macrophages with M2 phenotypic markers, defined as the number of M2 cells divided by total macrophages detected, was determined within each tumor. Staining for MGMT was performed at a dilution of 1:400 using anti-MGMT (Abcam, Cambridge, MA). MGMT expression was measured by determining the intensity of MGMT staining within the nuclei of cells within the tumor. Antibody titration was performed at multiple dilutions for optimized IL6R staining using anti-IL6R (Abcam, Cambridge, MA). These included 1:400, 1:1000, and 1:2000. The optimal staining intensity was unable to be confirmed after multiple attempts to establish a control in appropriate tissue and so IL6R staining was not performed on patient samples. LILRB4 staining was performed using direct anti-LILRB4

(Abcam, Cambridge, MA) diluted at 1:200. LILRB4 expression was measured by staining of peri-tumoral Kupffer cells and expressed as cell counts.

All cell counts were measured per 10 high-powered fields. Immunohistochemical analysis was performed using quPath software. Positive cell counts were determined for both tumor and stroma within each slide.

Statistical Analysis

Patients were stratified into overall survival groups with "low-risk" patients being those alive at last follow-up and "high-risk" being those in the poorest survival quartile. Mann-Whitney U Test was used to assess differences between groups. Significance was set at $p \leq 0.05$.

Results

Patient Demographics

Fifty-three patient tissue samples were analyzed in this study. Among these, 30 were included in final analysis based on survival quartiles: 21 were in the low-risk quartile, still alive at last follow-up, and 9 were high-risk and in the lowest survival quartile. The median age was 58 years (interquartile range 50-71) and 27% of patients were female. The median survival among high-risk patients was 15 months. The 21 survivors had a median follow-up of 49 months. Demographic data can be found in Table 1 in addition to data regarding known mutation status and primary tumor sidedness.

NanoString Analysis

NanoString analysis identified four targetable genes whose relative overexpression was associated with poor survival: CSF1, MGMT, IL6R, and LILRB4. Log 2-fold change and adjusted p-values are listed in Table 2. Further demographic and gene mutation-specific data,

stratified by genes of interest, can be found in Table 3. A heat map denoting differential gene expression associated with survival group is shown in Figure 2, where yellow shades represent under-expression relative to those in the poor survival group, and blue cells correspond to gene over-expression relative to patients still alive at last follow-up.

Survival Analysis

Kaplan-Meier survival analysis was subsequently performed to assess whether relative expression of each gene of interest was associated with 5-year overall survival. There was a statistically significant association between overall survival and relative expression of CSF1 (p=0.016), MGMT (p=0.03), and IL6R (p=0.005); LILRB4 expression was not significantly associated with survival on log-rank test (p=0.124) (Figure 3A-D).

Multivariable cox regression was performed to assess the association between expression of each gene of interest and overall survival. Regression analyses were adjusted for gender, age, and tumor sidedness as these factors have demonstrated clinical relevance in CRCLM. Increased CSF1 expression among poor survivors compared to those alive at last follow-up was significantly associated with worse survival on multivariable cox regression (HR 12.29, p=0.028, Table 4). Similarly, increased MGMT expression among poor survivors compared to those alive at last follow-up was associated with an over fivefold increased hazard of death in our cohort (HR 5.58, p=0.046, Table 5). IL6R expression was also significantly associated with increased hazard of death (HR 14.45, p=0.018, Table 6). Finally, LILRB4 expression did not reach statistical significance in its association with overall survival (HR 4.62, p=1.62, Table 7).

Immunohistochemistry Analysis

Cell counts were compared between groups using the Mann-Whitney U Test; these results are summarized in Table 8. All cell counts are per 10 high-powered fields.

CSF1

As CSF1 is a secreted molecule difficult to directly stain for on FFPE specimens, its expression was determined by the distribution of macrophages with an M2 phenotype as indicated by CD163-positive staining (red). Total macrophages were stained for using the pan-macrophage marker CD68 (brown). In the peritumoral space, the number of M2 macrophages was greater among patients in the poor survival quartile (mean 12.11, S.D. 3.18) compared with low-risk patients (mean 10.14, S.D. 7.26) although not statistically significant (p=0.062). Qualitatively, there was a visible difference between high-risk patients (Figure 4A) and low-risk patients (Figure 4B), with more red-staining M2 macrophages in the poor survival cohort. At the tumor-liver interface, the number of M2 macrophages was higher among high-risk patients (21.67 ±9.42) than low-risk patients (15.62 ± 7.27) but not statistically significant (p=0.117). Qualitatively, there was an expected aggregation of macrophages along the tumor-liver interface with greater red-staining M2 macrophages among high-risk patients (Figure 4C) compared with low-risk patients (figure 4D). Within the tumor, the number of intratumoral M2 macrophages was lower among high-risk patients (7.11 ± 5.56) compared to low-risk patients (12.05 ±7.77, p=0.063). However, the percentage of M2-differentiated macrophages out of total macrophages was higher among high-risk patients (72.39% ± 36.5% versus 60.8% ± 25.76%; p=0.226). Intratumoral macrophage expression is indicated in Figure 4E and 4F.

MGMT

MGMT expression was indicated by intratumoral intensity staining. Mean intensity did not differ significantly between high-risk patients (1.5 ± 0.76) and low-risk patients (1.9 ± 0.83, p=0.228). Qualitatively, MGMT staining was localized to the nucleus (Figure 5A, 5B).

LILRB4

LILRB4 staining with anti-LILRB4 antibody was performed. Positive staining cells were those with antibody detection within peri-tumoral Kupffer cells. LILRB4 expression did not differ significantly between groups (high-risk: 53.67 ± 46.61; low-risk: 69.2 ± 53.67; p=0.571). Qualitatively, LILRB4 expression was granular in nature and most localized to peri-tumoral Kupffer cells, a type of liver macrophage (Figure 6A, 6B).

Discussion

CRCLM represents an important area of research in immunotherapy owing to poor efficacy of presently available treatments. We demonstrate differential gene expression associated with poor survival among patients with resected CRCLM. Specific genes of interest include CSF1, MGMT, IL6R, and LILRB4. Importantly, these identified genes suggest a myeloid-predominant tumor immune microenvironment and potential therapeutic targets or prognostic indicators.

We saw increased levels of CSF1 expression among poor survivors relative to CSF1 expression among patients still alive at last follow-up. On immunohistochemical analysis, CSF1 expression, reflected in the distribution of M2-differentiated tumor associated macrophages, was consistent with expected findings: there was a predominance of M2-polarized macrophages among poor survivors, particularly in the peritumoral space and at the tumor-liver interface. Given the immunosuppressive and pro-tumorigenic effects of M2-polarized macrophages, our quantitative and qualitative observations are concordant with current literature.[12] Our results suggest that CSF1 expression did translate to phenotypic changes that were quantifiable in our tissue samples. Although not statistically significant, our investigational findings certainly support further research in this pathway.

Colony stimulating factor 1 (CSF1) binds to the CSF1 receptor (CSF1R) expressed on monocytes, macrophages, and myeloid dendritic cells and acts to regulate cellular development, survival, proliferation, and differentiation.[13] Within the tumor immune microenvironment, CSF1 signaling to tumor-associated macrophages (TAMs) leads to their polarization to the pro-tumorigenic M2 macrophage phenotype. This M2-polarized phenotype is associated with an immunosuppressive, pro-tumorigenic tumor immune microenvironment that facilitates tumor proliferation and spread.[12] Within the tumor immune microenvironment, these M2-TAMs support immune escape and tumor growth through increased production of growth factors, metastasis-promoting cytokines, and promotion of angiogenesis and tissue remodeling.[14] CSF1 signaling leading to M2-polarization and cancer has been studied most extensively in the context of treatment-resistant glioma, where CSF1R blockade can inhibit glioma progression; these findings have been replicated in preclinical sarcoma models.[15,16]

On NanoString analysis, there was a relative increase in MGMT expression among poor survivors compared to patients still alive at last follow-up. Further analysis with immunohistochemistry demonstrated that MGMT expression was not significantly different between among tumors from patients in high-risk and low-risk cohorts.

O^6-methylguanine DNA methyltransferase (MGMT) is a gene that encodes the MGMT DNA repair enzyme. MGMT acts by repairing alkylating DNA damage through alkyl group cleavage and its function is considered an innate protection of cells against carcinogenesis. It has been extensively studied in the context of colorectal cancer owing to its propensity for promoter methylation leading to loss of function. MGMT promoter methylation has been documented in 30-40% of metastatic colorectal cancers and is noted to have a paradoxical effect on

carcinogenesis. Active MGMT works to reduce cancer formation due to DNA repair, but owing to its ability to repair alkylating DNA damage, facilitates resistance to alkylating chemotherapy agents. Conversely, patients with silenced MGMT are at increased risk of cancer development but retain normal sensitivity to alkylating agents.[17] MGMT is an important prognostic indicator and can be used to predict patient response to chemotherapy.

It is important to note the paradoxical effect of MGMT expression on cancer development, with MGMT methylation-induced silencing associated with increased risk of cancer but intact MGMT associated with decreased response to alkylating chemotherapy agents. Notably, only one patient in our cohort received alkylating chemotherapy in the adjuvant setting hence the exclusion of this factor from analysis. Although the intensity of MGMT expression in intratumoral cells did not differ between groups, we still see translation of genetic changes to protein expression.

Interleukin 6 receptor (IL6R) affects tumor proliferation through signaling to tumor-associated macrophages, myeloid-derived suppressor cells (MDSCs), and T-cells. IL6 is secreted by tumor-associated macrophages and, by binding IL6R, activates the JAK/STAT3 cellular proliferation signaling pathway. Oncogenic STAT3 activation is frequently associated with adenocarcinoma through increasing cellular growth and supporting the ability of cancer cells to proliferate and spread.[18,19] Elevated serum levels of IL6 in patient serum is associated with more advanced stage and decreased survival among patients with many solid tumors including colorectal cancer.[20]

NanoString analysis demonstrated a relative increase in LILRB4 expression among patients in the poor survival group compared to LILRB4 expression among patients still alive at last follow-up. Leukocyte immunoglobulin-like receptor 4 (LILRB4) is expressed on multiple

immune cells including tumor-associated macrophages, monocytes, natural killer cells, myeloid-derived suppressor cells, and T cells.[21,22] Its signaling is associated with immune regulation and a pro-tumorigenic tumor immune microenvironment through myeloid-derived suppressor cell activation leading to T cell inhibition. LILRB4 blockade has recently been associated with improved survival in acute myelocytic leukemia (AML) leading to fast-tracked Food and Drug Administration (approval) of IO-202, a targeted drug that blocks LILRB4 signaling.[23] These results have led to a currently recruiting phase I clinical trial (NCT05309187) of IO-202 and pembrolizumab for the treatment of solid tumors.[24] LILRB4 represents a promising target in colorectal cancer.

Immunohistochemistry analysis showed that LILRB4 expression was observed in peritumoral Kupffer cells. Kupffer cells are liver-resident macrophages of myeloid origin that play an important role in the hepatic tumor immune microenvironment through signaling with myeloid derived suppressor cells and tumor-associated macrophages. Kupffer cells act to control liver metastasis through phagocytosis and secretion of cytotoxic cytokines. Conversely, Kupffer cells can also act in a tumor-tolerant state by promoting tumor cell adhesion, immune evasion, and metastatic proliferation.[25] Given the role of LILRB4 in myeloid-derived suppressor cell activation and subsequent T cell anergy to promote a pro-tumorigenic and immunosuppressive tumor immune microenvironment, our detection of LILRB4 expression in peritumoral Kupffer cells supports the role of LILRB4 in complex tumor-myeloid cell crosstalk within tissue.

Conclusions

Among patients with colorectal cancer liver metastases, expression of CSF1, MGMT, IL6R, and LILRB4 are associated with decreased overall survival. Immunohistochemical

analysis demonstrated protein expression consistent with CSF1 signaling, through M2-macrophage polarization, and LILRB4 signaling, through peritumoral Kupffer cell staining. These genes represent important myeloid effectors in the tumor immune microenvironment and support the role of myeloid cells in the development or persistence of CRCLM. Having established gene expression changes that translate to protein synthesis, further investigation is needed to define the effects on tumor growth *in vivo* using animal models.

Strengths and Limitations

We demonstrate differential gene expression associated with survival among patients with resected CRCLM. Gene expression was determined using the NanoString PanCancer IO360 770-gene panel. This comprehensive panel identifies upregulated and downregulated genes throughout the tumor immune microenvironment. Beyond the extensive gene panel, an additional strength of the IO360 is its ability to identify genetic changes present in the context of the natural immune response, which is critical to this study's aim to study the complex interactions between tumor and host immune system. Furthermore, this study uses real patient samples from resected CRCLM specimens in which both tumor and adjacent liver tissue are examined. This presents an important opportunity to identify changes present in tumor-adjacent tissue, a critical aspect of immunologic cancer studies.

We then used immunohistochemistry to provide protein validation of gene expression findings. Immunohistochemical analysis utilizes antibody staining to provide both quantitative and qualitative data on protein expression in the context of native tissue morphology. It allows for spatial phenotypic analysis: that is, the identification of protein expression and the spatial relationships of positive cells with respect to tissue. In this study, these characteristics are of particular importance when considering the tumor immune microenvironment, wherein tumors and the host immune cells interface. By analyzing protein expression both within the tumors and in the adjacent liver tissue, we can better assess the intricacies of the host response to tumor in addition to changes within the tumor itself. All immunohistochemical analysis in this study was reviewed by an expert Winship Cancer Institute Gastrointestinal pathologist, who approved the staining methods and dilutions and formulated an analysis plan with our team. This multidisciplinary approach to analysis ensures the highest quality of staining and interpretation

of results. All stained slides, as well as criteria for positive cellular staining, were reviewed as well.

This study is not without limitations. It is a single-surgeon pilot study of 53 patients, 30 of whom were included in analysis. All patients were treated at Winship Cancer Center, an NCI-designated cancer center affiliated with Emory University, a high-volume, quaternary referral center. The operating surgeon is a highly experienced, surgical oncology-trained physician who operates at a high annual volume of patients. Although this speaks highly to the quality of multidisciplinary cancer care given to patients in our cohort, it does limit study generalizability in that many patients in the United States are treated at community centers that potentially see a lower volume of patients and without the opportunity for NCI-designated cancer center care. Furthermore, within the analysis cohort, patient groups were imbalanced, with 21 patients in the low-risk group and 9 in the high-risk group. These factors affect both the generalizability of these results and the ability to generate clinical inferences from the statistical analysis performed. Furthermore, when considering survival analysis, there were only 9 death events, all of which were present in the high-risk group. This limitation was inherent in the patient stratification method of this study. However, as this study is an investigational pilot study, we believe that the data generated represents important preliminary data to serve as a foundation for further studies. Additionally, immunohistochemistry is limited by its subjectivity. The ability of immunohistochemistry to quantitate protein expression is limited by user experience and impressions; we addressed this limitation through review of findings with an expert pathologist. The issue of subjectivity of immunohistochemistry has been a topic of extensive research, and automated computer programs have been shown to improve this in certain studies.[26] In this

study, automated cellular counting was performed and then validated manually to ensure maximum accuracy.

Conclusions

Resected colorectal cancer liver metastases differentially express CSF1, MGMT, IL6R, and LILRB4. Upregulation of these genes is associated with worse survival, and these genetic findings are reflected in antibody staining with immunohistochemistry. These findings suggest a myeloid-predominant tumor immune microenvironment in which tumor-associated macrophages closely interact with myeloid derived suppressor cells, T cells, and M2-polarized macrophages leading to a pro-tumorigenic liver landscape.

Additional work is needed to better characterize the mechanistic role of CSF1 in M2-macrophage polarization and identify therapeutic targets. Similarly, further investigation is needed to identify the effect of LILRB4 blockade on CRCLM growth.

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Figures

Figure 1. Consort Diagram Delineating Patient Selection

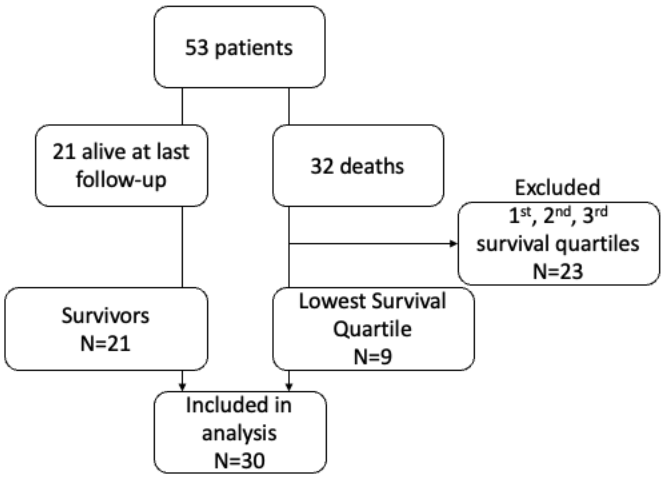


Figure 2. Heat Map Showing Differential Gene Expression Results of NanoString Analysis

Patient samples are represented by columns and boxes in this heat map. Cells below the purple panels represent patients from the high-risk, poor survival group, while cells below turquoise panels represent patients from the low-risk group of patients still alive at last follow-up. Within the heat map, blue cells correspond to increased gene expression relative to the median while yellow colors denote decreased gene expression relative to the median.

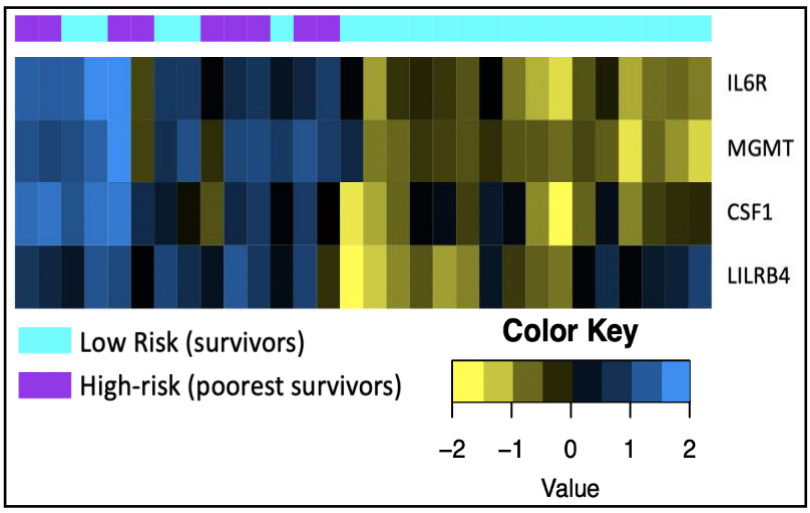
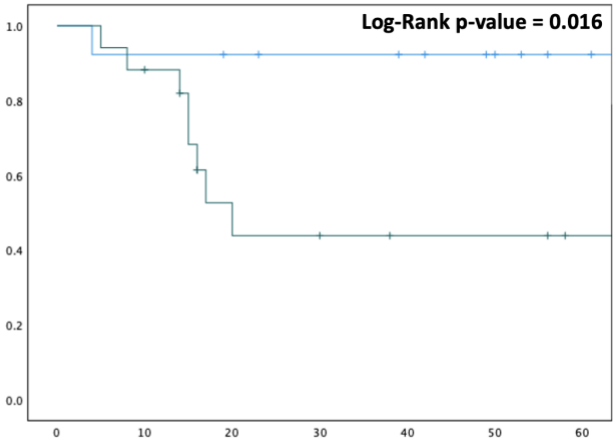
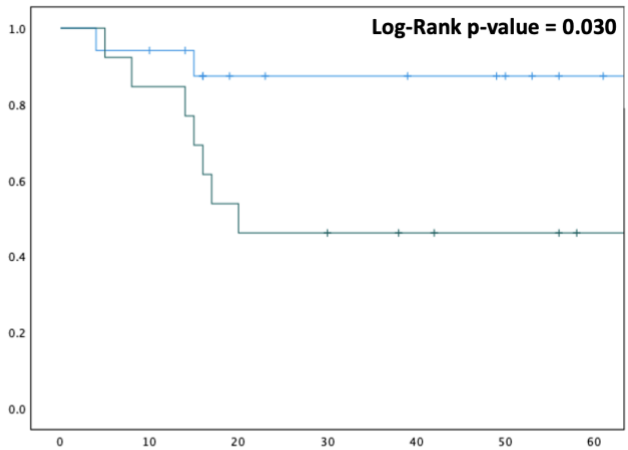


Figure 3A. Kaplan-Meier Analysis of Overall Survival Associated with CSF-1 Expression.



Number at Risk							
CSF1 Below Median	13	12	11	10	8	6	2
CSF1 Above Median	17	13	6	4	3	2	1

Figure 3B. Kaplan-Meier Analysis of Overall Survival Associated with MGMT Expression.



Number at Risk							
MGMT Below Median	17	14	10	9	8	6	2
MGMT Above Median	13	11	7	5	4	2	1

Figure 3C. Kaplan-Meier Analysis of Overall Survival Associated with IL6R Expression.

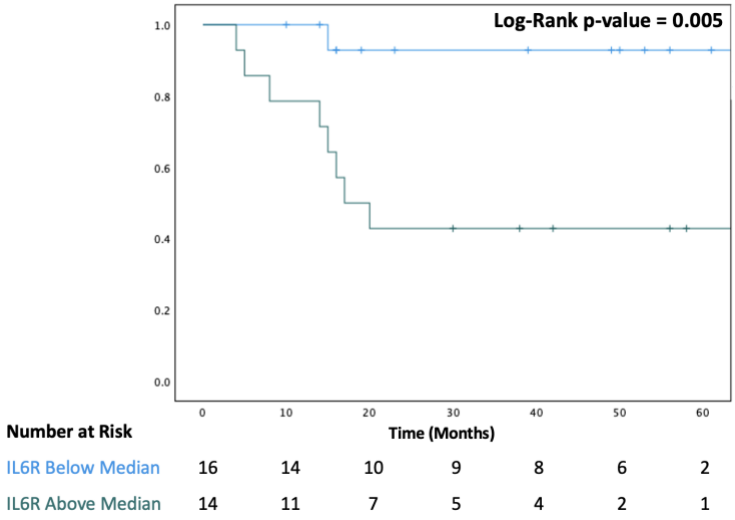


Figure 3D. Kaplan-Meier Analysis of Overall Survival Associated with LILRB4 Expression.

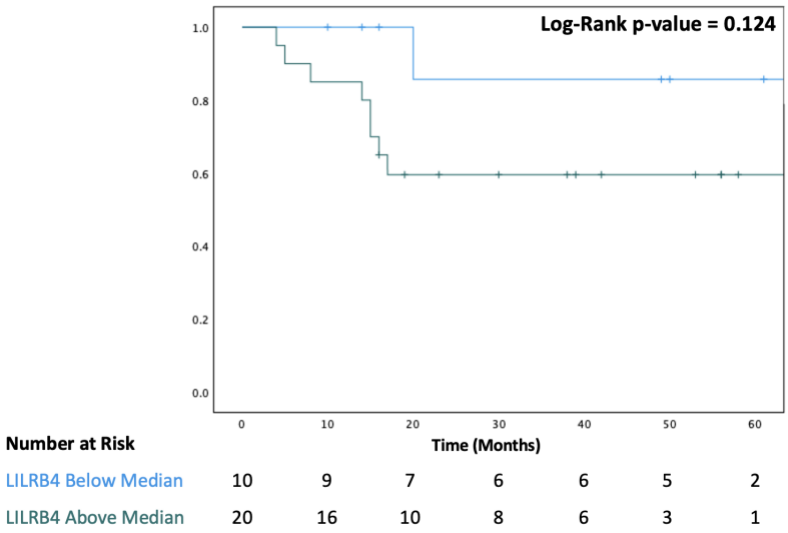


Figure 4. Immunohistochemistry Staining for CSF1.

Immunohistochemistry staining of CSF1 expression as M2 macrophages (red) and all macrophages (brown): 4A) Peritumoral, poor survival group; 4B) Peritumoral, high survival group; 4C) Tumor-liver interface, poor survival group; 4D) Tumor-liver interface, high survival group; 4E) Intratumoral, poor survival group; 4F) Intratumoral, high survival group. Magnification level: 8.11x; scale bar can be found at bottom-left of each image.

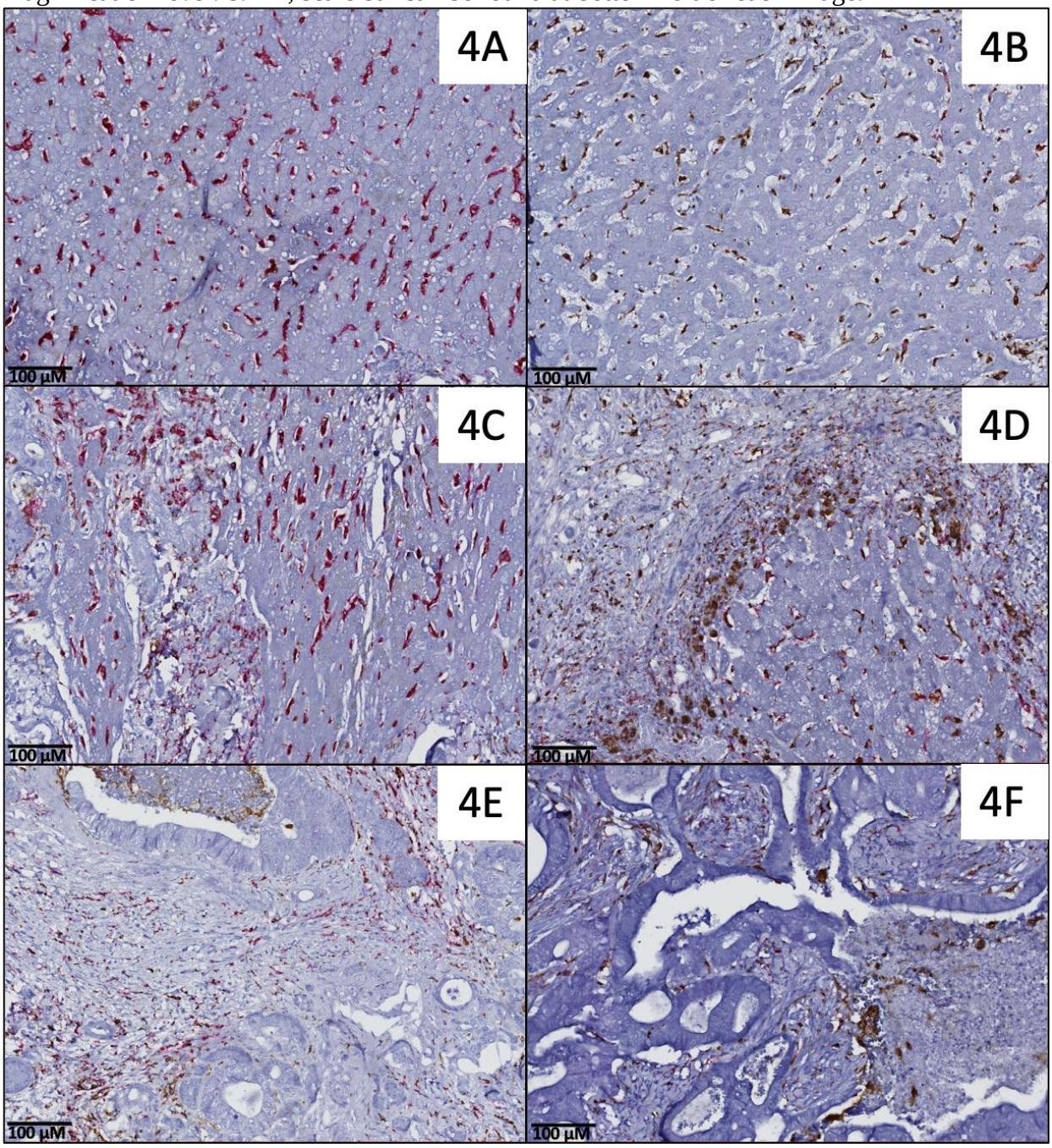


Figure 5. Immunohistochemistry Staining for MGMT.

Intratumoral MGMT staining (brown) of a high-risk patient (5A) and low-risk patient (5B). Magnification level: 2.5x (5A) and 5x (5B); scale bars can be found at the bottom-left of each slide.

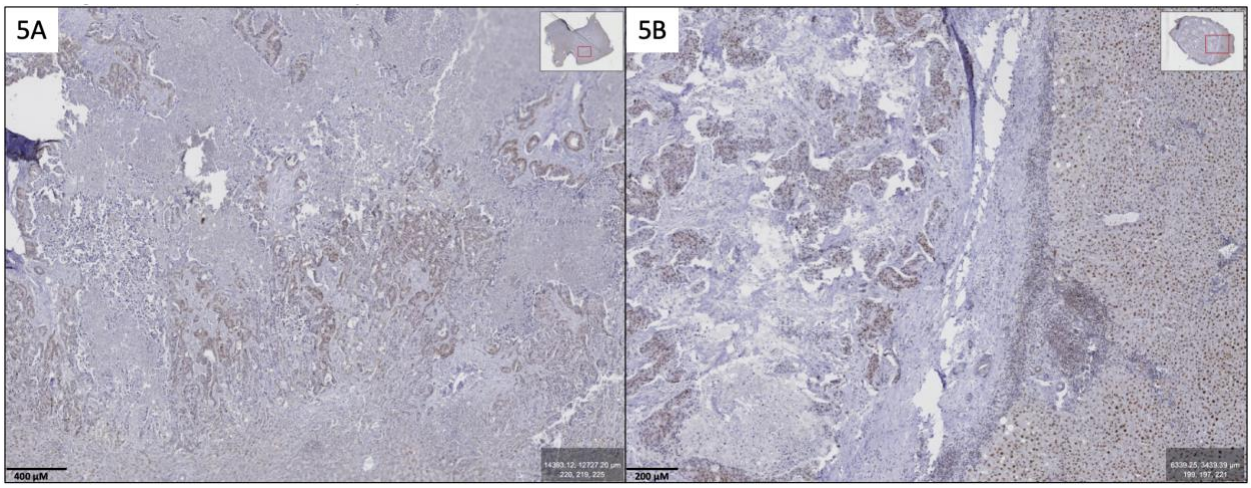
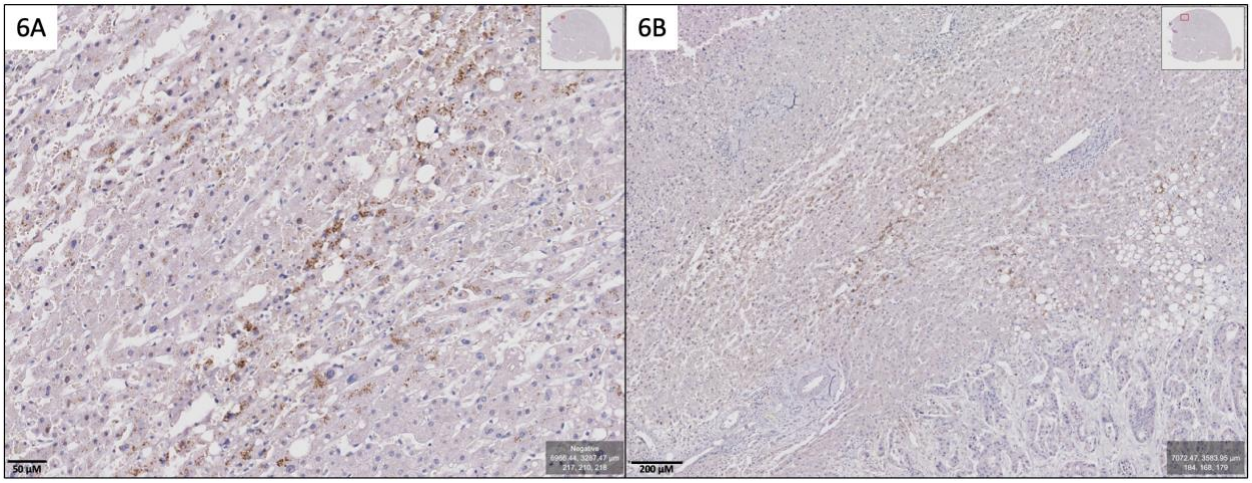


Figure 6. Immunohistochemistry Staining for LILRB4.

Peri-tumoral Kupffer cell staining for LILRB4 expression (brown) of a sample from a high-risk patient at different magnification levels to demonstrate granular cellular expression within peri-tumoral Kupffer cells (6A) and, at lower magnification, the spatial distribution of these cells with respect to the tumor (6B). Scale: 15x (6A) and 3.5x (6B). Scale bars can be found at the bottom-left of each slide.



Tables

Table 1. Patient Demographics

	Low-Risk N=21		High-Risk N=9		Total N=30	
	N	**(%)**	**N**	**(%)**	**N**	**(%)**
Age (median, IQR)	50	(50-70)	59	(51-72)	58	(50-71)
Gender						
Female	6	(28.6)	2	(22.2)	8	(26.7)
Male	15	(71.4)	7	(77.8)	22	(73.3)
BRAF						
Wild-type	3	(75)	1	(100)	4	(80)
Mutated	1	(25)	0	(0)	1	(20)
MSI						
Stable	9	(90)	4	(100)	13	(92.9)
High	1	(10)	0	(0)	1	(7.1)
KRAS						
Wild-type	6	(54.5)	4	(50)	10	(52.6)
Mutated	5	(45.5)	4	(50)	9	(47.4)
Primary Tumor Side						
Left	13	(61.9)	6	(66.7)	19	(63.3)
Right	8	(38.1)	3	(33.3)	11	(36.7)
Overall Survival (months)	49		15		27	
Follow-up (months)	49				27	

*BRAF status was missing for 25 (83%) patients. MSI status was missing for 16 (53%) patients. KRAS status was missing for 11 (37%) patients. There were no missing data for age, gender, primary tumor side, survival, or follow-up.

Table 2. Aggregate NanoString Analysis Data

	Aggregate Log 2fold Change*	Adjusted p-value
CSF1 (Colony Stimulating factor 1)	1.57	0.028
MGMT (O^6-Methylguanine DNA Methyltransferase)	1.73	0.034
IL6R (Interleukin-6 Receptor)	2.27	0.034
LILRB4 (Leukocyte Immunoglobulin-Like Receptor 4)	1.58	0.032

Table 3. Patient Demographics Stratified by Genes of Interest

	CSF1		MGMT		IL6R		LILRB4	
	Below-median* N (%)	Above-median* N (%)	Below-median* N (%)	Above-median* N (%)	Below-median* N (%)	Above-median* N (%)	Below-median* N (%)	Above-median* N (%)
Age (Median, IQR)	61 (51-71)	57 (50-62)	56 (50-65)	60 (55-71)	55 (50-63)	61 (55-71)	58 (46-70)	58 (51-71)
Gender								
Female	4 (30.8)	4 (23.5)	5 (29.4)	3 (23.1)	5 (31.3)	3 (21.4)	4 (40)	4 (20)
Male	9 (69.2)	13 (76.5)	12 (70.6)	10 (76.9)	11 (68.8)	11 (78.6)	6 (60)	16 (80)
Primary Tumor Side								
Left	6 (46.2)	13 (76.5)	10 (58.8)	9 (69.2)	9 (56.3)	10 (71.4)	6 (60)	13 (65)
Right	7 (53.8)	4 (23.5)	7 (41.2)	4 (30.8)	7 (43.8)	4 (28.6)	4 (40)	7 (35)

*Below- and above-median refer to relative gene expression based on NanoString analysis.

Table 4. Multivariable Cox Regression: Association between CSF1 Expression & Overall Survival

	Univariate		Multivariable	
	HR (95% CI)	P-value	HR (95% CI)	P-value
CSF1 Expression	**8.61 (1.06, 69.73)**	**0.044**	**12.29 (1.31, 115)**	**0.028**
Male Gender	1.29 (0.27, 6.19)	0.755	1.11 (0.22, 5.5)	0.901
Age	1 (0.94, 1.06)	0.985	1.01 (0.94, 1.07)	0.881
Right Side	0.81 (0.20, 3.24)	0.766	2.06 (0.44, 9.57)	0.358

Table 5. Multivariable Cox Regression: Association between MGMT Expression & Overall Survival

	Univariate		Multivariable	
	HR (95% CI)	P-value	HR (95% CI)	P-value
MGMT Expression	**4.78 (0.99, 22.99)**	**0.051**	**5.58 (1.03, 30.21)**	**0.046**
Male Gender	1.29 (0.27, 6.19)	0.755	0.95 (0.17, 5.16)	0.947
Age	1 (0.94, 1.06)	0.985	0.98 (0.91, 1.05)	0.535
Right Side	0.81 (0.20, 3.24)	0.766	1.18 (0.26, 5.34)	0.831

Table 6. Multivariable Cox Regression: Association between IL6R Expression & Overall Survival

	Univariate		Multivariable	
	HR (95% CI)	**P-value**	**HR (95% CI)**	**P-value**
IL6R Expression	**10.77 (1.35, 86.12)**	**0.025**	**14.45 (1.57, 133)**	**0.018**
Male Gender	1.29 (0.27, 6.19)	0.755	0.77 (0.13, 4.65)	0.775
Age	1 (0.94, 1.06)	0.985	0.97 (0.9, 1.04)	0.351
Right Side	0.81 (0.20, 3.24)	0.766	1.34 (0.29, 6.13)	0.707

Table 7. Multivariable Cox Regression: Association between LILRB4 Expression & Overall Survival

	Univariate		Multivariable	
	HR (95% CI)	**P-value**	**HR (95% CI)**	**P-value**
LILRB4 Expression	**4.41 (0.55, 35.29)**	**0.162**	**4.62 (0.54, 39.48)**	**0.162**
Male Gender	1.29 (0.27, 6.19)	0.755	0.87 (0.17, 4.43)	0.864
Age	1 (0.94, 1.06)	0.985	1 (0.93, 1.07)	0.889
Right Side	0.81 (0.20, 3.24)	0.766	0.86 (0.21, 3.52)	0.831

Table 8. Immunohistochemical Analysis of Targeted Antibody Staining.

		Alive at last follow-up N=21			Poor Survivors N=9		
Antibody Target	**P-value***	**Median**	**Mean**	**S.D.**	**Median**	**Mean**	**S.D.**
CSF1 Peritumoral M2 count	0.062	9	10.14	7.261	12	12.11	3.18
CSF1 Tumor-liver interface M2 count	0.117	16	15.62	7.27	24	21.67	9.421
CSF1 Intratumoral M2 count	0.063	10	12.05	7.768	6	7.11	5.555
CSF1 Percentage M2/total macrophage	0.226	55%	60.80%	25.76%	90%	72.39%	36.50%
MGMT Intratumoral intensity	0.228	2	1.9	0.831	1	1.5	0.756
LILRB4 Peritumoral cell count	0.571	64.5	69.2	53.671	29	53.67	46.607

*Obtained via Mann-Whitney U Test. There were no missing data.

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