A Distinct Innate Immune Signature of Early Onset Colorectal Cancer

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ABSTRACT

Despite a decrease in the prevalence of colorectal cancer (CRC) over the last 40 y, the prevalence of CRC in people under 50 y old is increasing around the globe. Early onset (<50 y old) and late onset (≥65 y old) CRC appear to have differences in their clinicopathological and genetic features, but it is unclear if there are differences in the tumor microenvironment. We hypothesized that the immune microenvironment of early onset CRC is distinct from late onset CRC and promotes tumor progression. We used NanoString immune profiling to analyze mRNA expression of immune genes in formalin-fixed paraffin-embedded surgical specimens from patients with early (n = 40) and late onset (n = 39) CRC. We found three genes, SAA1, C7, and CFD, have increased expression in early onset CRC and distinct immune signatures based on the tumor location. After adjusting for clinicopathological features, increased expression of CFD and SAA1 were associated with worse progression-free survival, and increased expression of C7 was associated with worse overall survival. We also performed gain-of-function experiments with CFD and SAA1 in s.c. tumor models and found that CFD is associated with higher tumor volumes, impacted several immune genes, and impacted three genes in mice that were also found to be differentially expressed in early onset CRC (EGR1, PSMB9, and CXCL9). Our data demonstrate that the immune microenvironment, characterized by a distinct innate immune response signature in early onset CRC, is unique, location dependent, and might contribute to worse outcomes. ImmunoHorizons, 2021, 5: 489–499.

INTRODUCTION

Colorectal cancer (CRC) is the second leading cause of cancer-related death in the United States (1). Over the last 40 y, there has been an overall decrease in the prevalence of CRC attributed to current screening guidelines (1, 2). Despite this decrease, there has been an increase in the prevalence of CRC in young adults under the age of 50 over the same time period (2, 3). In particular, between 1980 and 2010, the prevalence of rectal cancer in people aged 20–39 has quadrupled, and it appears that the
overall increase in early onset CRC (≥50 y old) is largely driven by an increase in rectal cancer in young patients (4). Based on these trends, it is predicted that by the year 2030, the prevalence of colon cancer will increase by 90%, and rectal cancer will increase by 124% in people aged 20–34 (2). In addition, the rates of advanced disease manifested as localized, regional, and distant CRC have increased in patients 40–49 y old. This indicates that the increase in prevalence is real and does not represent a shift in the age of diagnosis because of earlier detection with screening (5).

The cause of the rise in early onset CRC is unclear, but it appears to be global (6–8). The proportion of CRC cases occurring in young patients has risen more in the highest income quartile, urban population, and Western states in the United States (3, 9). This rise in early onset CRC does not correlate with patterns of obesity or heavy alcohol use (9). Young patients with CRC are more likely to present with regional or metastatic disease, high grade, mucinous, and signet ring tumors (3, 8, 10–15). Early onset is more commonly located in the rectum and left colon compared with late onset CRC (6, 11, 14). Patients with early onset CRC appear to have similar or improved stage-specific and overall survival (6, 10–12, 16).

The majority of early onset CRC is sporadic with only 16% of early onset CRC cases having genetic mutations (17). About 10–21% of early onset CRCs are mismatch repair deficient with 6–8% having Lynch syndrome, and these cancers are usually right sided (17–19). In addition to distinct clinicopathological features, early onset CRC appears to have molecular differences to late onset CRC. Compared with late onset CRC, early onset CRC has lower rates of BRAF mutations and methylator phenotype (20, 21). Early onset microsatellite stable CRC has more mutations in TP53 and CTNNB1 compared with late onset microsatellite stable CRC (22). Several genes and key pathways in early onset CRC have been identified and include PEG10, the Wnt/β catenin, MAP kinase, growth factor signaling, PI3KT–AKT, and TNFRI pathways (20, 23–25).

The immune microenvironment in CRC is a key player in disease progression, therapy response, and overall survival (26–29). The immune composition of CRC is heterogeneous and plays an important role in prognosis and potential for response to immunomodulatory agents (30). A subset of CRCs exhibit microsatellite instability (MSI) that correlates with higher tumor mutational burden, neoantigen load, and therefore, a more robust anti-tumor response. MSI status has served as a biomarker of CRC that is amenable to immunotherapies. Other emerging studies have introduced an “immunoscore” that quantifies CD3, CD8, and CD45R0 lymphocyte populations in the tumor core and the invasive margin (30, 31). Patients with a high density of CD45R0 and CD8 cells had better outcomes. There are efforts underway to enhance this score by including other markers of immune function such as PD-1 (30, 31). Similarly, specific cell types and gene signatures that feed into the Th1 type immune responses, such as CD8, CD4, dendritic cells, IFN-γ, and granzyme B, have been associated with good prognosis. Conversely, other cell types such as myeloid-derived suppressor cells or tumor-associated macrophages have been associated with poor prognosis (30, 31). Although many of these studies have evaluated the immune contexture across the spectrum of CRC, to our knowledge, the role of the immune microenvironment in early onset CRC has not been well studied or compared with late onset disease. We set out to characterize the differences between the immune microenvironment in early onset (≥50 y old) and late onset (≥65 y old) CRC in a well-annotated cohort of patients at our tertiary cancer center.

MATERIALS AND METHODS

The Oregon CRC Registry was queried to find patients with CRC who underwent surgical resection between January 2008 and October 2019 and were either under 50 or over 65 y old at the time of their diagnosis. In addition, six patients who underwent sigmoid colectomy for diverticulitis were identified to serve as a control group. The electronic medical record was used to obtain patient demographics, neoadjuvant treatment, pathology, genetic mutational status, adjuvant treatment, and outcomes.

Formalin-fixed paraffin-embedded (FFPE) slides were reviewed by pathology and only used if at least 70% of the tissue was tumor. The FFPE blocks were sectioned into 5 μm–thick slides of tissue for use. H&E-stained slides were prepared for each sample to identify the tumor and were used as guides to sharply dissect the tumor from the unstained slides to be used for RNA extraction. RNA was isolated using the RNeasy FFPE Kit (QIAGEN). RNA concentration and purity was confirmed with the NanoDrop spectrophotometer (Thermo Fisher Scientific).

NanoString immune profiling was performed using the human PanCancer Immune Panel (catalog [Cat] no. 115000132) or mouse cancer immune panel and run on the NanoString nCounter SPRINT Profiler. We chose to use the NanoString platform because this platform had better tolerance for moderate RNA integrity scores typical of archival FFPE specimens. Then, 100 ng of RNA extracted from the FFPE tissue was used per the manufacturer’s instructions. The NanoString datasets have been deposited to the Gene Expression Omnibus (accession no. GSE175433) (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE175433).

Quantitative PCR analysis was performed to validate the expression of two genes identified as differentially expressed based on the NanoString analysis using TaqMan Gene Expression Assays (Applied Biosystems, Cat no. 4448892, SAA1 Hs00761940_m1, and CXCL3 Hs00171061_m1) and the Viia 7 quantitative RT-PCR (qRT-PCR) machine (Applied Biosystems). Two housekeeping genes, GAPDH and ACTB (Applied Biosystems, Cat no. 433182 [Hs02758991_g1 and Hs001060665_g1]), were used for all samples. Gene expression was reported as the change in the cycle threshold (−2ΔCt, normalized to housekeeping genes).
The rectal cancer tissue microarray was generated from FFPE blocks. GeoMx Spatial proteomics were performed on the tissue array using NanoString workflows and protocols with the immune core, immune cell phenotyping, and immune activation panels. Slides were stained with pan-cytokeratin and CD45 Abs and imaged, and regions of interest were chosen in a blinded fashion for capture of probes. A total of 48 regions of interest was selected from at least seven patients from each group. Data were processed and analyzed with help from the Oregon Health & Science University (OHSU) NanoString core facility per established NanoString protocols. Background-corrected, normalized counts were used for comparisons between the sample groups.

All animal experiments were approved by the OHSU Institutional Animal Care and Use Committee. CT26 tumor cells were obtained from American Type Culture Collection and grown in DMEM with 10% FBS. The 8–12-wk-old BALB/C mice of both sexes were purchased from The Jackson Laboratory and were injected s.c. with \(3 \times 10^5\) tumor cells in Matrigel (Becton Dickinson) per each flank. Tumor growth was measured with callipers, with the volume computed as \(1/2 \times \text{length} \times \text{width}^2\). Tumors were harvested once the average tumor volume was verified using time-dependent covariates. Comparisons, and tumor grade. The proportional hazards assumption was used to compare the expression of our genes of interest in the entire CRC cohort and at least one location-specific cohort (right colon, left colon, or rectum) were designated genes of interest. This short list of 10 genes was then used for further analysis. The overall survival curves of these two groups were stratified into the high and low groups based on the top quantile and bottom quantile of the expression of each individual gene. The overall survival curves of these two groups of patients were estimated by the Kaplan–Meier method with statistical significance calculated by the log-rank test.

Statistics
Early and late onset patient cohorts were compared using a two-tailed unpaired \(t\) test for continuous variables, and \(\chi^2\) test was used for categorical data. Raw NanoString immune gene expression counts were normalized to the best 10 housekeeping genes. The nSolver Advanced Analysis module was used to derive differential expression signatures according to NanoString recommendations. Adjusted \(p\) values were obtained with a Benjamini–Hochberg post hoc correction. The genes with an adjusted \(p\) value of less than 0.2 in the entire CRC cohort and at least one location-specific cohort (right colon, left colon, or rectum) were designated genes of interest. This short list of 10 genes was then used for further analysis. Hazard ratios were calculated using multivariable Cox proportional hazards, and all estimates were adjusted for age, metastasis at diagnosis, neo-adjuvant therapy, angiolymphatic invasion or perineural invasion, and tumor grade. The proportional hazards assumption was verified using time-dependent covariates. Comparisons between gene expression for quantitative PCR results was done with a two-tailed unpaired \(t\) test with Welch correction. Statistical analysis was performed using GraphPad Prism version 8.4.2 (GraphPad Software, San Diego, CA) and JMP 14.3 (SAS Institute, Cary, NC).

Public datasets
The GSE87211 rectal cancer gene expression microarray dataset was used to compare the expression of our genes of interest in rectal tumor specimens and matched adjacent mucosa (32). This dataset was downloaded and divided into early (<50 y old) and late onset (>65 y old) groups. Tumor and mucosa specimens were compared between groups using a two-tailed unpaired \(t\) test with Welch corrections.

To further examine the correlation of the upregulated genes with the clinical survival outcomes in the independent and large datasets, we investigated their expressions in bulk RNA-sequencing data from The Cancer Genome Atlas. The processed gene expression data (fragments per kb per million fragments) as well as clinical data for primary colon adenocarcinoma (COAD) solid tumors were downloaded using the Bioconductor TCGAbiolinks package (version 2.14.0). Patients with overall survival less than 10 d were excluded. The remaining COAD tumor samples were stratified into the high and low groups based on the top quantile and bottom quantile of the expression of each individual gene. The overall survival curves of these two groups of patients were estimated by the Kaplan–Meier method with statistical significance calculated by the log-rank test.

Study approval
This study was approved by the OHSU Institutional Review Board (study no. 00021278). Only patients who had undergone informed consent to be included in the Oregon CRC Registry were included in this study.

RESULTS
Our institutional cohort consisted of 40 early onset and 39 late onset CRC patients. A higher percentage of the early onset CRC group had metastatic disease and therefore underwent more neo-adjuvant and adjuvant treatments (Supplemental Fig. 1A). Additionally, our early onset CRC group had less MSI compared with the late onset group (Supplemental Fig. 1B). We performed gene expression profiling for 770 immune response genes using the NanoString Immune Panel from RNA extracted from FFPE tissues. We found three immune genes significantly (adjusted \(p < 0.1\)) upregulated in early onset CRC (SAA1, C7, and CFD) and seven genes upregulated in late onset CRC (CXCL3, CCL18, IL-8, DMBT1, CEACAM6, CXCL5, and CXCL10) (Fig. 1A–D). Right colon, left colon, and rectal cancer have differentially expressed immune genes between early and late onset groups (Fig. 1B–D). The genes with an adjusted \(p\) value of less than 0.2 in the entire CRC cohort and at least one location-specific cohort (right colon, left colon, or rectum) were designated genes of interest (SAA1, C7, CFD, CXCL12, DMBT1, CEACAM6, CCL18, IL-8, CXCL3, and CXCL10). These genes were used for further analysis. The increased expression of SAA1, C7, and CFD in early onset CRC is largely driven by an increase in rectal cancer, although C7 was also increased in early onset right colon cancers. We identified 10 genes of interest that were differentially expressed between early and late onset CRC in the whole cohort and at least one location-specific group. After adjusting for age, metastatic disease, angiolymphatic...
FIGURE 1. Immune microenvironment differs with age in CRC and may contribute to survival.
Volcano plots depict log2 fold change in gene expression in primary tumors versus adjusted p values of early versus late onset CRC patients. Gene expression was profiled from FFPE sections using a NanoString immune profiling panel. (A) Early (n = 40) versus late onset (n = 39) in all CRC specimens. (B) Right colon cancer specimens only (early n = 10, late n = 14). (C) Left colon cancer specimens only (early n = 19, late n = 10). (D) Rectal cancer specimens only (early n = 11, late n = 15). Hazard ratios for (E) progression and (F) death were calculated using multivariable Cox proportional hazards and are adjusted for age, metastasis at diagnosis, angiolymphatic invasion, perineural invasion, neoadjuvant therapy, and tumor grade. Hazard ratios are per half of an SD from the mean of gene expression.

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invasion, perineural invasion, tumor grade, and neoadjuvant treatment, we found that increased expression of SAA1 and CFD is associated with worse progression-free survival (Fig. 1E), and increased expression of C7 is associated with worse overall survival (Fig. 1F). We found several associations between the expression of our genes of interest and the clinical characteristics, pathological features, and outcomes of our patient population. Increased expression of C7 is associated with neoadjuvant treatment, right colon location, metastatic disease, and angiolymphatic and perineural invasion (Supplemental Fig. 1C). Elevated CFD is only associated with neoadjuvant treatment, and SAA1 does not appear to be associated with any clinical or pathological features (Supplemental Fig. 1C). High expression of C7 and CFD was also associated with worse overall survival in The Cancer Genome Atlas COAD cancer cohort (Supplemental Fig. 2). The combination of SAA1, C7, and CFD as an immune signature may have a role in predicting poor progression-free and overall survival.

We validated the increased expression of CXCL3 in late onset CRC and SAA1, C7, and CFD in early onset rectal cancer using qRT-PCR assays from the same patient samples (Fig. 2A–D). We also found that SAA1 gene expression was higher in a group of liver metastases, but the significance of this finding is unclear because we did not have access to nontumor liver samples within our CRC registry to compare. Additionally, SAA1 expression was lower in a group of nontumor samples compared with early onset rectal cancer. We analyzed a publicly available dataset (GSE87211) of gene expression microarray profiles from rectal cancer (n = 105) and matched adjacent mucosa specimens (n = 84) to further validate our findings (32). In this rectal cancer dataset, we found a difference in CXCL3 expression between tumor and mucosa in both groups, but no difference in gene expression between early and late onset rectal tumor specimens (Fig. 2E). This is congruent with our findings because CXCL3 is not differentially expressed in rectal cancer in our cohort. We found that SAA1 levels were increased in the early onset tumors compared with late onset tumors, but there was no difference in expression between age groups in the adjacent mucosa (Fig. 2F). This suggests that the upregulation of this gene may not simply be a product of immune aging but more specifically because of the tumor immune microenvironment in early onset CRC.

We found that elements of complement (C7 and CFD) and SAA1 have increased expression in early onset CRC and are associated with worse outcomes. Analysis of gene set enrichment signatures from our profiles indicate complement and B cell function is the most enriched pathway in early onset CRC (data not shown). C7 and CFD play a role in the terminal and alternative pathways of complement, but components of complement also have a noncanonical role that modulates the immune system and plays a role in the tumor immune microenvironment (33).

Although our RNA-based profiling efforts capture the molecular signatures, it is challenging to infer any cellular composition changes in the immune contexture of these tumors. To address this, we performed a spatial proteomics assay on CD45-rich regions from a tissue microarray using the NanoString GeoMx Digital Spatial profiling platform. In an exploratory array with 10 duplicate cores each from our early onset rectal cohort, late onset rectal cohort, and noncancer tissues, we observed differences in expression levels of eight proteins. Pan-cytokeratin, CD163, β2-microglobulin, FOXP3, CD40, and CD14 were all higher in the late onset cohort, whereas CD54 and HLA-DR were expressed more in the early onset cohort (Supplemental Fig. 3). Given our limited sample size, we propose that future studies using this platform might also provide cellular differences in immune microenvironment between early and late onset cancers from archival FFPE tissues.

Because our two cohorts were not matched for sex, we compared our NanoString database on sex and found that the only gene differentially expressed by sex was USP9Y, which is found on the Y chromosome (Supplemental Fig. 4A). Although our gene signature as well as those from other cancer datasets indicate that there are distinct age-specific changes in these genes, it is possible that some of these changes are due to natural aging of the immune system. We observed that three genes from our signature (CXCL3, CXCL10, and CFD) were also profiled from normal PBMCs as part of a study on sexual dimorphism in human immune aging and showed little to no association with age or sex (Supplemental Fig. 4B–D) (34).

To evaluate the functional significance of these pathways in preclinical models, we first compared the expression of SAA1 and CFD in multiple murine and human CRC cell lines. The murine cell lines showed no detectable expression of these genes, making it feasible to perform gain-of-function studies in an immune-competent host (Fig. 3A). We transfected CT26 murine COAD cells with SAA1 and CFD to generate stable cell lines (Fig. 3B) and implanted these cells as s.c. flank tumors in syngeneic BALB/C mice. We confirmed that the tumors at end points still retained the expression of SAA1 and CFD compared with controls (Fig. 3C). We found that tumors with high SAA1 expression had similar growth rates as the tumors with low SAA1 expression, but CFD-expressing tumors had higher volumes compared with controls (Fig. 3D, 3E).

To understand whether ectopic expression of SAA1 or CFD altered the tumor immune microenvironment in mice, we used the same NanoString platform to evaluate the immune gene expression signatures. Interestingly, SAA1 gain of function impacted only a few genes in contrast to several genes that were differentially regulated in CFD-expressing tumors (Fig. 4A, 4B). We saw significant changes in specific pathways, including Ag presentation and IFN response. Importantly, we identified four genes that are commonly regulated either by early age of onset in human CRC or CFD expression in our preclinical tumor model. Three of these were concordant between the human and mouse datasets: a transcription factor EGRI, a proteasome component PSMB9, and the chemokine CXCL9 (Fig. 4C). Taken together, our results identify differences in immune microenvironments of early onset CRC versus
FIGURE 2. Validation of NanoString data with independent qRT-PCR assays and independent datasets. (A) CXCL3, (B) SAA1, (C) C7, and (D) CFD expression of individual tumor RNA samples from FFPE slides measured using TaqMan probes in a qRT-PCR assay. Change in gene expression (2^{-ΔΔCt}) are depicted as fold change over two housekeeping genes. Violin plots depict (E) CXCL3 and (F) SAA1 gene expression from a publicly available rectal cancer dataset GSE87211. Values are log gene expression measured via microarrays in tumors and matched adjacent mucosal samples. The p values are from a two-tailed unpaired t test with Welch correction or a Mann–Whitney U test (C and D). Each dot represents an individual patient sample.
late onset CRC and suggest that one of these genes, CFD, might have a contributing effect on the immune microenvironment.

**DISCUSSION**

In this work, we describe immune genes, in particular the complement genes C7 and CFD and the inflammatory gene SAA1, associated with early onset CRC and present some evidence for how these genes could shape a distinct immune microenvironment. Complement can play a protumorigenic or anti-tumor effect in the tumor immune microenvironment, and it is unclear what role C7 and CFD play in CRC (33). Complement activation in intestinal epithelial cells has been shown to be associated with inflammation; therefore, the increase in C7 and CFD in early onset CRC may be related to an underlying inflammatory process (35). Similar to complement, SAA1 has also been shown to be overexpressed in patients with active...
FIGURE 4. Ectopic expression of CFD alters the tumor immune microenvironment in mice.
(A and B) Volcano plots depicting differentially expressed immune genes (NanoString mouse tumor immune panel) in the mouse tumors from Fig. 3 (n = 6 tumors per group). (C) Venn diagram showing common genes between the human early versus late dataset and mouse CFD gain-of-function studies. Only significant genes at the indicated thresholds are depicted. Three of four genes have concordant expression between early onset human tumor samples and the mouse CFD-expressing tumors.
inflammatory bowel disease (36, 37). SAA1 is an acute phase reactant produced by the liver but is also present in multiple cancers and can be used as a biomarker for disease burden (34, 38, 39). SAA1 has been shown to direct pathogenic Th17 cell differentiation (36, 37). It is unclear if the increased expression of CFD, C7, and SAA1 in early onset CRC is the result of underlying immune dysregulation leading to a tumor-permissive microenvironment or an inappropriate immunological response to malignancy.

Our gain-of-function studies in the mouse tumor model and comparisons with the early onset CRC dataset yielded four candidates that are influenced causally by CFD and correlate with the early onset disease. Of these four genes, three were concordant (i.e., in the same direction of regulation in both human and mouse studies). EGR1 has been shown to be induced by various stimuli, including hypoxia, radiation, chemotherapy, and hyperglycemia, and shown to have both inhibitory and promoting effects on tumors (40). Although PSMB9 has been found to be expressed in CRC, it does not appear to be clinically relevant (41, 42). CXCL9, in contrast, has been shown to be associated with recruitment of CD8+ T cells, Th1 cells, and NK cells to the tumor microenvironment and is associated with improved patient outcomes in CRC (43–45). CXCL9 also functions as an inhibitor of angiogenesis (44). CXCL9 expression is decreased in both CFD-expressing tumors and in early onset CRC, which aligns with our finding of worse outcomes with increased CFD expression. These observations highlight the local microenvironment effects and suggest immune gene signatures can have considerable influence on the tumor microenvironment. Although other studies have shown differences across age in mouse tumors, we believe the immune signatures we have observed in our patient populations have been shaped by interactions with the host microbiome that are not recapitulated completely in preclinical models.

One of the hallmarks of immune aging is the phenomenon of “inflammaging,” which is characterized by low levels of persistent inflammation both systemically and within the tissue microenvironment. Although the majority of research on immune system aging has been on adaptive immunity, the phenomenon of inflammaging is now largely attributed to innate immune system dysfunction (16). Immune aging appears to be driven by a complex interaction between genetic and environmental factors and appears to exist along a trajectory toward an older immune system with people advancing across the trajectory at different speeds (46). Our findings paradoxically suggest that early onset CRC is associated with an inflammatory phenotype, which is what would be expected in an older population. It may be that early onset CRC is associated with expedited inflammaging that leads to a tumor-permissive microenvironment. We found that SAA1, an acute phase protein, is elevated in early onset CRC, which is akin to the elevation of another acute phase protein, C-reactive protein, in inflammaging. Studies have shown that older individuals have reduced microbiota diversity, decreased density of beneficial bacteria, and weakened integrity of the intestinal barrier (47). It is hypothesized that these factors allow leakage of proinflammatory microbial products into the systemic circulation and peripheral tissues, which leads to the chronic inflammation associated with aging (47, 48). The microbiome and dysregulation of the intestinal barrier may play a role in the cause of early onset CRC.

Our findings point to the innate immune system, specifically complement, as a component of the immune microenvironment that distinguishes early onset CRC. Although complement has been shown to have both protumor and anti-tumor effects, therapeutics targeting complement are currently being investigated, including small molecule inhibitors of CFD. The majority of studies on complement inhibition in the treatment of cancer have been done in mice. Both C3a and C5a receptors may be potential targets for cancer immunotherapy, and the inhibition of C5a in mice has been shown to decrease metastasis in colon cancer (33, 49, 50). Additionally, inhibition of the C5a receptor in mice reduces cancer-promoting inflammation that results from consumption of a high-fat diet (51). Of note, there is currently a phase I clinical trial for a C5aRI mAb in combination with durvalumab in advanced solid tumors (33). Our study suggests that early onset CRC may benefit from the addition of a complement inhibitor in addition to the standard use of chemotherapy and immunotherapy. Another possible therapeutic route in early onset CRC is manipulation of the microbiome. Mouse studies have shown that the microbiome appears to play a role in the effectiveness of chemotherapy and immunotherapy, including immune checkpoint inhibitors (52, 53). Manipulation of the microbiome by diet alterations, probiotics, antibiotics, or fecal transplant are being investigated as potential avenues to improve responsiveness to cancer treatments (52, 53).

Our findings suggest an inflammatory tumor-promoting phenotype in early onset CRC, and these treatments may be particularly useful in combating this immune microenvironment. A limitation of our study is its small clinical cohort of patient samples with early or late onset CRC, but we attempted to overcome this limitation by comparing our findings to publicly available gene expression datasets. Although our findings focused on C7, CFD, and SAA1, this picture does not adequately capture the complexity of the immune microenvironment, and we suspect that there may be other components that play a role in the pathogenesis and immune response to early onset CRC. Additionally, the role of neoadjuvant treatment is a potential confounder in our patient population. Our early onset CRC cohort had more neoadjuvant treatment, and C7 and CFD were found to be associated with both early onset CRC and neoadjuvant treatment. Neoadjuvant chemoradiation may play a role given that C7, CFD, and SAA1 were increased in early onset rectal cancer, and this population was most likely to receive neoadjuvant chemoradiation. The role of chemoradiation and its effect on C7, CFD, and SAA1 in early onset CRC requires further investigation.

Our data demonstrate that the immune microenvironment is different between early and late onset CRC, and these differences do not appear to solely be the result of age-related
immune changes. Additionally, differences in the immune microenvironment between early and late onset CRC are location specific. C7, CFD, and SAA1 are increased in early onset CRC and are associated with worse outcomes. These immune genes are associated with intestinal inflammation, suggesting that the immune microenvironment in early onset CRC may be proinflammatory and tumor permissive as a result of immune dysregulation.

**DISCLOSURES**

The authors have no financial conflicts of interest.

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**REFERENCES**

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**REFERENCES**


Clinical Characteristics of OHSU Patient Cohort

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<th>Late Onset</th>
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<td>22 (55%)</td>
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Pathological Characteristics of OHSU Patient Cohort

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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>2 (5%)</td>
<td>4 (10.3%)</td>
<td>0.7400</td>
</tr>
<tr>
<td>T2</td>
<td>5 (12.5%)</td>
<td>5 (12.8%)</td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>24 (60%)</td>
<td>24 (61.5%)</td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>9 (22.5%)</td>
<td>6 (15.4%)</td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>18 (45%)</td>
<td>21 (53.8%)</td>
<td>0.1550</td>
</tr>
<tr>
<td>N1</td>
<td>14 (35%)</td>
<td>11 (28.3%)</td>
<td></td>
</tr>
<tr>
<td>N2</td>
<td>8 (20%)</td>
<td>2 (5.1%)</td>
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<tr>
<td>Response to treatment</td>
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<tr>
<td>None</td>
<td>6 (15%)</td>
<td>2 (5.1%)</td>
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</tr>
<tr>
<td>Minimal</td>
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<tr>
<td>Partial</td>
<td>8 (20%)</td>
<td>5 (12.8%)</td>
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<tr>
<td>Complete</td>
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<td>0</td>
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<tr>
<td>Positive margins</td>
<td>3 (7.5%)</td>
<td>3 (7.7%)</td>
<td>0.9742</td>
</tr>
<tr>
<td>High grade tumor</td>
<td>4 (10%)</td>
<td>5 (12.8%)</td>
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<tr>
<td>Angiolympatic Invasion</td>
<td>19 (47.5%)</td>
<td>18 (46.2%)</td>
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</tr>
<tr>
<td>Perineural Invasion</td>
<td>13 (32.5%)</td>
<td>6 (15.4%)</td>
<td>0.0752</td>
</tr>
<tr>
<td>Perforation</td>
<td>2 (5%)</td>
<td>1 (2.6%)</td>
<td>0.5712</td>
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<td>Geneticsb</td>
<td></td>
<td></td>
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<tr>
<td>Kras mutant</td>
<td>9/20 (45%)</td>
<td>3/12 (25%)</td>
<td>0.2579</td>
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<tr>
<td>Braf mutant</td>
<td>2/20 (10%)</td>
<td>3/12 (25%)</td>
<td>0.2579</td>
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<tr>
<td>Microsatellite instability</td>
<td>2/36 (5.6%)</td>
<td>6/27 (22.2%)</td>
<td>0.0493</td>
</tr>
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</table>

4 late onset patients underwent transanal excisions so no N stage was available; Not all patients underwent genetic testing (denominator is number of patients who were tested).

Supplementary Figure 1: Clinical and pathological characteristics of institutional cohort.
Descriptive statistics of (A) clinical and (B) pathological characteristics of early (N=40) and late (N=39) onset colorectal cancer cohorts. Early and late onset patient cohorts were compared using a two-tailed unpaired T-test for continuous variables and chi-squared test was used for categorical data. C) Associations between immune gene expression and neoadjuvant treatment, tumor grade, tumor location, metastatic disease, angiolympatic invasion (AVI), and perineural invasion (PNI). Other clinicopathological features were not significantly associated with gene expression.
Supplemental Figure 2: Kaplan-Meier curves of colorectal adenocarcinoma patients from The Cancer Genome Atlas (TCGA). Overall survival of TCGA colorectal adenocarcinoma patients in high (brown color line) and low (blue color line) expression groups of (A) C7 and (B) CFD. p-value was calculated by log-rank test. NA indicates that the median overall survival time is not reached. OS: overall survival.
Supplemental Figure 3: Multiplex protein analysis of OHSU rectal cancer TMA. A tissue microarray was generated from samples analyzed for immune signature genes in Figure 1 (n=10 samples per group) and subjected to multiplex immunostaining using the Nanostring DSP platform. A) Imaging of cytokeratin (green) and CD45 to visualize regions of interest for analysis. Nanostring probes were cleaved and captured from regions as indicated and probe signals were counted after hybridization. B) Regions from multiple cores across same tumor were combined and averaged. Normalized counts are shown for the early and late onset groups. * indicate significant differences (Mann-Whitney U-test, p<0.2).
Supplemental Figure 4: The effect of sex and age on our immune gene signature. A) Volcano plot of immune gene expression in our patient population comparing female (N=42) and male (N=43) patients. USP9Y is expressed on the Y chromosome. Analysis of gene expression from RNA seq dataset (Marquez et al. Nat Communications 2020) of normal human aging associations of (B) CXCL3, (C) CXCL10, and (D) CFD from peripheral blood mononuclear cells in 39 healthy donors. Of our ten genes of interest, only CXCL3, CXCL10, and CFD were available in the data and analysis obtained from Marquez, E.J., Chung, C., Marches, R. et al. Sexual-dimorphism in human immune system aging. Nat Comm 11, 751 (2020).