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Research Article

Characteristics of Immune Cell Infiltration Landscape in Colorectal Cancer to Aid in Immunotherapy

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Abstract

Background: The tumor microenvironment (TME) is a complex environment composed of a variety of stromal cells and immune cells that infiltrate the tumor space. Recent clinical work has shown intratumoral immune cell infiltration (ICI) to be closely related to colorectal cancer (CRC) patient survival, yet the specific landscape of infiltrating immune cells associated with this cancer type remains to be clarified.

Methods: We utilized two computational algorithms to evaluate the ICI status of 712 CRC patients, stratifying these patients into two ICI status-based patterns and assigning ICI scores through the use of principal component analyses.

Results: We found that the overall survival (OS) of patients with higher ICI scores was significantly longer than that of patients with low ICI scores. When ICI scores were combined with the results of tumor mutational burden (TMB) analyses, we determined that CRC patients with both high ICI scores and low TMB exhibited the best survival outcomes. High expression of MARCO in those patients with low ICI scores was correlated with reduced natural killer (NK) and effector T cell infiltration and with increased regulatory T cell infiltration, suggesting these factors may be linked to poor patient prognosis.

Conclusions: These results suggest ICI scores to be a valuable biomarker for the prognostic evaluation of CRC patients. Future efforts to analyze the ICI patterns of larger patient sample cohorts will help to extend these analyses, offering new insights into the role of the TME in cancer progression while highlighting novel immunotherapeutic approaches to treating this cancer type.

Keywords: immune cell infiltration, the tumor microenvironment, prognostic biomarker, colorectal cancer, score.

List of Abbreviations: TME: The Tumor Microenvironment CRC: Colorectal Cancer; ICI: Immune Cell Infiltration; NK Cells: Natural Killer cells TMB: Tumor Mutation Burden; TAMs: Tumor-Associated Macrophages TLSs: Tumor-Infiltrating Lymphocytes; TCGA: The Genomic Data Commons Data Portal; GEO: Gene Expression Omnibus PCA: Principal Component Analysis DEGs: Differentially Expressed Genes GSEA: Gene Set Enrichment Analysis OS: Overall Survival; Tregs: T Cells Regulatory

Introduction

Colorectal cancer (CRC) is among the most common

and deadliest cancers in developed nations, with an estimated 400,000 CRC diagnoses and 212,000 deaths globally each year [1]. Immunotherapeutic treatments serve to augment the ability of natural host defense mechanisms to recognize and remove tumor cells, and such regimens have proven effective in synergistically enhancing the survival of patients with a range of tumor types [2-4]. However, immunotherapies generally only benefit a small percentage of patients, and there is thus a clear need to better understand which therapeutic markers can be analyzed to gauge CRC patient responses to immunotherapeutic treatment [2-5].

The tumor microenvironment (TME) is a complex setting that influences cancer development and progression [6]. The TME contains a diverse array of stromal cells, lymphatic structures, and blood vessels, all of which can influence and be influenced by the oncogenic mutations driving the onset and evolution of a given cancer. The infiltration of various immune cell types into the TME has been shown to be an effective prognostic biomarker for tumor invasion status, offering insights into cancer grade, stage, and metastasis [7,8]. Tumor-associated macrophages (TAMs), for example, can produce cytokines including interleukin-10 (IL-10) and transforming growth factor-B (TGF-B) that can suppress immune cell activation, thereby promoting tumor proliferation and contributing to poorer patient prognosis [9-11]. In contrast, higher levels of tumor-infiltrating lymphocytes (TILs) including both CD4+ and CD8+ T cells are related to improved patient survival and immunotherapeutic responsiveness [12].

Immune checkpoint blockade therapies rely on the activation of extant TILs, enhancing their ability to recognize tumor cells and to thereby engage appropriate immunotherapeutic responses [13,14]. Merely identifying these TILs, however, is not sufficient to reliably characterize the complexities of the TME, as many patients with high TIL levels nonetheless exhibit resistance to immunotherapy treatment [15,16]. Such effects may be in part attributable to TAM-derived immunosuppressive cytokine production, or to stromal infiltration of the tumor, as this can interfere with TIL accumulation [14,15]. These prior results suggest that dynamic interactions between cell types are more important in the TME than are any individual cell population from a prognostic perspective. Prior studies have not performed a high-level analysis of the prognostic relevance of different immune cell infiltration (ICI) populations in CRC.

Herein, we employed the CIBERSORT and ESTIMATE algorithms to characterize CRC patient tumor gene expression profiles in an effort to comprehensively characterize intra-tumor immune cell landscape characteristics within these tumors [17,18]. We then stratified CRC patient tumors into two subtypes based upon the observed degree of ICI, and we established ICI scores that were successfully used to gauge patient prognosis and which may offer value for the design of novel immunotherapeutic regimens.

Materials and Methods

Dataset selection

All data used in the present study were derived from The Cancer Genome Atlas (TCGA) (https://portal.gdc.cancer.gov/) and Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih. gov/geo/) databases under the accession number GSE17538 (Figure 1A).

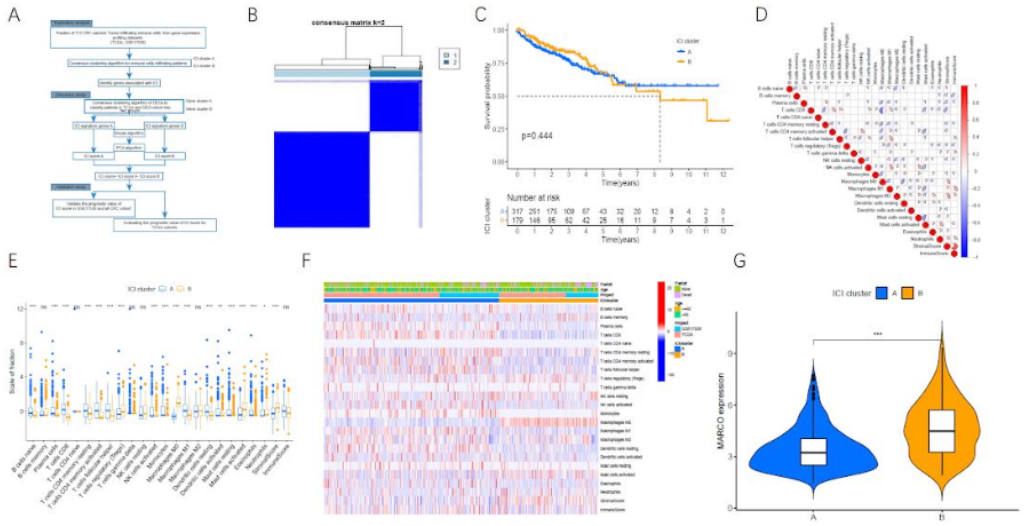

Figure 1: The TME-associated immune cell landscape of CRC

(A) An overview of CRC sample selection. (B) Immune cell infiltration (ICI)-based CRC sample matrices. (C) CRC patient OS for individuals with different ICI subtypes was assessed using Kaplan-Meier curves and log-rank tests ($p = 0.047$). (D) Tumor-infiltrating cell interactions. (E) Relative frequencies of different tumor-infiltrating immune cell types in the two ICI clusters (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; Wilcoxon test). (F) ICI populations in CRC cohorts were subjected to an unsupervised clustering analysis, with columns corresponding to samples and rows corresponding to tumor-infiltrating immune cells. (G) MARCO expression levels in different ICI clusters (Wilcoxon test, $p < 0.001$).

* Figure No. 1(B, D, F), 2(A, B, C, D), 3(A, D, E) were generated by R software (R-4.0.3-win), and URL link: https://github.com/tim-lebedkov/packages/releases/download/2020_10/r-4.0.3-R-4.0.3-win.exe

Tumor-Infiltrating Immune Cell Consensus Clustering

The CIBERSORT R package 21 was used to estimate tumor infiltration by different immune cell populations based upon 1000 permutations and LM22 markers, with both immune cell and matrix contents being evaluated to yield specific immune and matrix scores. CRC patient samples were then clustered according to sample-specific ICI patterns. A PAM-based approach relying upon Euclidean and Ward's linkages was utilized for unsupervised clustering with the ConsensuClusterPlus R package, and clustering was conducted 1000 times to ensure stable classification.

Identification of ICI-related gene expression patterns

ICI profiles were used to group CRC patients into different ICI clusters, after which differentially expressed genes (DEGs) between these clusters were compared with the following cutoff criteria using the limma R package: adjusted $p < 0.05$; absolute fold-change > 1.4.

Dimensional Reduction and ICI Score Calculation

An unsupervised clustering approach was initially used to classify patients according to DEG expression values, with DEGs that were positively and negatively correlated with key clustering-related features being respectively referred to as ICI gene features A and B. Dimensional reduction was then performed for these ICI-related genetic signatures using a Boruta algorithm, and principal component 1 in the resultant principal component analysis (PCA) was then extracted as a signature score. A method similar to that previously used to compute a gene expression grade index was then employed to calculate individual patient ICI scores as follows:

$$\text{ICI score} = \sum \text{PC1}_A - \sum \text{PC1}_B$$

Somatic Alteration Data Compilation

Mutational data corresponding to CRC patients in the TCGA database was downloaded and used to estimate tumor mutational burden (TMB). CRC driver genes were identified with the maftool R package, and somatic mutations therein were evaluated for those with high and low ICI scores. The top 20 most frequently mutated driver genes were then retained for further analysis.

Statistical Analyses

GraphPad Prism 7.0 and SPSS 21.0 (IBM, NY, USA) were used for all statistical testing. Data between groups were compared via the Wilcoxon test. Patient classification into two ICI subtypes was achieved with the X-tile software via an iterative approach

aimed at reducing computational batch effects. Kaplan-Meier plotter was employed to generate all survival curves, which were analyzed with log-rank tests. Chi-squared tests were employed to assess correlations between ICI score subgroups and somatic mutation frequencies, while correlations were assessed via Spearman's correlation analyses. A two-tailed $p < 0.05$ was the significance threshold for this study.

Results

Assessment of the CRC-related TME Immune Cell Infiltration Landscape

We began by employing the ESTIMATE and CIBERSORT algorithms to gauge the relative enrichment of different immune cell populations in samples of tumors from 712 CRC patients (Figure1A). Data were obtained from the TCGA and GEO databases (Accession number: GSE17538), and analyzed tumor cells exhibited matched immune cell infiltration (ICI) profiles. The ConsesusClusterPlus R package was then used to classify these CRC patients into distinct ICI-related subtypes via an unsupervised clustering approach (Figure 1B).

No differences in overall survival (OS) were evident between patients with the two independent ICI-related CRC subtypes identified in this study. ($p = 0.444$; Figure 1C). To better understand the biological basis for these prognostic relationships, we further assessed the immune cell makeup of the TME in these patient clusters (Clusters A and B). Patients in cluster A contain higher levels of naive B cells, M1 macrophages, memory CD4 T cells, CD8 T cells, NK cells, monocytes, and plasma cells. Patients in ICI cluster B contain higher levels of regulatory T cells (Tregs), M0 macrophages, and activated mast cells (Figure 1D-E). Although there was a significant difference in immune cell invasion between cluster A and cluster B, there was no significant difference in survival rate between the two groups, indicating that the microenvironment affecting CRC survival was complex, which was not only related to immune cell invasion, but also possibly related to other components of the immune microenvironment. Correlation coefficient heatmaps were also prepared to visualize the immune cell infiltration landscape of the TME in these CRC patient cohorts (Figure 1F).

We further assessed the relative expression of the key immune-related gene MARCO in these two ICI patient clusters, revealing significantly increased MARCO expression among patients in cluster B relative to those in cluster A. Differences between immune cell types and MARCO expression levels in these patient cohorts were then evaluated (Figure. 1G).

Immune Gene-Related Subtype Analyses

To better understand the biological basis for the ICI subtypes of CRC patients identified above, we conducted a differential transcriptomic analysis of these patient cohorts with the R limma package. Through unsupervised clustering of 34 identified DEGs, these patients were stratified into two genomic clusters (clusters A and B) (Figure 2A). In total, 23 of these DEGs were positively correlated with this gene cluster and were denoted as 'ICI gene signature A', whereas the remaining DEGs were referred to as 'ICI gene signature B'. The Boruta algorithm was then used to conduct a dimensionality reduction for these two ICI gene signatures in order to reduce computational noise associated with these analyses [19]. The clusterProfiler R package was then used to prepare a heatmap separating the transcriptomic profiles of these 34 DEGs across genomic clusters (Figure 2B) as described previously [20], with significantly enriched biological processes associated with these genes being shown in Figures 2C and 2D.

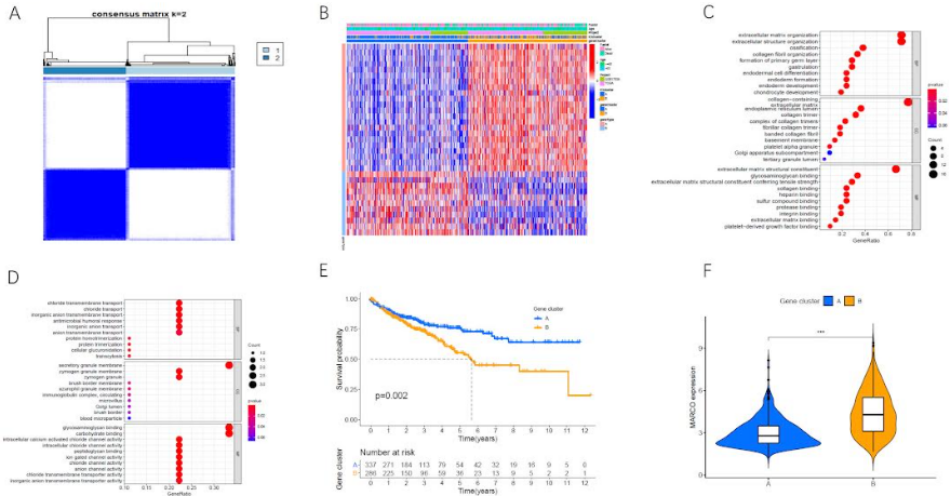

Figure 2: Immune Gene-Related Subtype Identification

(A) Consensus matrices for CRC samples grouped according to DEG profiles. (B) Common DEGs between the two ICI cluster groups were subjected to unsupervised clustering to yield clusters A and B. (C and D) Gene Ontology (GO) enrichment analyses of ICI-related signature genes in clusters A (C) and B (D). Numbers of genes associated with the indicated GO terms are shown on the x-axis. (E) Survival outcomes were compared between patient groups with a Kaplan-Meier curve (p=0.002; log-rank test). (F) MARCO expression levels in different ICI clustersWilcoxon test, $p < 0.001$).

The prognostic relevance of these ICI gene clusters was next evaluated using the Kaplan-Meier plotter tool, revealing that patients in cluster A had a better prognosis relative to patients in cluster B (p=0.002; Figure 2E). Significant differences in MARCO gene expression were also detected between these two genomic clusters ($p < 0.001$; Figure 2F), with the expression of this key immunological gene being significantly higher in samples from patients in cluster B relative to those from patients in cluster A.

Evaluating Correlations between ICI Scores and Somatic Variation

A PCA approach was next employed to compute aggregate ICI scores for the two ICI gene signatures detailed above in an effort to quantitatively evaluate the ICI landscape of CRC. The resultant scores (ISA and ISB for genes in signatures A and B, respectively) were then calculated for each patient by summing the relevant individual scores, yielding a prognostic signature which was defined as an ICI score. The patients in our study cohort were then separated into two groups based upon whether they had high or low ICI scores, with the cutoff value used to differentiate these two groups being established with the X-tile software. Patient distributions in these two gene clusters are shown in Figure 3A. We then evaluated immune activity in each patient group before assessing the prognostic relevance of our ICI scores, revealing that most immune checkpoint- and immune activity-related genes were significantly overexpressed in samples from the low ICI group with the exception of PIGR, IGLJ3, CLCA1, ITLN1, IGHM, PLA2G2A, CLCA4, ZG16, UGT2B17, REG4, and SPINK4 (Figure 3B).

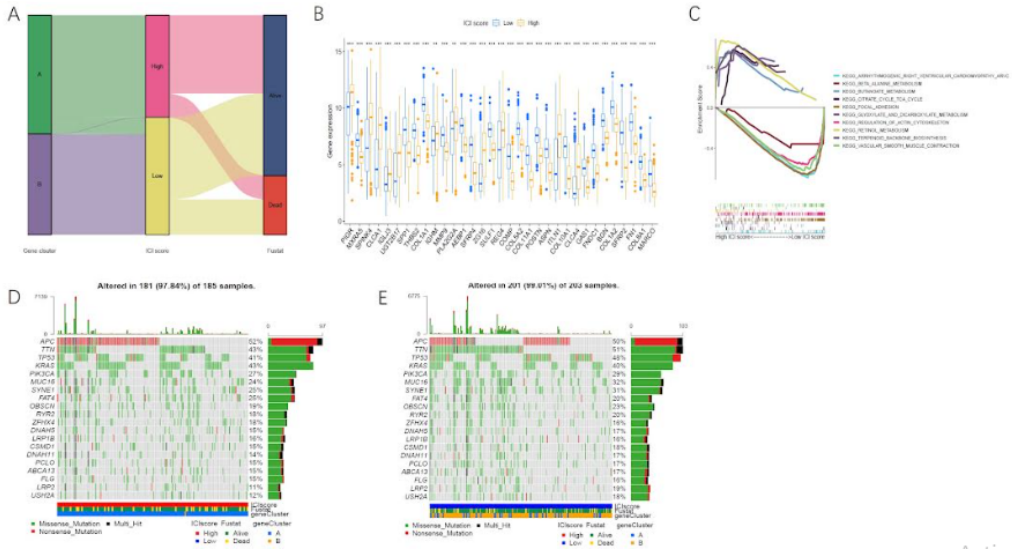

Figure 3: Correlations Between ICI Scores and Somatic Variants.

(A) ICI gene cluster distributions in groups with different ICI scores and survival outcomes are shown using an alluvial diagram. (B) Immune-checkpoint-related and immune activation-related genes present in low and high ICI score subgroups. (C) Enrichment plots demonstrating the enrichment of regulation- and vascular-related signaling pathways in the low ICI score subgroup, and butanoate- and retinol-related signaling pathways in the high ICI score subgroup. (D and E) High (red) and low (blue) ICI scores were used to construct oncoPrint, with columns corresponding to individual patients.

TAMs expressing MARCO can interfere with the activation of NK and T cells, suppressing their cytokine production, proliferation, and cytotoxicity, while simultaneously aiding Treg proliferation [21]. In line with such a model, we found that high MARCO expression in individuals with low ICI score subtypes was correlated with higher Treg infiltration and reduced NK and effector T cell levels, potentially explaining the poorer prognosis for these patients. A gene set enrichment analysis (GSEA) further showed the regulation and vascular signaling pathways to be significantly enriched among patients in the low ICI score group, while the butanoate and retinol signaling pathways were significantly enriched among those in the high ICI group (Figures 3C).

Many prior studies have shown that tumors bearing a high mutational burden are more likely to exhibit increased CD8+ T cell infiltration, aiding in their elimination. As such, TMB may predict a given cancer patient's responsiveness to immunotherapy treatment [22,23]. We therefore evaluated potential relationships between TMB and ICI scores in CRC patients, evaluating somatic variant distributions in CRC driver genes in the low and high ICI score patient subgroups using maftools [24]. We then retained the top 20 driver genes with the highest mutational frequency for further analysis (Figure 3D-3E).

Assessment of the Prognostic Value of ICI Scores

Finally, we conducted an in-depth analysis of the prognostic relevance of the ICI scores computed above using the Kaplan-Meier plotter tool. The OS of patients in the high ICI score group was significantly better than that of those in the low ICI score group (p=0.004; Figure 4A).

We then evaluated the synergistic value of ICI and TMB scores as predictors of CRC patient survival in a stratified survival analysis which found TMB status to be unrelated to ICI score-based prognostic analyses such that higher ICI scores were linked to better patient OS regardless of TMB status (High TMB + High ICI score (HH) vs. High TMB + Low ICI score (HL), Low TMB + high ICI score (LH) vs. Low TMB + Low ICI score (LL), p = 0.006; Figure 4B). Together these data suggested that ICI scores serve as prognostic biomarkers that are independent of TMB status. The mortality rate for patients with high ICI scores was also decreased relative to that of patients with low ICI scores (Figure 4C), and the ICI scores of surviving patients were significantly higher than those of non-surviving patients (p=0.027; Figure 4D).

We additionally evaluated the relationship between the prognostic value of ICI scores and other clinically relevant parameters in CRC patients. This approach revealed high ICI

scores to be associated with significantly better survival among individuals ≤ 65-years-old (p=0.024; Figure 4E) and those with advanced CRC (p =0.01; Figure 4F),. This also remained true regardless of patient gender (male p=0.04; female p=0.026; Figure 4G-4H).

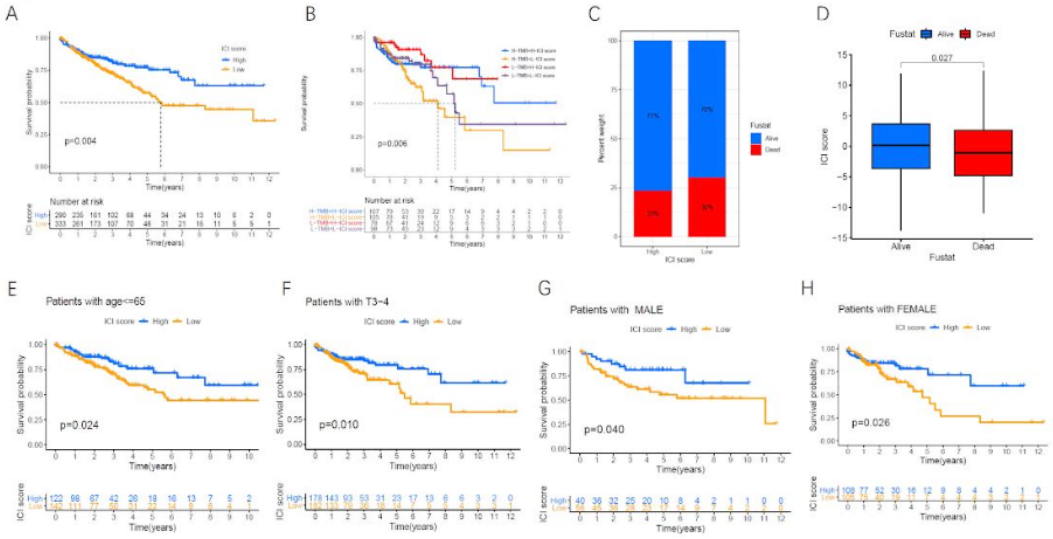

Figure 4: Evaluation of the prognostic value of ICI scores

(A) The survival outcomes of CRC patients in the low and high ICI score groups in the TCGA dataset were evaluated via Kaplan-Meier curves and log-rank tests (p=0.004). (B) TCGA-CRC patients were stratified according to TMB and ICI scores, after which differences in OS outcomes among cohorts were analyzed (p=0.006, log-rank test). (C) Survival outcomes and clinical responsiveness in those with low and high ICI scores. (D) Differences in ICI scores between surviving and non-surviving patients (Wilcoxon test, p = 0.027). (E) Survival outcomes for patients ≤ 65-years-old with low and high ICI scores were evaluated (p=0.024; log-rank test). (F) Survival outcomes for CRC patients with advanced disease with low and high ICI scores were evaluated (p=0.01; log-rank test). (G and H) The relationship between survival and ICI scores in male or female patients was evaluated (p=0.04; log-rank test).

Discussion

Immunotherapeutic treatment represents a highly efficacious approach to controlling the growth of many tumors, and can improve the quality of life for advanced CRC patients. However, many patients fail to respond to immunotherapies, and the Association for Cancer Immunotherapy has emphasized the importance of identifying those patients most likely to benefit from these therapeutic regimens [25]. Although promising immunotherapy advancements in CRC continue to evolve and generate enthusiasm, to optimize treatment efficacy, overcome resistance to immune checkpoint blockade, and appropriately select for patients who will likely benefit from immunotherapy, the development of rational therapeutic combinations remains critical [45]. There are several ongoing studies investigating potential targetable pathways involved in the host immune response to CRC. For this field to substantively evolve, correlative studies from clinical trials will be essential to identify biomarkers that can serve as immune response surrogates in CRC, biomarkers of Immune Response contains MSI-H:dMMR, PD-L1 and immunoscore [45].

A growing body of translational and clinical research has identified multiple molecular regulators of lymphocyte activation and suppression that can be therapeutically targeted, the most notable checkpoints in CRC under active clinical investigation include those that inhibit T-cell activation (CTLA-4, PD-1, and PD-L1) [45]. Another pragmatic approach is to exploit the inherent immunomodulatory properties of conventional CRC therapies in combination with immune-stimulatory agents, these combinations include chemotherapy, small molecule tyrosine kinase inhibitors, targeted mAbs, and radiotherapy (RT) [45]. VEGF inhibition has been shown to suppress activation of tumor-associated macrophages, enhance interactions between APCs and dendritic cells, as well as augment vasculature endothelium to enhance lymphocyte chemotaxis and T-cell tumor infiltration, The results will help to further define the optimal way to incorporate immunotherapy in MSI-H:dMMR CRC, particularly in the initial treatment planning for this patient population [45]. Another novel treatment option with the potential to augment the host antitumor immune response and enhance the therapeutic efficacy of vaccines is adoptive T-cell therapy [45]. Immunotherapy for CRC is rapidly evolving with the potential to revolutionize the treatment of this common disease. As our knowledge of the immune system and its intricacies continues to grow, so will our ability to harness its potential. We have already begun to see the potential of

immunotherapy with the breakthrough of anti–PD-1 therapy for the MSI-H:dMMR patient population, such as pembrolizumab and nivolumab. The challenge remains making the therapies effective for all patients, regardless of MSI:MMR status. Efforts are underway to exploit the immune system using traditional and novel therapies. The results of this study indicate that ICI scores represent a valuable prognostic biomarker that can be used to predict outcomes in these patients, and we found that higher ICI scores as well as increased intratumoral CD4+, CD8+, plasma cell, and macrophage infiltration were linked to better patient outcomes, in line with prior research [26,27]. This underscores the potential for already extant immune responses to achieve anti-tumor effects and to thereby affect the way cancer patients respond to immunotherapy. CRC tumors are thought to exhibit among the highest levels of immune cell infiltration on average [28]. We thus hypothesized that comprehensively characterizing ICI profiles and related patterns of gene expression would represent a novel approach to developing patient-specific evaluation and treatment strategies. We began by characterizing the TME associated with CRC tumors at a molecular level, enabling us to identify immune-related genes based upon ICI gene clusters. ICI gene cluster B was associated with lower immune scores, matrix scores, and with decreased immune cell infiltration consistent with what is often referred to as a 'cold' immune phenotype. In contrast, ICI gene cluster A was associated with higher immune scores and inflammatory cell infiltration, and these patients had a more favorable prognosis as well as increased CD8+ T cell, activated CD4+ T cell, and plasma cell infiltration [29,30]. Indeed, many prior studies have highlighted the effects of the TME on cancer patient OS [31]. The anti-tumor immune responses observed for patients in ICI gene cluster A suggest that they are likely to attain more benefits from immunotherapeutic treatment, and these clusters may thus offer value for the development of more efficacious immunotherapies. However, given the significant heterogeneity associated with the immunological TME in a given patient, it is vital that ICI patterns be evaluated on a patient-by-patient basis. Consistent with such an approach, one research group has reported to use of an individually-tailored tumor-specific biomarker model that can more reliably gauge breast cancer patient prognosis [32].

Using the Boruta algorithm, we established an ICI score that we were then able to analyze as a biomarker of CRC patient prognosis. GSEA analyses of these genes composing these ICI-related patterns revealed the regulation and vascular signaling pathways to be significantly enriched among samples from those with low ICI scores, while butanoate and retinol signaling pathways were enriched among those with high ICI scores. Recent work has clarified the relationship between genetic mutations and patient sensitivity to immunotherapy [33,34]. By leveraging this fact and evaluating key immunological parameters including consensus ICI scores, tumor driver mutation analyses [35], TMB measurements [36], LOH HLA (loss of heterozygosity at the HLA locus) assessments [37,38], PD-L1 expression analyses [39], and the detection of key immune gene expression-related signatures [40-43], it is possible to more reliably classify particular cancers. Integrated ICI scores offered new insights regarding differences in variant frequencies in many genes when comparing samples from the low and high ICI score groups, with some of these genes being directly linked to therapeutic sensitivity or resistance. Keytruda (pembrolizumab) works by targeting the cellular pathway of proteins found on the body's immune cells and some cancer cells, known as PD-1/PD-L1. By blocking this pathway, Keytruda may help the body's immune system fight the cancer cells and provide a benefit in patients with MSI-H or dMMR metastatic colorectal cancer [44]. However, not all MSI-H or DMMR metastatic colorectal cancers show differences in PD-1/PD-L1 expression, so ICI score can be used as a new marker for colorectal cancer with no differences in PD-1/PD-L1 expression. When we performed a stratified analysis, we determined the prognostic value of ICI scores to be independent of TMB status for CRC patients, suggesting that these two metrics offer distinct insights into the immunobiology of patient tumors, enabling the more robust assessment of patient outcomes. After employing two different algorithms to classify 712 CRC patients based upon their ICI profiles, we identified two ICI patterns and were able to employ a PCA approach to derive ICI scores therefrom. Higher ICI scores were associated with significantly higher patient OS relative to lower ICI scores, and this remained true in patients with advanced disease and in those ≤ 65-years-old. Higher levels of tumor-infiltrating lymphocytes (TILs) including both CD4+ and CD8+ T cells are related to improved patient survival and immunotherapeutic responsiveness [12]. Immune checkpoint blockade therapies rely on the activation of extant TILs, enhancing their ability to recognize tumor cells and to thereby engage appropriate immunotherapeutic responses [13,14]. Merely identifying these TILs, however, is not sufficient to reliably characterize the complexities of the TME, as many patients with high TIL levels nonetheless exhibit resistance to immunotherapy treatment [15,16]. TAMs expressing MARCO can interfere with the activation of NK and T cells, suppressing their cytokine production, proliferation, and cytotoxicity, while simultaneously aiding Treg proliferation [21]. In line with such a model, we found that high MARCO expression in individuals with low ICI score subtypes was correlated with higher Treg infiltration and reduced CD8+ T cell levels, potentially explaining the poorer prognosis for these patients.

Lastly, we explored ICI scores and associated patient characteristics, and found that simultaneous analyses of ICI scores and TMB status may improve our ability to reliably gauge CRC patient prognosis, offering a means of potentially identifying patients at a higher risk of tumor recurrence, although further research is required to test this possibility. ICI scores nonetheless

represent a powerful tool for estimating tumor-specific immune fitness, and can be used to predict which patients are most likely to benefit from immunotherapy, thereby helping to improve CRC patient survival.

Nevertheless, there are limitations to this study. First of all, this study is an analysis based on TCGA and GEO data. Although the analysis results suggest that ICI score can be used as a prognostic indicator to guide immunotherapy which provides theoretical basis for further clinical application, its clinical application needs further verification. Secondly, the TCGA and GEO dataset is limited, so more data is needed to improve this study.

Conclusion

Overall, we found that ICI scores can be reliably leveraged to assess CRC patient prognosis, with higher ICI scores being associated with prolonged OS. Stratified analyses revealed the prognostic value of ICI scores to be independent of TMB status for this cancer type, and this, together with observed GSEA and predictive outcomes, suggests that TMB and ICI status are independent tumor characteristics that may predict patient responses to immunotherapeutic treatment. We further found that that high MARCO expression in CRC patients with low ICI scores was correlated with reduced NK and effector T cell infiltration and increased Treg infiltration, potentially thereby contributing to poorer patient outcomes. Future studies of larger CRC patient cohorts will continue to offer new insights into the role of the TME as a driver of cancer progression, and may aid in the design of novel immunotherapeutic approaches to CRC treatment.

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Author Contributions

F.K und J.S conceived and designed the project. F.K and M.J.Z analyzed the data and wrote the manuscript. J.D and J.S carried out data interpretations and data analysis. F.K and J. S revised the manuscript. All authors read and approved the final manuscript.

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