IJC
International Journal of Cancer

Low tumor infiltrating mast cell density confers prognostic benefit and reflects immunoactivation in colorectal cancer

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The role of mast cells (MCs) in colorectal cancer (CRC) progression was controversial. Thus, our study was designed to evaluate the prognostic value of MCs as well as their correlation with immune microenvironment. A retrospective cohort of CRC patients of stages I–IV was enrolled in our study. Consecutive patients (854) were divided into training set (427 patients) and validation set (427 patients) randomly. The findings were further validated in a GEO cohort, GSE39582 (556 patients). The mast cell density (MCD) was measured by immunohistochemical staining of tryptase or by CIBERSORT algorithm. Low MCD predicted prolonged overall survival (OS) in training and validation set. Moreover, MCD was identified as an independent prognostic indicator in both sets. Better stratification for CRC prognosis can be achieved by building a MCD based nomogram. The prognostic role of MCD was further validated in GSE39582. In addition, MCD predicted improved survival in stages II and III CRC patients receiving adjuvant chemotherapy (ACT). Multiple immune pathways were enriched in low MCD group while cytokines/chemokines promoting anti-tumor immunity were highly expressed in such group. Furthermore, MCD was negatively correlated with CD8+ T cells infiltration. In conclusion, MCD was identified as an independent prognostic factor, as well as a potential biomarker for ACT benefit in stages II and III CRC. Better stratification of CRC prognosis could be achieved by building a MCD based nomogram. Moreover, immunoactivation in low MCD tumors may contributed to improved prognosis.

Introduction

Colorectal cancer (CRC) was estimated to be the third most commonly diagnosed and the third most common causes of cancer death in the USA for both men and women in 2016.[1] As a highly heterogeneous disease, tumor microenvironment (TME), a collection of cancer cells and neighboring mesenchymal stromal cells,[2] is recognized as a crucial aspect of tumor biology. The functional interaction between cancer cells and stromal cells impacts CRC progression and disease outcome.[3] For example, CD8+ T cells directly attack cancer cells by detecting abnormal antigens expressed by cancer cells thereby inhibiting the tumor progression. Higher CD8+ T cells infiltration in CRC tissue predicts better survival.[4] Other than CD8+ T cells, various tumor infiltrating immune cells exhibited prognostic value in previous studies.[5,6] Moreover, drugs targeting CRC microenvironment components emerge rapidly. Therefore, better understanding of the TME benefits CRC management.

As a classic immune cell, the role of mast cells (MCs) in allergy and inflammation has been well recognized.[7] MCs are highly versatile cells which secret numerous vasoactive and proinflammatory mediators,[8] exosomes,[9] proteases (including chymase, tryptase and carboxypeptidase), cytokines (including IL-6, IL-9, IL-13 and TNF) and chemokines (CXCL8, CCL2 and CCL5).[7] However, the function of MCs in modulating TME was remained largely unknown.

Heterogeneous prognostic roles of MCs were found in different cancers.[10–12] In CRC, several studies have investigated the relationship between MCs and prognosis with conflicting conclusions.[13–18] Acikalin *et al.*, Gulubova and Vlaykova and Malfettone *et al.* reported MCs infiltration conferred survival advantage[15,16,18] whereas Mehdawi *et al.*, Nielsen *et al.*, Tan *et al.* and Welsh *et al.* supported that MCs infiltration was associated with worse prognosis.[11,13,14,17] Therefore, studies

Key words: colorectal cancer, mast cell, prognosis, adjuvant chemotherapy

Abbreviations: ACT: adjuvant chemotherapy; CRC: colorectal cancer; GEO: Gene Expression Omnibus; IHC: immunohistochemistry; MC: mast cell; MCD: mast cell density; TMA: tissue microarray; TME: tumor microenvironment

Additional Supporting Information may be found in the online version of this article.

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Grant sponsor: National Natural Science Foundation of China; **Grant numbers:** 81372315, 81472228, 81602040; **Grant sponsor:** Youth Foundation of Zhongshan Hospital, Fudan University; **Grant number:** 2016ZSQN50

DOI: 10.1002/ijc.31613

History: Received 3 Jan 2018; Accepted 14 May 2018; Online 6 June 2017

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What's new?

Mast cells are inflammatory mediators that tend to congregate in the tumor periphery. Little is known, however, about their function in the tumor microenvironment, or whether their density is associated with prognosis. Here, in colorectal cancer (CRC) patients, low tumor-infiltrating mast cell density (MCD) was associated with increased overall survival, with MCD identified as an independent prognostic indicator. MCD was also found to predict survival among stage II and III CRC patients on adjuvant chemotherapy. Furthermore, low mast cell infiltration was associated with a more intense immune response, which may contribute to prolonged survival in low MCD patients.

that can unveil the relationship between mast cell density (MCD) and CRC prognosis are urgently needed.

Here, we accessed the relationship between MCD and prognosis in a large CRC cohort. The prognostic role of MCD was further validated in a GEO cohort. A MCD based nomogram was built for better stratification of CRC prognosis. MCD's predictive value of adjuvant chemotherapy (ACT) benefit in stages II and III CRC was also investigated. The crosstalk between MCs and tumor immune microenvironment was then explored and the immune response was activated extensively in low MCD patients, which indicated MCD as a potential biomarker in CRC management.

Materials and Methods

Study population

Eight hundred and fifty-four consecutive CRC patients who underwent radical primary tumor resections without prior treatment in Zhongshan Hospital (Shanghai, China) from 2008 to 2011 were divided into training set (427 patients) and validation set (427 patients) randomly. Demographics and clinical data were collected retrospectively. Cancer stages were determined referring to the 8th edition of the International Union Against Cancer (UICC)/American Joint Committee on Cancer (AJCC) TNM classification. Postoperative adjuvant chemotherapy was administered to patients according to the Chinese and NCCN CRC guidelines. For patients with resectable synchronous distant metastases, radical resections were also performed on the metastases.

The selection criterion of public dataset for external validation was as follows: (1) transcriptomic data (microarray data or RNA-Seq data) were available; (2) the basic clinicopathological information (detailed TNM stage and survival information) was available; (3) the sample size was larger than 100; (4) the median follow-up time was longer than 36 months. Therefore, GSE39582 dataset was selected from Gene Expression Omnibus (GEO) repository (https://www.ncbi.nlm.nih.gov/gds/).[19] Patients (566) from GSE39582 datasets were collected for The French national Cartes d'Identite' des Tumeurs program of stages I–IV colon cancer who underwent surgery between 1987 and 2007. After excluding patients with missing follow-up information (6 patients) or at stage 0 (4 patients), 556 patients from GSE39582 were included in the subsequent analyses.

The median follow-up time was 47.1 months for training set, 48.8 months for validation set and 52.0 months for GSE39582, respectively. Our study was approved by Clinical Research Ethics Committee of Zhongshan Hospital, Fudan University. Informed consent was obtained from all patients in Zhongshan cohort for the acquisition and use of tissue samples and clinical data. As a public dataset, neither ethics committee approval nor patient informed consent was needed for analyzing GSE39582 data.

Immunohistochemistry

Formalin-fixed paraffin-embedded surgical specimens were used for tissue microarray (TMA) construction and subsequent immunohistochemistry study as described previously.[20] The TMA slide was dried 2 hr at 60°C, the sections were dewaxed in xylene and graded alcohols, hydrated and washed in phosphate-buffered saline. After the endogenous peroxidase was inhibited by 3% H_2O_2 for 30 min, the sections were pretreated in a microwave oven (15 min in sodium citrate buffer, pH 6) and then incubated with 10% normal goat serum for 60 min. Primary antibodies composed of mouse anti-human monoclonal mast cell tryptase (diluted 1:1,000, Clone AA1, Abcam, Cambridge, UK) or rabbit anti-human polyclonal CD8 (diluted 1:200, ab4055, Abcam, Cambridge, UK), were applied overnight in a moist chamber at 4°C. Then the tissues were incubated with secondary antibodies, stained with diaminobenzidine (DAB) and counterstained with hematoxylin. Positive staining was calculated using Image Pro plus 6.0 (Media Cybernetics, Inc., Bethesda, MD) under high power filed (HPF, 200×). The MCD or CD8-positive T cell infiltration was recorded as the mean number of tryptase-positive/HPF or CD8-positive/HPF from three randomized fields. Two independent pathologists who were blinded to the clinical data evaluated the immunostaining and the results were averaged.

CIBERSORT

CIBERSORT is an accurate and robust algorithm for calculating the cell composition of a tissue from gene expression profile.[21] By applying CIBERSORT on microarray data, the estimated proportion of immune cell types can be obtained for each tumor sample. Specifically, we used LM22 as a reference expression signature with 100 permutations.

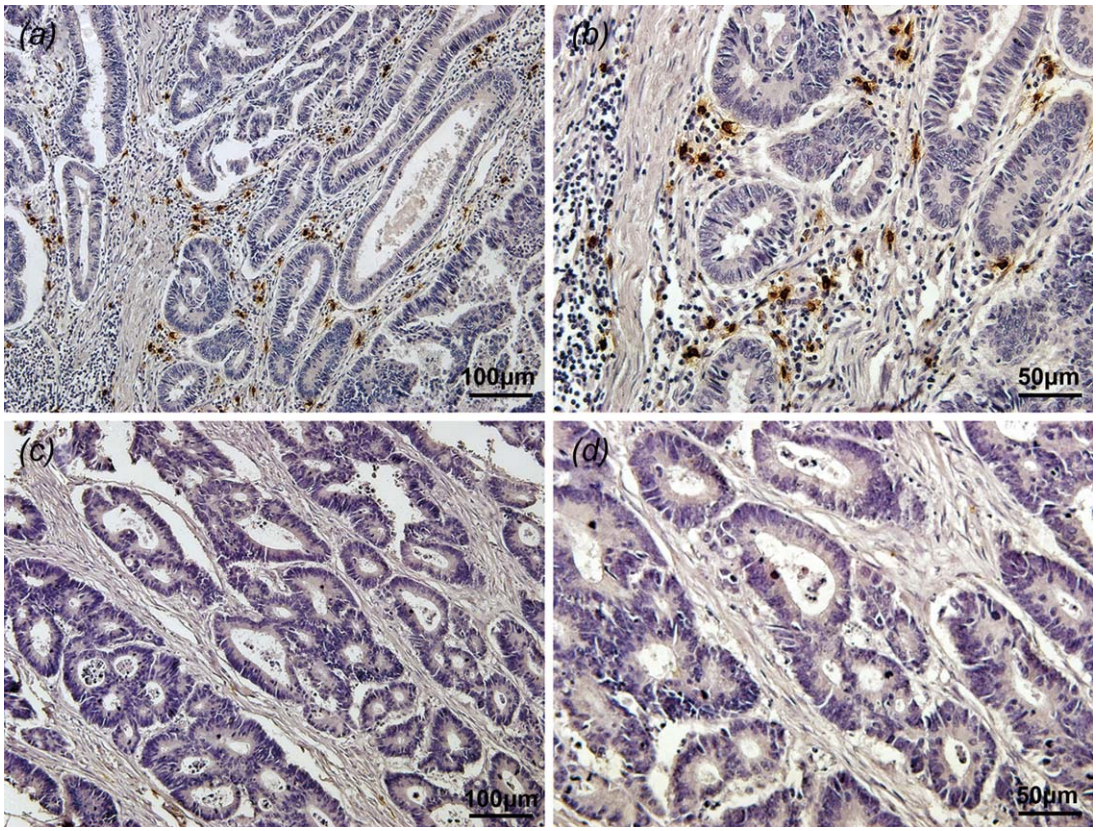

Figure 1. Representative images of tryptase+ MCs staining. Tumor tissue with high MC infiltration were presented in 200× (*a*) and 400× (*b*), and tumor tissue with low MC infiltration were presented in 200× (*c*) and 400× (*d*).

Differential expression analysis

Microarray data were downloaded from GEO repository. An R package, limma,[22] was utilized to perform differential expression analysis on microarray data. Significantly up and downregulated genes between high and low MCD groups were defined as fold change of at least 1.5 × and adjusted *p*-values ≤ 0.05. The results were visualized as a volcano plot using ggplot2 package.[23]

Gene set enrichment analysis

Gene set enrichment analysis (GSEA) was performed by the GSEA desktop application v.3.0 with 1,000 permutations.[24,25] Molecular Signatures Database (MSigDB) v6.0, *a priori* defined set of genes, was applied as a reference to determine pathways differentially enriched between low and high MCD groups.[25]

Statistical analysis

All statistical analyses were performed using SPSS 22.0 (SPSS, Inc., Chicago, IL) and R software, version 3.3.3 (The R Foundation for Statistical Computing, http://www.r-project.org/). MCD between normal and cancer tissues was compared by paired Wilcoxon signed rank test. Association between clinicopathological features and MCD were accessed by χ^2 test or Fisher exact test. Kaplan-Meier analysis and Log-rank test were used to evaluate the relationship between MCD and overall survival (OS). The cut-off value of training set was calculated by X-tile as 9.3[26] and was applied in the validation set. In GSE39582, the cut-off value of mast cell proportions was calculated by X-tile as well. Univariate and multivariate regression analyses were performed to identify independent prognostic factors, factors with $p < 0.1$ in univariate regression analyses were entered in the multivariate regression models. Nomogram models were constructed and validated using "rms" package in R software. Prognostic models were compared by Harrell index of concordance (C-index) and Akaike information criterion (AIC). A two-sided $p < 0.05$ was considered statistically significant.

Results

Association between MCD and clinical characteristics

MCs were identified by immunohistochemical staining of mast cell tryptase (MCT). To ensure the robustness of TMA based MCD evaluation, we randomly selected standard tumor sections of 100 patients in Zhongshan cohort for MCD evaluation and comparing the results with the TMA based MCD. The TMA based and standard section based MCD values were highly correlated ($r = 0.716$; $p < 0.001$; Supporting Information Fig. S1), which indicated TMA based method was suitable for evaluating MCD in CRC. The MCT positive MCs were located in the tumor tissues in a diffused manner with different density (Fig. 1). Moreover, MCs were found

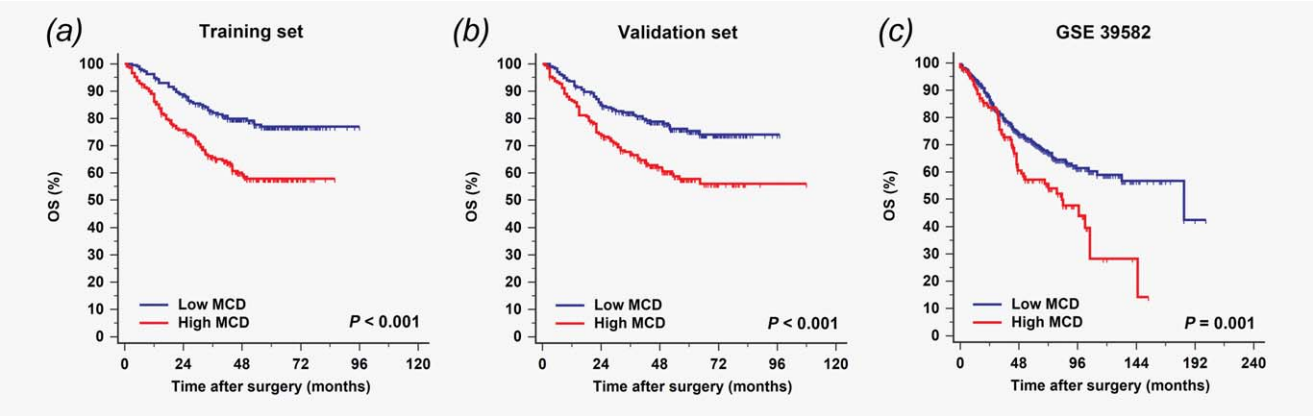

Figure 2. Kaplan-Meier analysis of OS in training set (*a*), validation set (*b*) and GSE39582 (*c*). [Color figure can be viewed at wileyonlinelibrary.com]

significantly more infiltrated in normal mucosae comparing to the paired tumor tissues in 845 paired samples of Zhongshan cohort (Supporting Information Fig. S2).

Baseline clinical characteristics of training and validation sets are presented in Supporting Information Table S1. The relationship between MCD and clinicopathological features was further accessed (Supporting Information Table S2). MCD was significantly associated with M1 stage in both sets ($p = 0.032$ and $p = 0.038$).

Correlation between MCD and CRC prognosis

To investigate the prognostic value of MCD, we performed Kaplan-Meier survival analyses and Log-rank tests in the training and validation sets (Figs. 2*a* and 2*b*). The high MCD group showed a worse OS in both sets (both $p < 0.001$). In univariate Cox regression analysis, preoperative CEA level, primary differentiation, T stage, N stage, vascular invasion, nerve invasion, M stage and MCD were significantly correlated with OS in both training and validation sets (all $p < 0.05$) whereas tumor location shows prognostic value only in training set ($p = 0.015$) (Table 1). In multivariate Cox regression analyses, MCD was identified as an independent prognostic factor of OS in both training set ($p = 0.001$, HR = 1.951, 95% CI = 1.333–2.856) and validation set ($p = 0.002$, HR = 1.750, 95% CI = 1.223–2.506) (Table 2).

MCD based nomogram construction

By accessing Harrell's Concordance index (C-index) and Akaike Information Criterion (AIC), incorporating MCD into TNM staging system yielded higher prediction accuracies than TNM alone in both training and validation sets (Supporting Information Table S3). Furthermore, we constructed a nomogram based on independent prognostic indicators in multivariate Cox regression analysis of training set, including MCD, tumor location, primary differentiation, N stage and M stage (Supporting Information Fig. S3A). In training set, the C-index for MCD based nomogram was improved to 0.815 with the AIC decreasing to 1,334.61. The similar results were observed in the validation set (Supporting Information Table S3). Also, the calibration curves for internal and external validation presented high agreement between the nomogram-predicted survival and actual survival (Figs. S3*b* and S3*c*). The above results suggested that applying MCD based nomogram better stratified CRC prognosis.

Validation of MCD's prognostic role in GSE39582

We further validated our findings in GSE39582. The relationship between MCD and clinicopathological characteristics is detailed in Supporting Information Table S4. In Kaplan-Meier analysis and Log-rank test, MCD was found significantly inversely correlated with OS ($p = 0.001$) (Fig. 2*c*). In multivariate Cox regression analysis, MCD, along with age and M stage, was identified as an independent prognostic factor of OS in the GSE39582 ($p = 0.013$, HR = 1.489, 95% CI = 1.088–2.039) (Supporting Information Table S5).

MCD and adjuvant chemotherapy in stages II and III CRC

Previous reports indicated tumor infiltrating immune cells, including macrophages and neutrophils could predict the benefit of post-operative adjuvant chemotherapy (ACT).[6,27] Therefore, we investigated the relationship between MCD and patients' prognosis receiving ACT. For stage IV CRC patients, not all patients underwent radical resection for both primary tumor and metastases and the situation is more complicated in terms of chemotherapy regimen and following treatment. To be more precise, we focused on the ACT benefit in stages II and III patients who received 5-FU based ACT. The detailed information and regimen of patients receiving ACT in three sets is listed in Supporting Information Tables S6 and S7. For patients without ACT, more MCs infiltration conferred significant survival benefit in training set, validation set and GSE39582 ($p = 0.031$, $p < 0.001$ and $p < 0.001$, respectively) (Figs. 3*a*, 3*b* and 3*d*). For patients underwent ACT, high MCD group was associated with worse OS in training set, validation set and GSE39582 ($p = 0.014$, $p = 0.138$ and $p = 0.043$, respectively) (Figs. 3*e*, 3*f* and 3*h*). In pooled analysis of Zhongshan cohort, high MCD also reduce the risk of poor survival in patients with ACT

Table 1. Univariate Cox regression analyses for overall survival in training and validation set

Factors	Overall survival			
	Training set		Internal validation set	
	HR (95% CI)	*p*	HR (95% CI)	*p*
Age (years)		0.240		0.319
≤60	1 (reference)		1 (reference)	
>60	1.233 (0.869–1.750)		0.840 (0.595–1.184)	
Gender		0.054		0.526
Male	1 (reference)		1 (reference)	
Female	0.692 (0.476–1.007)		1.119 (0.791–1.581)	
CEA (ng/ml)		**<0.001**		**<0.001**
≤5	1 (reference)		1 (reference)	
>5	2.828 (1.943–4.116)		2.779 (1.919–4.025)	
Tumor location		**0.015**		0.997
Right-sided colon	1 (reference)		1 (reference)	
Left-sided colon	0.917 (0.600–1.401)		1.018 (0.632–1.639)	
Rectum	0.558 (0.366–0.850)		1.007 (0.668–1.518)	
Tumor size		0.539		0.536
≤4.0 cm	1 (reference)		1 (reference)	
>4.0 cm	1.117 (0.785–1.588)		1.115 (0.790–1.574)	
Primary histological type		0.088		0.577
Non-mucinous	1 (reference)		1 (reference)	
Mucinous	1.504 (0.941–2.403)		1.152 (0.700–1.895)	
Primary differentiation		**0.007**		**0.001**
Well/moderate	1 (reference)		1 (reference)	
Poor/anaplastic	1.642 (1.143–2.360)		1.827 (1.286–2.596)	
T stage		**0.002**		**0.001**
T1/T2	1 (reference)		1 (reference)	
T3/T4	2.730 (1.431–5.208)		3.280 (1.604–6.708)	
N stage		**<0.001**		**<0.001**
N0	1 (reference)		1 (reference)	
N1–N2	3.085 (2.134–4.459)		3.180 (2.178–4.644)	
Vascular invasion		**<0.001**		**<0.001**
No	1 (reference)		1 (reference)	
Yes	2.499 (1.632–3.827)		2.596 (1.699–3.968)	
Nerve invasion		**<0.001**		**<0.001**
No	1 (reference)		1 (reference)	
Yes	2.780 (1.705–4.533)		3.002 (1.777–5.073)	
M stage		**<0.001**		**<0.001**
M0	1 (reference)		1 (reference)	
M1	9.737 (6.791–13.963)		10.164 (7.076–14.600)	
TNM stage		**<0.001**		**<0.001**
I	1 (reference)		1 (reference)	
II	1.661 (0.565–4.882)		1.762 (0.599–5.180)	
III	3.667 (1.292–10.411)		3.199 (1.124–9.099)	
IV	21.917 (7.988–60.134)		22.187 (8.093–60.823)	
MCD		**<0.001**		**<0.001**

Table 1. Univariate Cox regression analyses for overall survival in training and validation set (Continued)

Factors	Overall survival			
	Training set		Internal validation set	
	HR (95% CI)	*p*	HR (95% CI)	*p*
Low	1 (reference)		1 (reference)	
High	2.170 (1.511–3.118)		1.958 (1.381–2.776)	

Note: bold values present *p* values where $p < 0.05$. Abbreviations: HR: hazard ratio; 95% CI: 95% confidence interval; T: tumor invasion depth; N: lymph node involvement; M: metastasis; TNM: tumor node metastasis; MCD: mast cell density.

Table 2. Multivariate Cox regression analyses for overall survival in training and validation set

Factors	Overall Survival			
	Training set		Validation set	
	HR (95% CI)	*p*	HR (95% CI)	*p*
Gender		0.133		
Male	1 (reference)			
Female	0.740 (0.500–1.096)			
CEA (ng/ml)		0.160		0.271
≤5	1 (reference)		1 (reference)	
>5	1.493 (0.978–2.279)		1.394 (0.929–2.091)	
Tumor location		**0.038**		
Right-sided colon	1 (reference)			
Left-sided colon	0.602 (0.375–0.965)			
Rectum	0.587 (0.371–0.929)			
Primary histological type		0.073		
Non-mucinous	1 (reference)			
Mucinous	1.638 (0.955–2.809)			
Primary differentiation		**0.025**		0.854
Well/moderate	1 (reference)		1 (reference)	
Poor/anaplastic	1.479 (1.050–2.083)		1.035 (0.717–1.494)	
T stage		0.723		0.316
T1/T2	1 (reference)		1 (reference)	
T3/T4	1.099 (0.535–2.258)		1.464 (0.695–3.084)	
N stage		**0.019**		**0.012**
N0	1 (reference)		1 (reference)	
N1/N2	1.634 (1.086–2.458)		1.668 (1.117–2.493)	
Vascular invasion		0.132		**<0.001**
No	1 (reference)		1 (reference)	
Yes	1.455 (0.893–2.370)		2.267 (1.460–3.520)	
Nerve invasion		0.300		0.276
No	1 (reference)		1 (reference)	
Yes	1.361 (0.760–2.439)		1.350 (0.787–2.315)	
M stage		**<0.001**		**<0.001**
M0	1 (reference)		1 (reference)	
M1	7.276 (4.862–10.888)		6.875 (4.600–10.277)	
MCD		**0.001**		**0.002**
Low	1 (reference)		1 (reference)	
High	1.951 (1.333–2.856)		1.750 (1.223–2.506)	

Note: bold values present *p* values where $p < 0.05$. Abbreviations: HR: hazard ratio; 95% CI: 95% confidence interval; T: tumor invasion depth; N: lymph node involvement; M: metastasis; TNM: tumor node metastasis; MCD: mast cell density.

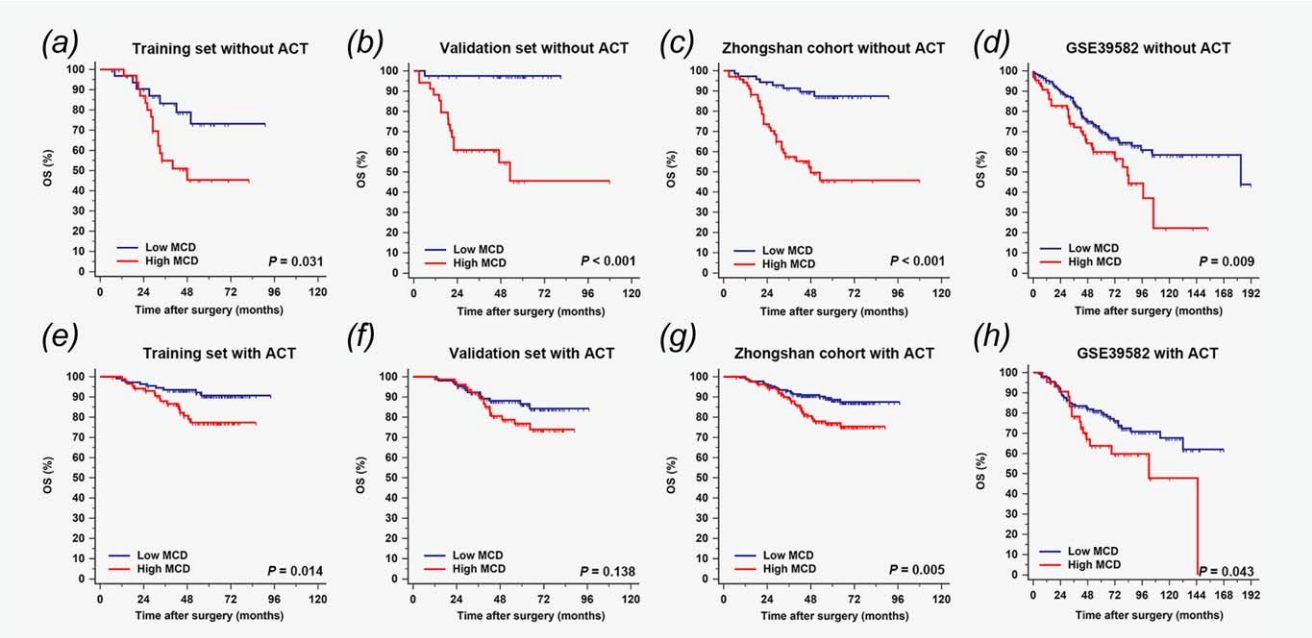

Figure 3. Subgroup analysis evaluating the predictive value of MCD for ACT benefit in stages II and III CRC patients. (*a*)–(*d*) Survival curves for patients without ACT in training set, validation set, Zhongshan cohort and GSE39582, respectively. (*e*)–(*h*) Survival curves for patients receiving ACT in training set, validation set, Zhongshan cohort and GSE39582. [Color figure can be viewed at wileyonlinelibrary.com]

significantly ($p = 0.005$) (Fig. 3*g*). Such results suggested that MCD might be a potential predictor of ACT benefit for stages II and III CRC patients, which needs further investigation.

MCD related cytokines and chemokines production

By performing differential expression analysis on the microarray data of GSE39582, we identified numerous genes up or downregulated between high and low MCD groups. Notably, several important cytokines and chemokines were differentially regulated (Fig. 4*a*).

IL-1β, which can induce CRC proliferation[28] and promote MDSC recruitment,[29] was upregulated in high MCD samples. Moreover, CXCL1, which causes increased tumor microvessel formation in VEGF-independent way[30] and promotes CRC cell growth, invasion and pre-metastatic niche formation of liver metastasis,[31] showed an increased expression in high MCD group. CCL20 and CXCL8 reported previously acting synergistically promoting CRC progression by inducing epithelial–mesenchymal transition[32] were also highly expressed in such group. Above findings implied an impaired prognosis of high MCD patients.

However, interferon-stimulated, T helper 1- (Th1-) type chemokines CXCL9 and CXCL10 were found upregulated in low MCD tumors, which can recruit T cells and NK cells as well as inhibit angiogenesis,[33,34] thereby boosting anti-tumor immune response. CCL19 expressed from dendritic cells binds to CCR7+ T cells, which may differentiate into cytotoxic lymphocytes and acquire anti-tumor ability.[35] In addition, CCL5 acts on the CCR5+ cytotoxic lymphocytes and Th1 at the invasive front of CRC tissue may enhance antitumor immunity.[36] Therefore, high expression of above cytokines and chemokines suggested immunoactivation in low MCD CRC patients.

MCD related immune microenvironment

By using GSEA to compare the expression profile between low and high MCD groups, multiple immune-related pathways were found enriched in low MCD group (Supporting Information Table S8), including antigen receptor mediated signaling pathway, T cell differentiation, T cell receptor signaling pathway, lymphocyte costimulation, etc. (Fig. 4*b*). The activation of numerous crucial immunological pathways suggested that tumors with less MC infiltration had more intense immune response, which explained the better prognosis in such patients.

The results of GSEA analysis suggested a close relationship between MCD and T cell related pathways. Interestingly, we found that MCD was negatively correlated with tumor infiltrating CD8+ T cell density. In Zhongshan cohort, MCD was inversely associated with CD8+ T cell infiltration evaluated by immunohistochemistry ($r = -0.122$, $p < 0.001$) (Figs. 4*c* and 4*d*). In addition, by performing CIBERSORT algorithm on GSE39582 and other two independent GEO datasets (GSE12945[37] and GSE17536[38]), the proportion of CD8+ T cells was validated to be negatively correlated with the proportion of MCs in CRC tissue (GSE39582: $r = -0.294$, $p < 0.001$; GSE17536: $r = -0.164$, $p = 0.030$; GSE12945: $r = -0.310$, $p = 0.014$) (Figs. 4*e*–4*g*). These

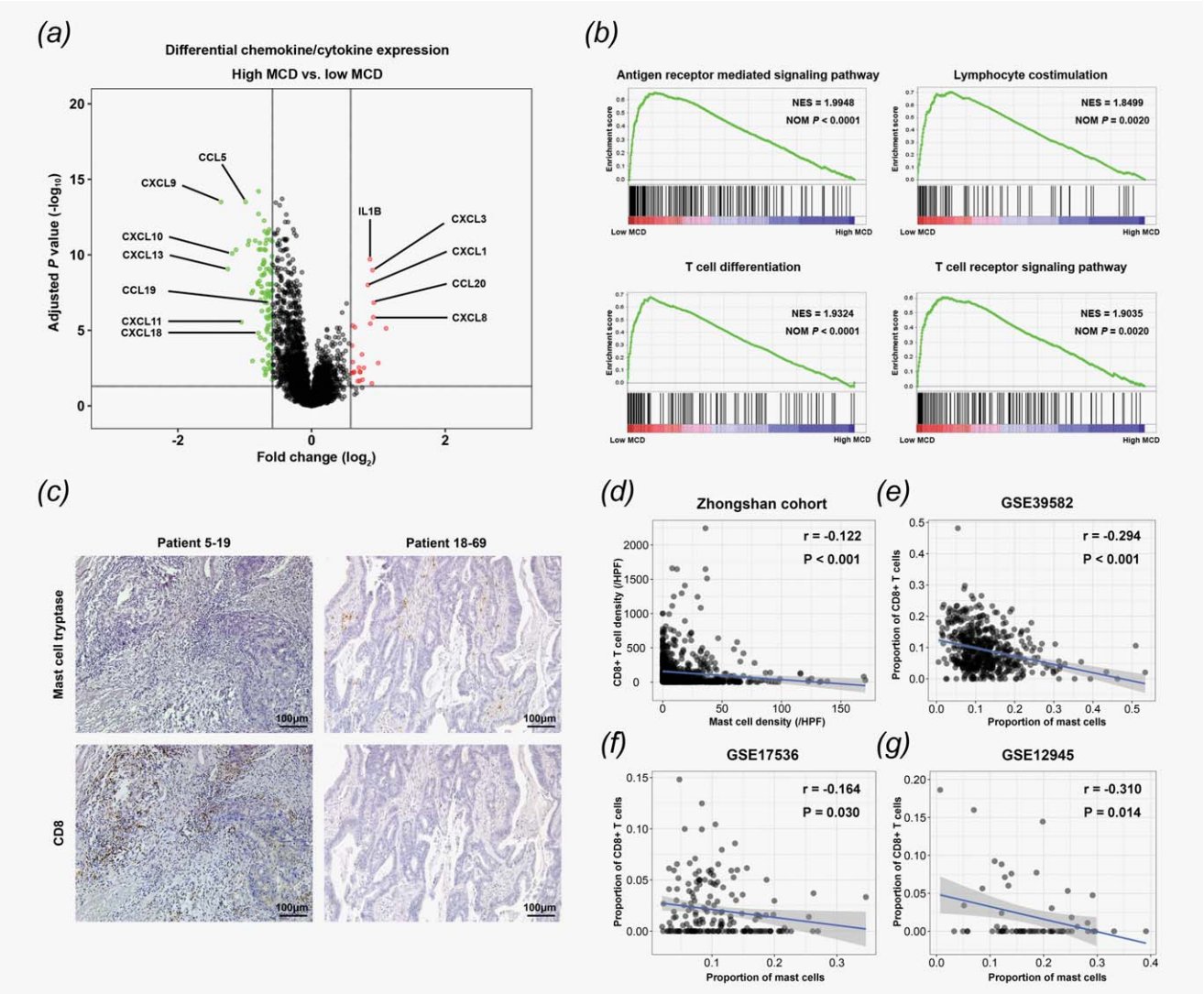

Figure 4. (*a*) Differentially expressed chemokines and cytokines between high MCD and low MCD groups in GSE39582. Genes labeled in red or green are significantly differentially up or downregulated, respectively. (*b*) Top pathways enriched in the low MCD *vs*. high MCD groups. (*c*) Representative images of immunohistochemical staining of a patient with low MCD and high CD8+ T cell density (Patients 5–19) and a patient with high MCD and low CD8+ T cell density (Patients 18–69) in Zhongshan cohort. (*d*)–(*g*) The infiltration of CD8+ T cells was negatively correlated with the infiltration of MCs (*d*, Zhongshan cohort: $r = -0.122$, $p < 0.001$; *e*, GSE39582: $r = -0.294$, $p < 0.001$; *f*, GSE17536: $r = -0.164$, $p = 0.030$; *g*, GSE12945: $r = -0.310$, $p = 0.014$).

results further indicated that the anti-tumor immunity was attenuated when more MCs infiltrated.

Discussion

MCs have long been identified and well described in allergic reaction and inflammation. However, the role of MCs in the progression of tumor remains largely unknown. Studies on MCs and prognosis in different cancers reached heterogeneous conclusions.[10–12] Similarly, both anti-tumor and pro-tumor effect of MCs in CRC was observed in previous studies.[13–18]

The feasibility of evaluating tumor markers using TMA has been discussed for years. Numerous studies found the association between molecular markers and prognosis was reproducible accessed by either tissue microarray (TMA) or conventional standard sections.[39] In addition, many previous studies evaluated immune cells infiltration of tumor tissue using TMA.[21,40,41] We accessed the validity of TMA based MCD calculation by comparing the results of these two methods. Highly correlated results suggest the TMA based method is suitable for evaluating MCD in CRC.

In our study, a large cohort was divided into training and validation sets and was utilized to evaluate the prognostic value of MCD in CRC. The negative association between MCD and OS was identified in both training and validation sets. Also, MCD was found to be an independent prognostic indicator of OS. Furthermore, the prognostic value of MCD was validated in an external public dataset, GSE39582. In

addition, MCD proved to be valuable for prognostic stratification by constructing a MCD based nomogram. Both improved C-index and reduced AIC value comparing to TNM staging system and TNM + MCD model indicated its potential usage in practical CRC management.

Post-operative ACT is a crucial treatment modality for lowering the risk of recurrence in high risk stages II and III CRC patients. For such patients, identifying sensitive individuals and predicting ACT benefit are important aspects of clinical management. The immune microenvironment not only reflects and impacts tumor progression but also influences the therapeutic response.[42] In our study, we found patients with lower MCD had better survival outcome after receiving 5-FU based ACT in both Zhongshan cohort and a French cohort, GSE39582. Notably, higher percentage of stage II patients receiving ACT and more patients were given FOLFOX/CapeOX regimen in Zhongshan cohort comparing to GSE39582. Since patients in GSE39582 underwent surgery between 1987 and 2007 whereas patients in Zhongshan cohort were enrolled from 2008, the variance of ACT percentage and regimen was largely due to the treatment evolution over two decades. Such heterogeneities may help to reach an unbiased conclusion. However, a well-designed, multi-centered, prospective study was needed to further validate this finding.

The detailed mechanism of MCs in TME remains largely unknown. In our study, pathways relating to immune activation were highly enriched in low MCD group. Antigen receptor mediated signaling pathway, T cell differentiation, T cell receptor signaling pathway, lymphocyte costimulation were identified as top pathways enriched in low MCD samples. Furthermore, the infiltration of tumor CD8+ T cell was found negatively correlated with the infiltration of MCs in both Zhongshan cohort evaluated by immunohistochemistry and public GEO datasets accessed by CIBERSORT. In addition, cytokines and chemokines, which recruit T cells and NK cells and promote cytotoxicity, were highly upregulated in low MCD tumor microenvironment, such as Th1-type chemokines CXCL9 and CXCL10. The upregulation of chemokines promoting anti-tumor immunity and increased tumor infiltrating CD8+ T cells density in low MCD patients indicates immunoactivation, which may explain the better survival and better response to chemotherapy in such individuals.[43,44]

However, several limitations of our study need to be noticed. The major one is the retrospective design of our study. A multicenter, prospective research is needed to validate the conclusions. Also, in-depth *in vitro* and *in vivo* experiments are urgently needed to unveil the hidden mechanism of MCs in TME.

In summary, MCD was identified as an independent prognostic factor, as well as a potential biomarker for ACT benefit in stages II and III CRC. Better prediction of CRC prognosis could be achieved by applying MCD based nomogram. CRC with low MCs infiltration conferred survival benefit may because of immunoactivation.

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