Original Article

Mast Cells in Adjacent Normal Colon Mucosa rather than Those in Invasive Margin are Related to Progression of Colon Cancer

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ABSTRACT

Objective: Mast cells (MC) reside in the mucosa of the digestive tract as the first line against bacteria and toxins. Clinical evidence has implied that the infiltration of mast cells in colorectal cancers is related to malignant phenotypes and a poor prognosis. This study compared the role of mast cells in adjacent normal colon mucosa and in the invasive margin during the progression of colon cancer.

Methods: Specimens were obtained from 39 patients with colon adenomas and 155 patients with colon cancers treated at the Sun Yat-sen University Cancer Center between January 1999 and July 2004. The density of mast cells was scored by an immunohistochemical assay. The pattern of mast cell distribution and its relationship with clinicopathologic parameters and 5-year survival were analyzed.

Results: The majority of mast cells were located in the adjacent normal colon mucosa, followed by the invasive margin and least in the cancer stroma. Mast cell count in adjacent normal colon mucosa ($MCC_{adjacent}$) was associated with pathologic classification, distant metastases and hepatic metastases, although it was not a prognostic factor. In contrast, mast cell count in the invasive margin ($MCC_{invasive}$) was associated with neither the clinicopathlogic parameters nor overall survival.

Conclusion: Mast cells in the adjacent normal colon mucosa were related to the progression of colon cancer, suggesting that mast cells might modulate tumor progression via a long-distance mechanism.

Key words: Mast cell; Colon cancer; Mucosa; Invasive margin; Prognosis

INTRODUCTION

In addition to genetic alterations of cancer cells, the infiltration of immune cells, such as dendritic cells, T cells, macrophages, and mast cells (MC) is believed to be involved in tumor progression[1-3]. For example, mast cells might impact tumor progression by induction of angiogenesis, tissue remodeling, and immune cell recruitment[4]. Although the experimental data support the notion that infiltration of mast cells in tumor tissue plays an important role in tumor progression, the relevant clinical evidence is complicated; the infiltrated mast cells might positively, negatively, or irrespectively impact tumor progression[5-7]. With respect to colorectal cancers, the relationship between the infiltration of mast cells and tumor progression is also controversial[8-17]. As the function of mast cells may be related to its phenotype and location in cancer tissue[18], the current study examined the role of mast cells in the adjacent normal colon mucosa and in the invasive margin during the progression of colon cancer.

MATERIALS AND METHODS

Materials

Paraffin-embedded specimens, including tumor tissues

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and adjacent normal tissues, were obtained from 39 patients with pathologic evaluation-confirmed colon adenomas and 155 patients with colon cancers who underwent radical resection or biopsy between January 1999 and July 2004 at the Sun Yat-sen University Cancer Center in Guangzhou, China (Table 1). The TNM classification system of the American Joint Committee on Cancer (edition 6) was used for clinical staging, and the World Health Organization classification of tumors (2000 version) was used for histological tumor grading. Patients did not receive chemotherapy or radiation therapy before surgery.

Follow-up

Follow-up was provided to stage I–IV colon cancer patients. Patients were observed on an every-3-month basis during the 1st year, once every 6 months in the 2nd year, and by telephone or mail communication once every year thereafter, for a total of 5 years. Patients received adjuvant or palliative 5-FU-based chemotherapy according to the NCCN guidelines. Overall survival (OS) was defined as the time from diagnosis to death or was censored at the last known alive data.

Immunohistochemical Assay

Tissue sections (5 μm thickness) were cut, dried, deparaffinized, and rehydrated in graded alcohol and xylene before antigen retrieval by pressure cooker treatment in citrate buffer (pH 6.0) for 3 min. Endogenous peroxidase was blocked with 3% hydrogen peroxide incubation. Mouse anti-human mast cell tryptase monoclonal antibody (1:160,000 dilution, Serotec, Oxford, UK), mouse anti-human mast cell chymase monoclonal antibody (1:8,000 dilution, Serotec, Oxford, UK), and mouse anti-human CD31 monoclonal antibody (working solution, catalog number: ZM-0044, Zhongshan Goldenbridge Biotechnology, Beijing, China) were used. Immunostaining was performed using the EnVision+ Dual Link Kit (Dako Cytomation, Denmark) according to the manufacturer's instructions. The development was performed with a substrate-chromogen solution (3,3'- diaminobenzidine dihydrochloride [DAB]) for 3–5 min. Sections were then counterstained with hematoxylin and mounted in non-aqueous mounting medium.

Mast Cell Evaluation

The mast cell count in the invasive margin ($MCC_{invasive}$) was defined as the number of tryptase-positive mast cells localized in the invasive margin of the colon cancer, as tryptase expression occurs universally in mast cells. The stained sections were first screened under a low power objective (100×) to identify the areas with the highest number of mast cells in the invasive margin. The $MCC_{invasive}$ was then recorded under 400× magnification [1 mm^2 per high power field (HP)] in 5 fields of vision with an ocular micrometer. The number of mast cells in every field was expressed as MC/HP, and for each case, the mean $MCC_{invasive}$ was noted. The mean $MCC_{invasive}$ = the total number of mast cells in the five fields divided by five. The mast cell count in adjacent normal colon mucosa ($MCC_{adjacent}$) was also evaluated in five consecutive fields, similarly to $MCC_{invasive}$. The mast cell count (MCC) in each section was scored separately by two independent observers with no prior knowledge of clinicopathologic parameters. The inter-observer agreement for the MCC was 81%. Disagreements were re-evaluated until a consensus was reached.

Statistical Analysis

Statistical analyses were performed using SPSS 13.0 software for Windows (SPSS Inc, Chicago, IL, USA). The descriptive statistical tests, including the mean, standard deviation, and median were calculated according to the standard methods. The Kolmogorov-Smirnov test and Shapiro-Wilks' W-test were used to analyze the normality of the distribution. The relationship between the various clinicopathologic characteristics and the MCC parameters were compared and analyzed using Chi-square tests, likelihood ratio, and linear-by-linear association, as appropriate. The non-parametric Mann-Whitney U test and Kruskal-Wallis test were used to evaluate the significance of the differences of the mean ranks. Cumulative survival curves were drawn by the Kaplan-Meier method, and the difference between the curves was analyzed by the log-rank test. Multivariate analyses were based on the Cox proportional hazards regression model. A two-tailed $P<0.05$ was considered statistically significant.

RESULTS

Phenotypes and Distribution of Mast Cells

Tryptase and chymase staining were used to define the phenotypes of mast cells. Most of the mast cells in the mucosa and invasive margin showed similar tryptase and chymase staining, indicating that these cells display an MC_{TC} phenotype (Figure 1). The density of mast cells was counted; the majority of the cells were located in adjacent normal colon mucosa, followed by the invasive margin, and least in the cancer stroma (Figure 2). $MCC_{adjacent}$ significantly increased when tumors developed from adenomas to advanced colon cancers (7.20±2.72 vs. 6.60±3.31 vs. 7.70±3.48 vs. 7.70±3.17 vs. 9.00±2.83, $P<0.05$), whereas no statistically significant difference existed for $MCC_{invasive}$ among patients with stages I-IV colon cancer (Table 1). Finally, the relationship between mast cells and tumor microvessels was examined. It failed to show the related distribution of mast cells and microvessels in consecutive sections (Figure 3).

Relationship between MCC and Clinicopathologic Characteristics

The relationship between MCC and clinicopathologic parameters was analyzed. The results showed that $MCC_{invasive}$ was not related to the conventional clinicopathologic parameters, such as TNM classification characteristics and hepatic metastases (Table 2). However, $MCC_{adjacent}$ was associated with pathologic classification, distant metastases, and the synchronous and meta- chronous hepatic metastases of colon cancer (Table 2).

Relationship between MCC and OS

Among the 155 colon cancer patients, 93 patients were alive after the 5-year follow-up. Thus, the 5-year survival rate was 60%. The $MCC_{adjacent}$ ranged from 1.40 to 20.20 MC/HP,

with a median of 8.00 MC/HP. The $MCC_{invasive}$ ranged from 0.20 to 15.00 MC/HP with a median of 4.00 MC/HP. According to Gulubova's method, patients were then divided into high and low $MCC_{adjacent}$ and $MCC_{invasive}$ groups by median values (8.00 MC/HP and 4.00 MC/HP, respectively)[9]. Based on univariate and multivariate analyses, neither $MCC_{adjacent}$ nor $MCC_{invasive}$ was related to OS (Figures 4, 5 and Tables 3, 4).

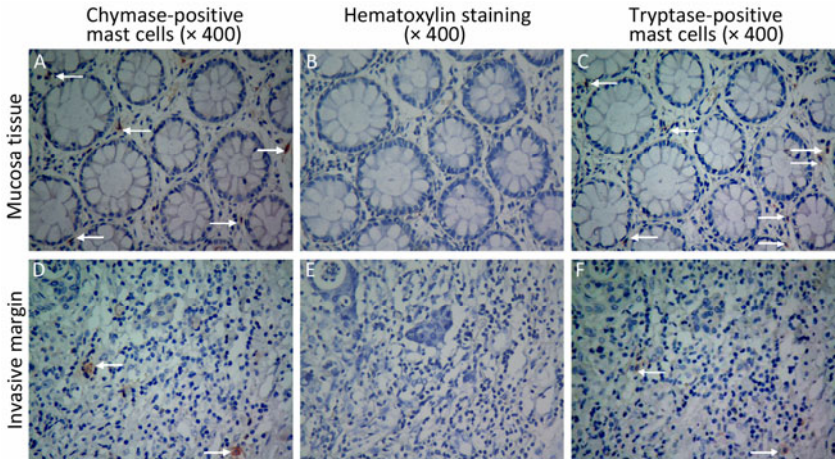

Figure 1. The phenotypes of mast cells. Mast cells were stained with chymase monoclonal antibody (×400 A and D), and tryptase monoclonal antibody (×400 C and F). B and E are negative controls (×400). Arrows indicate chymase-positive mast cells (A and D) and tryptase-postive mast cells (C and F).

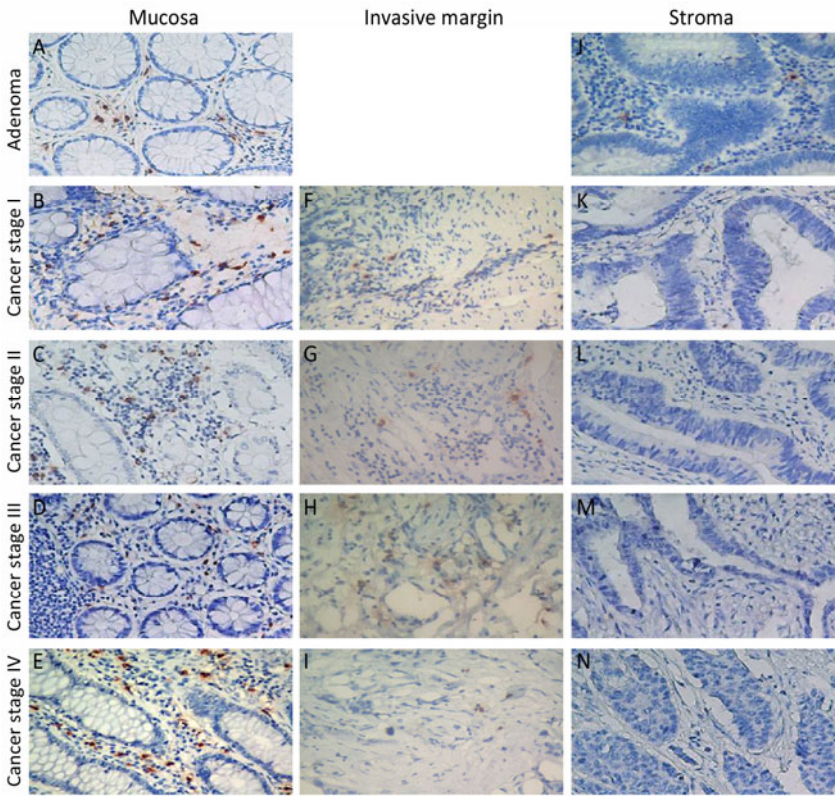

Figure 2. The distribution of mast cells in adenomas and cancers of the colon. The tryptase-positive mast cells were stained with an immunohistochemical assay (×400). $MCC_{adjacent}$ increased when tumors developed from adenomas to advanced colon cancer (A–E). Whereas no significant difference for $MCC_{invasive}$ among patients with stages I–IV was observed (F–I). Rare mast cells were observed in the tumor stroma (J–N).

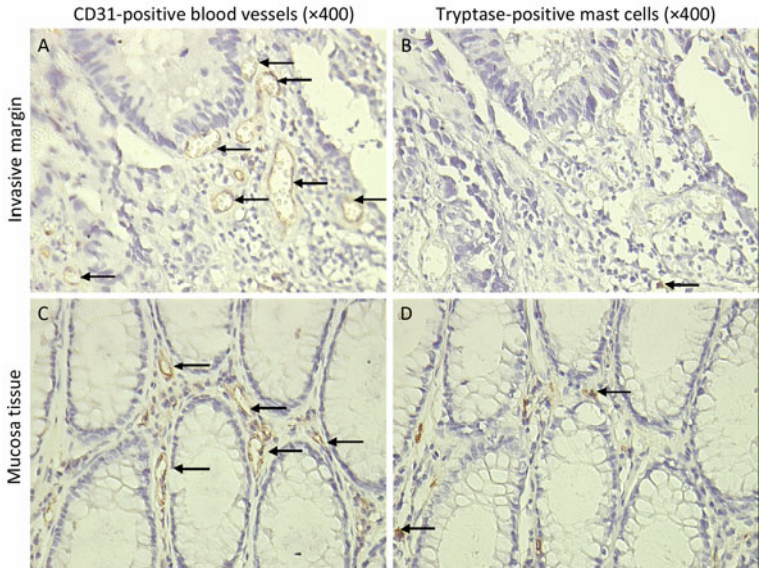

Figure 3. The relationship between mast cells and blood vessels. Blood vessels were stained with CD31 monoclonal antibody (×400 A and C). Mast cells were stained with tryptase monoclonal antibody (×400 B and D). Tryptase-positive mast cell count was not positively related with CD-31 positive blood vessel count. Neither was the distribution. Arrows indicate CD-31 positive blood vessels (A and C), and tryptase-positive mast cells (B and D).

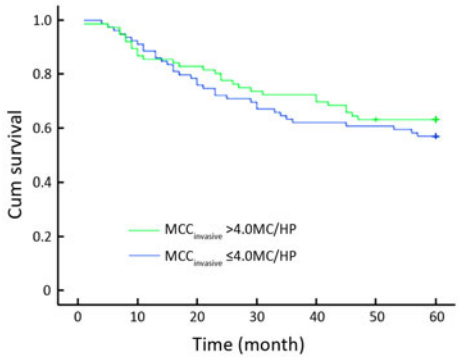

Figure 4. The relationship between the $MCC_{invasive}$ and OS. No statistical significance was observed between the patients with $MCC_{invasive}$ >4.0 MC/HP and those with $MCC_{invasive}$ ≤4.0 MC/HP. $MCC_{invasive}$: the number of tryptase-positive mast cells localized in the invasive margin.

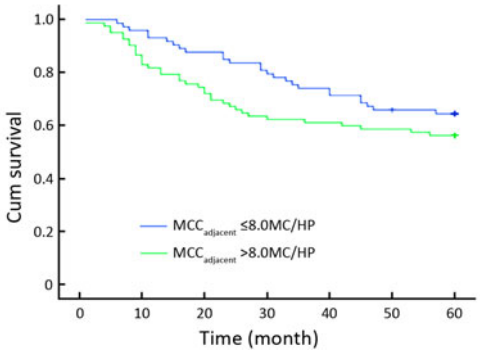

Figure 5. The relationship between the $MCC_{adjacent}$ and OS. No statistical significance was observed between the patients with $MCC_{adjacent}$ >8.0 MC/HP and those with $MCC_{adjacent}$ ≤8.0 MC/HP. $MCC_{adjacent}$: the number of tryptase-positive mast cells localized in adjacent normal colon mucosa.

Table 1. The count of mast cells in colon adenomas and colon cancers

Tumor	n	$MCC_{adjacent}$ (median±interquartile range)	$MCC_{invasive}$ (median±interquartile range)
Adenoma	39	7.20±2.72	---
Colon Cancer Stage I	38	6.60±3.31	3.80±2.36
Colon Cancer Stage II	38	7.70±3.48	4.80±2.47
Colon Cancer Stage III	38	7.70±3.17	3.80±2.73
Colon Cancer Stage IV	41	9.00±2.83	3.70±1.89
P		[a]0.003**	0.092**

** Kruskal-Wallis test. [a]$P<0.05$, statistically significant.

Table 2. The association between MCC and clinicopathologic characteristics

Variable	n	$MCC_{invasive}$		*P*	$MCC_{adjacent}$		*P*
		≤4	>4		≤8	>8	
Age(year)				0.093			0.389
≤59	80	46	34		35	45	
>59	75	33	42		38	37	
Gender				0.153			0.484
Male	91	42	49		45	46	
Female	64	37	27		28	36	
Location of primary tumor				0.536			0.663
Left	92	45	47		42	50	
Right	63	34	29		31	32	
Pathologic classification				0.436			[a]0.029
Papillary + tubular	132	69	63		67	65	
Other	23	10	13		6	17	
Grade				0.847			0.726
G1	15	7	8		8	7	
G2	109	56	53		53	56	
G3	25	12	13		10	15	
G4	6	4	2		2	4	
Depth of penetration				0.396			0.054
T1	6	3	3		1	5	
T2	32	20	12		21	11	
T3	92	46	46		41	51	
T4	25	10	15		10	15	
Lymph node involvement				0.352			0.089
N0	76	35	41		42	34	
N1	62	36	26		26	36	
N2	17	8	9		5	12	
Distant metastases				0.970			[a]0.008
M0	114	58	56		61	53	
M1	41	21	20		12	29	
Hepatic metastases				0.997			[a]0.027
No	100	51	49		54	46	
Metachronous	14	7	7		7	7	
Synchronous	41	21	20		12	29	

Chi-square tests: [a]*P*<0.05, statistically significant.

Table 3. Univariate analysis of factors associated with OS (n=155)

Variable	OS	
	HR, (95% CI)	*P*
Gender (female vs. male)	0.831 (0.496–1.391)	0.481
Age (>59 y vs. ≤59 y)	0.986 (0.599–1.624)	0.956
Location of primary tumor (right vs. left)	0.919 (0.551–1.532)	0.746
Grade (G4+G3 vs. G2+G1)	2.238 (1.303–3.844)	0.004[a]
Pathologic classification (papillary+tubular vs. other)	1.759 (0.758–4.083)	0.189
Stage (IV+III vs. II+I)	12.904 (5.850–28.465)	0.000[b]
Hepatic metastases (no vs. yes)	0.037 (0.018–0.076)	0.000[b]
$MCC_{adjacent}$ (high vs. low)[A]	1.427 (0.862–2.364)	0.167
$MCC_{invasive}$(high vs. low) [B]	0.821 (0.498–1.355)	0.441

A: $MCC_{adjacent}$ high: $MCC_{adjacent}$ >8.0 MC/HP; $MCC_{adjacent}$ low: $MCC_{adjacent}$ ≤8.0 MC/HP. B: $MCC_{invasive}$ high: $MCC_{invasive}$ >4.0 MC/HP; $MCC_{invasive}$ low: $MCC_{invasive}$ ≤4.0 MC/HP. 95% CI: 95% confidence interval. [a]*P*<0.05, [b]*P*<0.001, statistically significant.

Table 4. Multivariate Cox analysis of factors associated with OS (n=155)

Variable	OS	
	HR, (95% CI)	*P*
Gender (female vs. male)	1.054 (0.627–1.770)	0.843
Age (>59 y vs. ≤59 y)	1.142 (0.692–1.884)	0.604
Location of primary tumor (right vs. left)	1.368 (0.806–2.324)	0.246
Grade (G4+G3 vs. G2+G1)	1.680 (0.968–2.915)	0.065
Pathologic classification (papillary+tubular vs. other)	1.216 (0.518–2.853)	0.653
Stage (IV+III vs. II+I)	3.385 (1.372–8.348)	0.000[b]
Hepatic metastases (no vs. yes)	0.069 (0.031–0.152)	0.000[b]
$MCC_{adjacent}$ (high vs. low) [A]	0.985 (0.590–1.645)	0.953
$MCC_{invasive}$(high vs. low) [B]	0.639 (0.381–1.072)	0.090

A: $MCC_{adjacent}$ high: $MCC_{adjacent}$ >8.0 MC/HP; $MCC_{adjacent}$ low: $MCC_{adjacent}$ ≤8.0 MC/HP. B: $MCC_{invasive}$ high: $MCC_{invasive}$ >4.0 MC/HP; $MCC_{invasive}$ low: $MCC_{invasive}$ ≤4.0 MC/HP. 95% CI: 95% confidence interval. [b]*P*<0.001, statistically significant.

DISCUSSION

Using immunohistochemistry, this study analyzed the distribution of mast cells in adenomas, colon cancers, and matched adjacent normal colon tissue. The results showed that $MCC_{adjacent}$ increased when tumors developed from adenomas to advanced colon cancers, although $MCC_{adjacent}$ was not a prognostic factor.

Most previous studies have shown that mast cell is an early and persistent infiltrating immune cell in colorectal cancers, acting before significant tumor growth and angiogenesis have occurred. After the switch to angiogenesis, mast cell assembles in the invasive margin or around the vessels, which is related to the malignant phenotypes or poor prognosis[8-17]. In contrast to those studies, this study showed that $MCC_{invasive}$ was associated with neither the malignant phenotypes nor the survival of colon cancer patients. Additionally, in consecutive sections, the spatial relationship between mast cells and endothelial cells also failed to support the idea that mast cells could regulate angiogenesis. Both pieces of evidence supported the randomized distribution hypothesis of mast cells in tumor tissue, which had been observed in endometrial adenocarcinomas, non-small cell lung carcinomas, cutaneous mastocytomas, and melanomas[19,20].

Because the relationship between mast cells in the invasive margin and tumor progression was not observed, this study further analyzed the relationship between $MCC_{adjacent}$ and tumor progression. Mast cells reside in the mucosa in the digestive tract[21-23]. After activation, mast cells can produce and release mediators and cytokines, which are involved in the modulation of a wide variety of gastrointestinal, physiologic, and pathologic processes, such as the regulation of epithelial barriers, mucosal immune function, bacterial defense, motility, and visceral sensitivity[24-28]. As the role of mast cells in the adjacent normal colon mucosa in the progression of colon cancers had not been examined, this study examined the phenotypes of mast cells with tryptase and chymase staining. The results showed that mast cells in normal colon mucosa were mostly MC_{TC} phenotype. Secondly, this study observed that the increased $MCC_{adjacent}$ was associated with pathologic classification, distant metastases, and the synchronous and metachronous hepatic metastases of colon cancer. This suggested that mast cells in the adjacent normal colon mucosa have the potential for long-distance regulation, as peripheral mature mast cells ordinarily do not circulate in the blood. It remains unknown how mast cells in the mucosa interact with colon cancers. Therefore, it is important to analyze the underlying mechanism by which mast cells in adjacent normal colon mucosa affect colon cancer progression, especially with hepatic metastases. This mechanism may hold targets for new therapeutics to be developed in the future[29-31].

This study showed that mast cells in the adjacent normal colon mucosa rather than mast cells in the invasive margin were associated with the progression of colon cancer, indicating that mast cells might be involved in tumor progression via a long-distance regulatory mechanism.

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