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Digital quantitative analysis of mast cell infiltration in interstitial cystitis

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Aims: To evaluate the significance of mast cell infiltration in interstitial cystitis (IC) by comparison with equally inflamed controls using a digital quantification technique.
Methods: Bladder biopsy specimens from 31 patients with Hunner type IC and 38 patients with non-Hunner type IC were analyzed. Bladder biopsy specimens from 37 patients without IC, including 19 non-specific chronic cystitis ("non-IC cystitis") specimens and 18 non-inflamed bladder ("normal bladder") specimens, were used as controls. Mast cell tryptase-, CD3-, CD20-, and CD138-immunoreactive cells were quantified using digital image analysis software to evaluate both mast cell and lymphoplasmacytic cell densities. Mast cell and lymphoplasmacytic cell densities were counted independently in the entire lamina propria and detrusor areas and compared among the four groups.
Results: In the lamina propria, there were no significant differences in mast cell and lymphoplasmacytic cell densities between Hunner type IC and non-IC cystitis or between non-Hunner type IC and normal bladder specimens. In the detrusor, the mast cell densities were not significantly different among the four groups. Mast cell density was correlated with lymphoplasmacytic cell density, but not with clinical parameters.
Conclusions: Mast cell density is not significantly different between IC specimens and non-IC control specimens with a similar degree of background inflammation. The intensity of mast cell infiltration generally correlated with that of lymphoplasmacytic cells. We conclude that mast cell count is of no value in the differential diagnosis between IC and other etiologies.

KEYWORDS
cystitis, detrusor, diagnosis, differential, interstitial, mast cells, mastocytosis, urinary bladder

Abbreviation: ESSIC, European society for the study of IC/PBS; HIC, Hunner type interstitial cystitis; IC, interstitial cystitis; NHIC, non-Hunner type interstitial cystitis; ROI, region of interest.

Mickey Karram led the peer-review process as the Associate Editor responsible for the paper.

1 | INTRODUCTION

Interstitial cystitis (IC) is a chronic bladder disease of ill-defined etiology, characterized by lower urinary tract symptoms such as urinary frequency, urgency, bladder discomfort, and/or bladder pain.[1–3] The clinical diagnosis of IC is based on the presence of these symptoms, exclusion of confusable diseases, and cystoscopy.[1–3] Based on cystoscopic findings, IC can be classified into two subtypes: IC with Hunner lesions (Hunner type IC; HIC) and IC without Hunner lesions (non-Hunner type IC; NHIC).[1,2]

Recently, we distinguished HIC from NHIC pathophysiologically: HIC is an inflammatory disorder characterized by lymphoplasmacytic infiltration and epithelial denudation, while NHIC is generally a non-inflammatory disorder with minimal inflammatory changes.[4,5] However, the diagnostic significance of the histopathological findings of IC has long been controversial. In fact, histopathology is not included in the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) criteria.[3] By contrast, the European Society for the Study of IC/PBS (ESSIC) includes histological features, such as inflammatory infiltrates, detrusor mastocytosis, and intrafascicular fibrosis, in their criteria for IC/bladder pain syndrome (BPS).[2]

Mast cell infiltration has been studied in depth. While increased mast cell density in interstitial cystitis has repeatedly been documented,[6–16] other studies have shown that mastocytosis is not a specific histological finding.[17–19] In these earlier studies, mast cells were counted manually, and no study compared the mast cell densities of IC with those of non-IC bladder specimens with a similar degree of background inflammation.[6–19]

In this study, we accurately analyzed the mast cell density in IC biopsy specimens and non-IC controls using quantitative image analysis software. The degree of background lymphoplasmacytic inflammation was also assessed to evaluate its correlation with mast cell infiltration. This study sought to clarify whether mast cell infiltration is a specific feature of IC, and to evaluate the diagnostic significance of the mast cell count.

2 | MATERIALS AND METHODS

Ethics approval was obtained from the institutional review board of the University of Tokyo (Reference Nos. 3124 and 2381). All methods were performed in accordance with approved guidelines. Written informed consent was obtained from all patients.

2.1 | Study samples

Bladder biopsy samples obtained from 2008 to 2015 were retrieved from the archives of the Department of Pathology at the University of Tokyo Hospital. Specifically, biopsies were obtained from 31 patients with HIC, 38 patients with NHIC, 19 patients with chronic inflammation but no IC ("non-IC cystitis"), and 18 patients with no IC and minimal inflammatory changes ("normal bladder"). Two samples were taken from each of the 31 patients with HIC, one from the Hunner lesion and the other from a non-lesion area. The diagnosis of IC, as well as the differential diagnosis of HIC and NHIC, were made according to the clinical guidelines for IC and hypersensitive bladder.[1] In this study, all NHIC patients had cystoscopic evidence of glomerulation or mucosal bleeding after hydrodistention and corresponded to ESSIC type 2. Likewise, all HIC patients corresponded to ESSIC type 3.[2] All IC patients met the NIDDK criteria.[3]

An experienced pathologist reviewed the histology of a series of non-IC biopsy specimens that were arbitrarily selected from the archives of the Pathology Department. Non-IC biopsies that showed histological evidence of chronic inflammation represented by substantial stromal infiltration of lymphoplasmacytic cells were designated as the "non-IC cystitis" group. Those non-IC biopsies that revealed no evidence of stromal inflammation were designated as the "normal bladder" group. Patients who had undergone intravesical administration of anti-cancer agents for bladder cancer were excluded.

2.2 | Histopathology and immunohistochemistry

Conventional histological assessment of hematoxylin- and eosin-stained slides was performed routinely. All tissue samples were fixed in formalin and embedded in paraffin. These samples were used to prepare 4-μm serial sections. Immunohistochemistry (IHC) was performed according to standard procedures using an autostainer (Ventana BenchMark XT or Discovery XT, Ventana Medical Systems, Tucson, AZ). Anti-human CD3 (1:50, Clone LN10, Novocastra, Newcastle upon Tyne, UK), CD20 (1:100, Clone L26, Dako, Glostrup, Denmark), CD138 (prediluted, Clone B-A38, Nichirei Bioscience, Tokyo, Japan), and mast cell tryptase (1:4000, Clone AA1, Dako, Glostrup, Denmark) antibodies were used to detect T-lymphocytes, B-lymphocytes, plasma cells, and mast cells, respectively. Appropriate controls for each antibody were included.

2.3 | Quantitative analysis

Images of the immunostained slides were digitized by the NanoZoomer Digital Pathology system (Hamamatsu Photonics, Hamamatsu, Japan), followed by digital quantification using the image analysis software Tissue Studio®, version 3.5 (Definiens AG, Munich, Germany). The region of interest (ROI) was established by freehand drawing on the

screen of a polygon encircling the entire subepithelial area (lamina propria and detrusor) or detrusor area only (Figure 1). After establishing the ROI, we performed a manual ROI-nuclei (positive vs negative) analysis using the Tissue Studio® software as described in our previous studies.[4,5] IHC thresholds were used to detect CD3-, CD20-, CD138, and mast cell tryptase-positive cells (thresholds were set at 0.3, 0.3, 0.5, and 0.8, respectively). The ROI area (mm^2) was measured, and the numbers of CD3-, CD20-, CD138-, and mast cell tryptase-positive cells in the ROI were automatically counted. The sum of CD3-, CD20-, and CD138-positive cells was considered as total lymphoplasmacytic cells. The cell density ($cell/mm^2$) of mast cells or lymphoplasmacytic cells was evaluated in the lamina propria and detrusor areas separately. The density for the lamina propria was calculated as follows: (subepithelial cell number − detrusor cell number)/(subepithelial area − detrusor area). Of the two specimens from each patient with HIC, the one with higher mast cell density in the entire subepithelial area was selected and used for the study.

2.4 | Correlation of mast cell density with lymphoplasmacytic cell density and with the clinical parameters of IC

To investigate the association between mast cell infiltration and background inflammation, the mast and lymphoplasmacytic cell densities in the entire subepithelial area were assessed in all specimens by the correlation coefficient test. We also explored the correlations between mast cell density in the lamina propria or the detrusor area and clinical parameters of IC cases, including age, years from onset to biopsy, O'Leary and Sant's symptom index and problem index (OSSI/OSPI), visual analogue scale for pain (VAS), urinary frequency, maximum voided volume, and bladder capacity measured at biopsy.

2.5 | Statistical analysis

The Wilcoxon rank sum test for two-group comparison, and the Kruskal-Wallis test followed by a post hoc Mann-Whitney

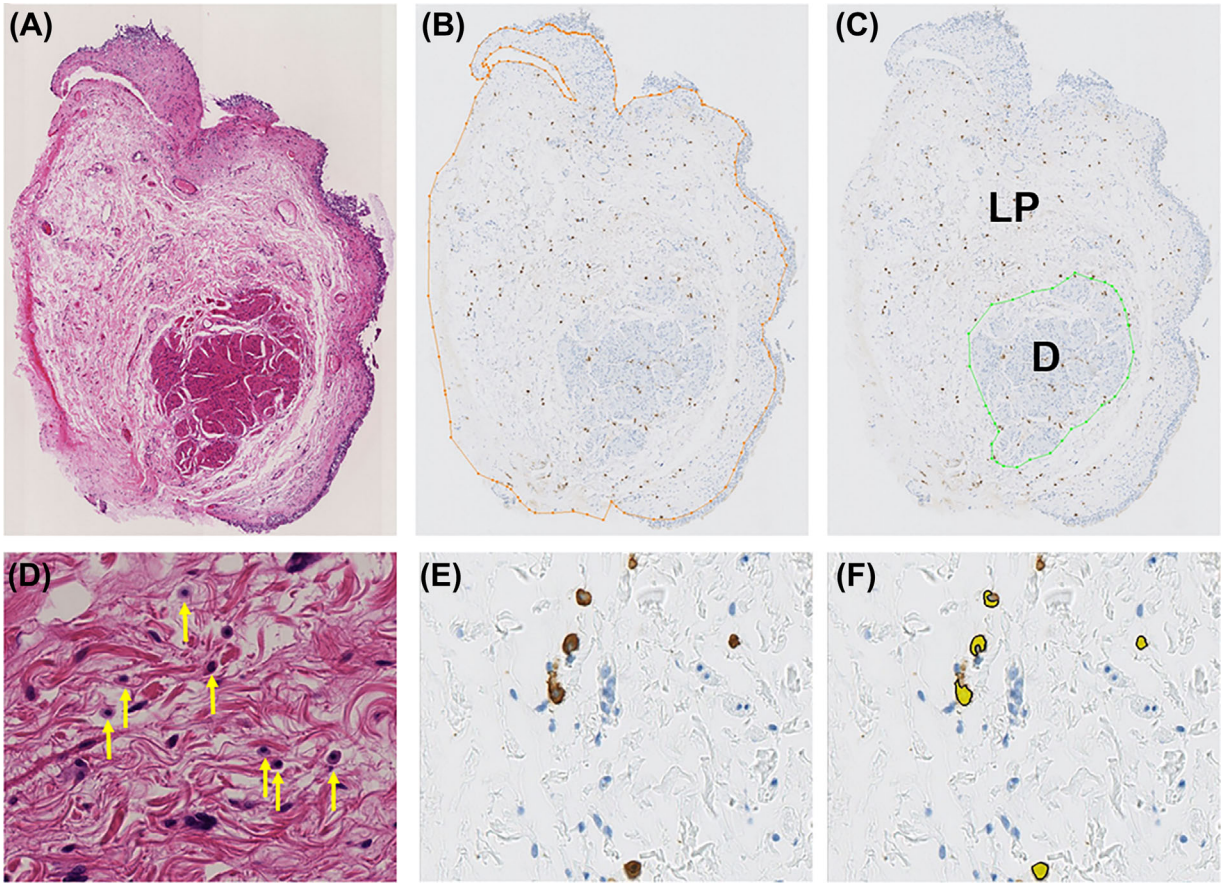

FIGURE 1 Digital quantification of mast cell infiltration. A, A representative hematoxylin- and eosin-stained section of a urinary bladder biopsy specimen. B, ROI in the subepithelial area. C, ROI in the detrusor area (all original magnification, ×5). **LP**, lamina propria area; **D**, detrusor area. D, Mast cells stained with hematoxylin and eosin. Some mast cells were identified readily (yellow arrow), while others were inconspicuous morphologically. E, Mast cells immunostained with mast cell tryptase. F, Mast cell tryptase-positive cells evaluated by imaging analysis software (all original magnification, ×10). Mast cells were identified by the intensity and area of the immunostained region

U-test with the Bonferroni correction for multiple (four-group) comparison were used for continuous variables. Fisher's exact test was used for categorical variables. The Spearman rank correlation coefficient test was applied to explore the correlation between continuous variables. A *P*-value less than 0.05 (or 0.0083 for multiple comparison) was considered statistically significant. All statistical calculations were carried out using JMP® Pro software, ver. 11 (SAS Institute, Cary, NC).

3 | RESULTS

3.1 | Characteristics of the study samples

The demographics of patients with HIC and NHIC are shown in Table 1. Patients with HIC were significantly older at the time of symptom onset and biopsy, and had a significantly smaller bladder capacity at hydrodistension compared with those with NHIC ($P < 0.01$). However, symptom severity did not differ significantly between the two subtypes of IC. The non-IC cystitis specimens were obtained from patients with non-specific chronic cystitis ($n = 7$) and from non-cancerous bladder regions of patients with non-muscle invasive bladder cancer ($n = 12$). The normal bladder specimens were obtained from normal bladder regions of patients with non-muscle invasive bladder cancer ($n = 16$), inverted papilloma ($n = 1$), or leiomyoma of the urinary bladder ($n = 1$). The mean ages of patients with non-IC cystitis and normal bladders were 72.5 (range 54-85) and 64.3 (range 40-78) years, respectively. Females comprised 42% (8/19) of the non-IC cystitis and 28% (5/18) of the normal bladder patients.

3.2 | Histological findings

Figure 2 shows representative examples of the histology of HIC, non-IC cystitis, NHIC, and normal bladder specimens. Detrusor muscle was observed in 17 of 31 (55%), 13 of 19 (68%), 20 of 38 (53%), and 13 of 18 (72%) specimens in the HIC, non-IC cystitis, NHIC, and normal bladder groups, respectively.

3.3 | Quantitative analysis

The density of lymphoplasmacytic cells or mast cells did not differ significantly between HIC and non-IC cystitis or between NHIC and normal bladder specimens in the entire lamina propria area (Figure 3A and 3B).

In the detrusor area, lymphoplasmacytic cell density was much lower than that in the entire lamina propria area (supplementary Figure S1). Mast cell density did not differ significantly among the four groups (Figure 3C).

The density of lymphoplasmacytic cells or mast cells in non-IC specimens was not significantly affected by either coexisting or a past history of bladder cancer (supplementary Figure S2).

3.4 | Correlation of mast cell density with lymphoplasmacytic cell density and clinical parameters

Mast cell density in the entire subepithelial area was significantly correlated with lymphoplasmacytic cell density (Spearman's $\rho = 0.46$, $P < 0.001$). No significant correlations between mast cell density and clinical parameters, including symptom severity, were observed (Table 2).

TABLE 1 Demographics of patients with IC

	HIC	NHIC	P value
No. (male/female)	31 (3/28)	38 (11/27)	0.07
Mean age at the time of biopsy (years)	68.5 ± 11.1 [38-88][a]	55.8 ± 17.8 [20-83]	<0.01*
Age at onset of IC (years)	65.3 ± 10.3 [38-80]	52.6 ± 17.6 [15-81]	<0.01*
Years from symptom onset to biopsy (years)	3.3 ± 2.6 [0-11]	3.3 ± 3.7 [0-15]	0.50
OSSI	13.2 ± 4.0 [7-20]	12.0 ± 3.6 [3-20]	0.30
OSPI	11.6 ± 3.6 [3-16]	10.8 ± 3.8 [1-16]	0.42
VAS	6.7 ± 2.4 [1-10]	6.0 ± 2.9 [0-10]	0.46
Urinary frequency (/day)	16.3 ± 5.4 [7-30]	15.6 ± 7.4 [4-42]	0.36
Average voided volume (mL)	109.8 ± 41.7 [40-220]	125.4 ± 81.1 [30-400]	0.92
Maximum voided volume (mL)	168.0 ± 58.6 [50-300]	219.3 ± 112.9 [50-500]	0.08
Maximum bladder capacity at hydrodistension (mL)	526.7 ± 180.4 [200-900]	698.7 ± 181.0 [400-1200]	<0.001*

IC, Interstitial cystitis; HIC, Hunner type interstitial cystitis; NHIC, non-Hunner type interstitial cystitis; OSSI/OSPI, O'Leary and Sant's symptom index/O'Leary and Sant's problem index; VAS, visual analogue scale (for pain).

[a]Mean ± SD [range].

*$P < 0.05$, statistically significant difference by Wilcoxon rank-sum test.

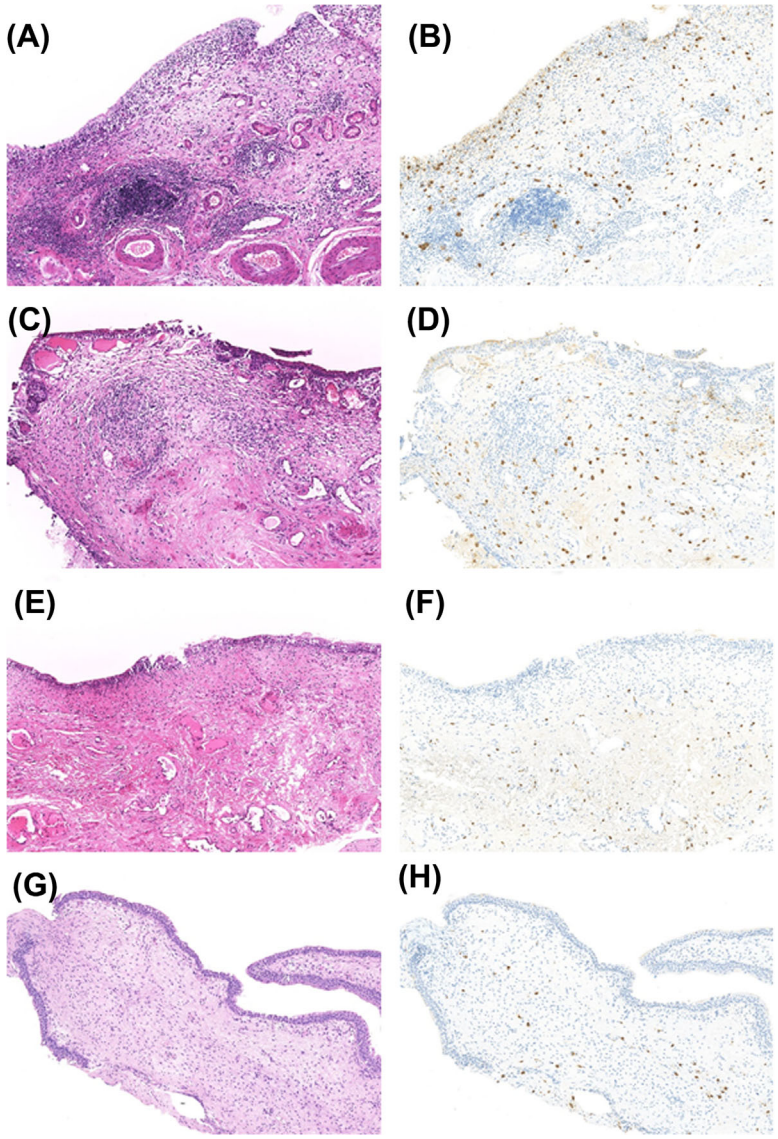

FIGURE 2 Representative histology of HIC, non-IC cystitis, NHIC, and normal bladder. A, HIC specimen stained with hematoxylin and eosin. B, HIC specimen immunostained with mast cell tryptase. C, Non-IC cystitis specimen stained with hematoxylin and eosin. D, Non-IC cystitis specimen immunostained with mast cell tryptase. E, NHIC specimen stained with hematoxylin and eosin. F, NHIC specimen immunostained with mast cell tryptase. G, Normal bladder specimen stained with hematoxylin and eosin. H, Normal bladder specimen immunostained with mast cell tryptase. Dense inflammatory cell infiltration was observed in HIC and non-IC cystitis, whereas subtle inflammation was observed in NHIC and normal bladder. Mast cell tryptase-positive cells appeared more prominent in HIC and non-IC cystitis compared with NHIC and normal bladder (all original magnification, ×10)

4 | DISCUSSION

To our knowledge, this is the first study to enumerate mast cells in IC specimens and compare mast cell density between IC and non-IC bladders with equivalent lymphoplasmacytic infiltration using digital quantification software. As a result, we found that mast cell infiltration correlated with the background lymphoplasmacytic infiltration in all specimens, but that mast cell count was of no value otherwise in differentiating IC from non-IC controls.

There has been a long controversy regarding the significance of mast cell infiltration in IC specimens. Some studies have reported increased mast cell density in IC, while others have found no difference between IC and controls.[6–19] We believe that these inconsistent results were due to the following factors: (1) non-standardized staining methods used for mast cell identification,[9,20] including c-kit IHC, naphthol esterase, toluidine blue, Alcian blue-PAS, and Giemsa staining; (2) limited areas selected for evaluation,[21] such as a 1-2 mm^2 area defined by grids, 5-10 high-power fields in areas with the highest mast cell density, or randomly selected areas; (3) uncertain cohort settings[13,16] caused by combining HIC and NHIC samples or by not reporting the

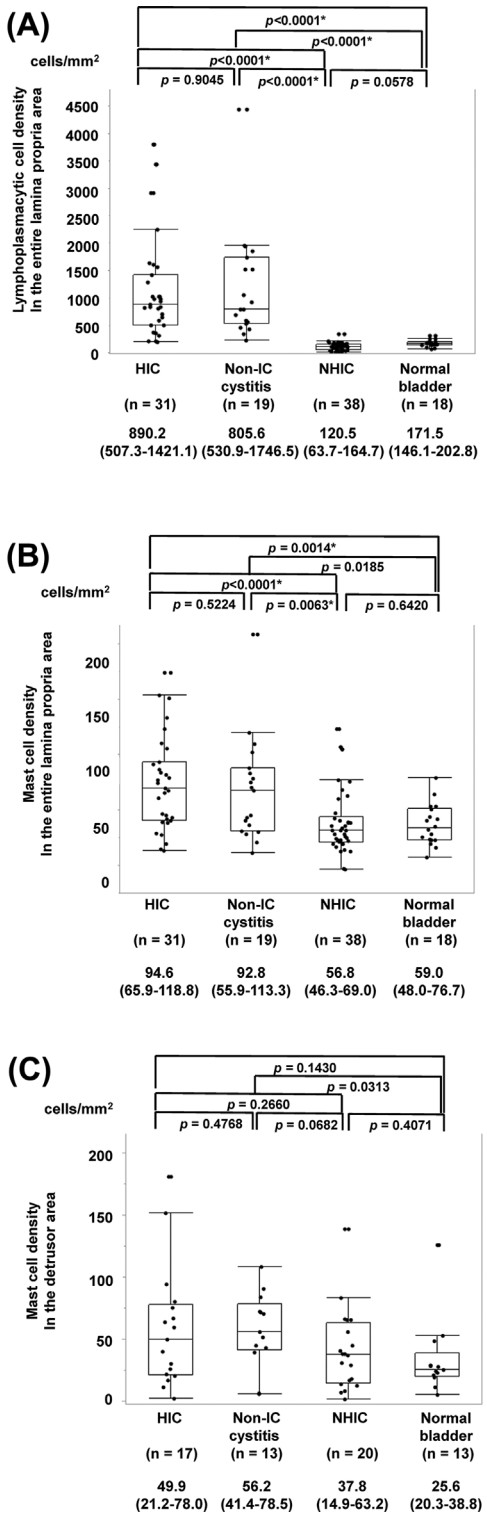

FIGURE 3 Quantitative analysis of lymphoplasmacytic cells and mast cells. A, Lymphoplasmacytic cell density in the lamina propria. B, Mast cell density in the lamina propria. C, Mast cell density in the detrusor. Values are expressed as medians (interquartile range). *$P < 0.0083$, statistically significant difference. In the lamina propria area, no significant differences in the cell (lymphoplasmacytic or mast) densities were observed between HIC and non-IC cystitis or between NHIC and normal bladder. In the detrusor, there was no significant difference in mast cell density among the four groups

biopsy sites; and (4) ignorance of background inflammation that could influence mast cell infiltration.[6–10,12,14–16,18] In this study, we used a sensitive immunohistochemical stain for human mast cell tryptase (equivalent to the ESSIC-proposed naphthol esterase staining) to detect infiltrating mast cells. We also utilized imaging analysis software to count lymphoplasmacytic and mast cells objectively and analyzed the lamina propria and detrusor areas independently.

We have recently shown that HIC and NHIC are histologically distinct.[4] HIC is an inflammatory disease characterized by frequent clonal B-cell expansion, whereas NHIC exhibits no or only subtle inflammatory changes. Other studies reported a significant increase in mast cell density in HIC alone.[6,8,14,15] Thus, we chose to use different controls for HIC and NHIC; specifically, we compared HIC with non-IC cystitis with a similar degree of lymphoplasmacytic infiltration and compared NHIC with normal bladder. Consequently, we found no significant difference in mast cell density between IC and inflammation-matched controls (ie between HIC and non-IC cystitis or between NHIC and normal bladder). Considering that mast cell and lymphoplasmacytic cell densities are significantly correlated, and that mast cells reside in bladder tissue to some degree,[22] mast cells in IC may be recruited along with other inflammatory infiltrates. In a cluster analysis of IC/BPS biopsy samples, Leiby et al have also demonstrated that the degree of lymphocytosis varied among the identified clusters, and paralleled that of mastocytosis.[23]

The ESSIC proposes using detrusor mastocytosis (defined as 28 mast cells/mm^2 area or greater) as the criterion threshold. Specifically, greater than 1.47 mm^2 (seven squares using 0.21 mm^2 square grids) of detrusor muscle is recommended for evaluation of detrusor mastocytosis.[2,21] However, in our study, the median mast cell density in detrusor muscle of non-IC cystitis was 56.2/mm^2, which is twice as high as the criterion threshold. Furthermore, the median mast cell density of normal bladder specimens (25/mm^2) was equivalent to that of ESSIC-defined detrusor mastocytosis. In addition, detrusor muscle was included in 63 of 106 (59.4%) specimens in our study, and the average area was only 0.88 mm^2, far less than the recommended 1.47 mm^2. Obtaining a "sufficient" amount of detrusor muscle sample during routine biopsy procedures is not always feasible. The results of the current study are aligned with a previous work by Gamper and her colleagues.[17] They found that detrusor or submucosal mast cell counts had poor diagnostic value to differentiate patients with BPS/IC from those with overactive bladder syndrome or healthy controls. In addition, only 30% of the analyzed samples in their study included detrusors more than 1 mm^2. Collectively, mastocytosis in the bladder biopsy specimens appears to be of limited value in histopathological diagnosis of IC.

TABLE 2 Correlation between mast cell density and clinical parameters in IC

	Mast cell density			
	HIC		NHIC	
	Lamina propria ($n = 31$)	Detrusor ($n = 17$)	Lamina propria ($n = 38$)	Detrusor ($n = 20$)
Age (years)	$\rho = -0.32$, ($P = 0.08$)[a]	$\rho = 0.25$, ($P = 0.34$)	$\rho = 0.18$, ($P = 0.27$)	$\rho = 0.33$, ($P = 0.15$)
Years from onset to biopsy (years)	$\rho = 0.12$, ($P = 0.51$)	$\rho = 0.59$, ($P = 0.10$)	$\rho = -0.09$, ($P = 0.57$)	$\rho = -0.29$, ($P = 0.22$)
OSSI	$\rho = -0.22$, ($P = 0.25$)	$\rho = 0.01$, ($P = 0.99$)	$\rho = -0.003$, ($P = 0.99$)	$\rho = -0.14$, ($P = 0.56$)
OSPI	$\rho = 0.03$, ($P = 0.87$)	$\rho = 0.07$, ($P = 0.77$)	$\rho = 0.07$, ($P = 0.69$)	$\rho = -0.04$, ($P = 0.88$)
VAS	$\rho = -0.29$, ($P = 0.12$)	$\rho = 0.15$, ($P = 0.57$)	$\rho = -0.27$, ($P = 0.13$)	$\rho = -0.55$, ($P = 0.20$)
Urinary frequency	$\rho = -0.11$, ($P = 0.57$)	$\rho = -0.05$, ($P = 0.86$)	$\rho = -0.02$, ($P = 0.92$)	$\rho = -0.14$, ($P = 0.55$)
Average voided volume (mL)	$\rho = 0.02$, ($P = 0.90$)	$\rho = 0.22$, ($P = 0.42$)	$\rho = 0.12$, ($P = 0.47$)	$\rho = 0.41$, ($P = 0.07$)
Maximum voided volume (mL)	$\rho = -0.07$, ($P = 0.70$)	$\rho = -0.02$, ($P = 0.95$)	$\rho = 0.01$, ($P = 0.97$)	$\rho = 0.51$, ($P = 0.02$)
Maximum bladder capacity at hydrodistensio (mL)	$\rho = -0.15$, ($P = 0.42$)	$\rho = 0.16$, ($P = 0.55$)	$\rho = 0.19$, ($P = 0.25$)	$\rho = 0.34$, ($P = 0.14$)

IC, Interstitial cystitis: HIC, Hunner type IC: NHIC, non-Hunner type IC: OSSI/OSPI, O'Leary and Sant's symptom index/ O'Leary and Sant's problem index: VAS, visual analogue scale (for pain).

[a]Spearman's correlation coefficient ρ and P-value (in parentheses).

The limitations of this study should be noted. First, a general limitation of all IC research is that study results cannot be compared directly because of different guidelines, definitions, and inclusion criteria. Second, we measured the number of mast cells but did not assess their function and location. Activated perineural mast cells sensitize peripheral sensory nerves via mast cell granules and neurotransmitters, such as substance P or nerve growth factor (known as "mast cell-nerve interaction").[24] This sensitization has been implicated in the pathogenesis of the hypersensitive symptoms associated with IC.[25] Third, we counted the number of mast cells infiltrating the superficial detrusor layer in less than 1 mm^2 areas. Further functional analysis of mast cells using whole sections of the detrusor layer may be needed to precisely elucidate the pathophysiological role of mast cell infiltration in IC. Fourth, the non-IC controls had different qualities from the previous studies; they were recruited from the histopathology archives. Thus, our study results cannot be directly compared with previous studies. In addition, the use of samples associated with bladder cancer as controls in investigations of mast cells is controversial,[7,9,26] although we observed no significant difference in mast cell density between non-IC samples from cancer patients and those from non-cancer patients in the present study. Our analyses yielded similar results regardless of whether the control cohort was limited to bladder cancer, non-cancer, or combined cases.

5 | CONCLUSIONS

Mast cell density is not significantly different between IC specimens and non-IC control specimens with a similar degree of background inflammation. The mast cell count is of no value in the differential diagnosis of IC and other etiologies.

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AUTHORS' CONTRIBUTION

YA and DM conceived and designed the experiments; YA, A. Niimi, A. Nomiya, and YY performed the experiments; YA and DM analyzed the data and wrote the paper; TM, YI, AG, MF, and YH revised the manuscript critically; and YI, MF, and YH contibuted to the final approval of the version to be published.

CONFLICTS OF INTEREST

All authors declare that they have no relevant financial interests regarding this study.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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