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Close Association of Intraepithelial Accumulation of M2-Skewed Macrophages with Neoplastic Epithelia of the Esophagus

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Tumor-associated macrophages (TAMs) are the most abundant cancer stromal cells and are directed by the tumor microenvironment to acquire trophic functions facilitating angiogenesis, matrix breakdown and cancer cell motility. TAMs have anti-inflammatory or alternatively activated (M2) phenotypes expressing CD204 and/or CD163. We previously reported that infiltration of a large number of CD204-positive TAMs are associated with angiogenesis, progression and poor disease-free survival of human esophageal squamous cell carcinomas (ESCCs). In this study, we investigated the initraepithelial distribution of TAMs in the early human esophageal carcinogenesis. We found that the numbers of CD68-, CD163- or CD204-positive macrophages within the unit length of 38 lesions of carcinoma *in situ* (CIS) excised by endoscopic mucosal dissection were significantly higher than those in the corresponding non-neoplastic squamous epithelia. Mapping of the infiltrating number of CD204-positive macrophages per 5 mm unit length within the whole epithelial area of 5 resected cancer laden esophagi demonstrated that the areas with high CD204-positive macrophage infiltration were significantly associated with CIS or squamous intraepithelial neoplasia. These results may suggest that macrophages with the M2-skewed phenotype have some biological roles in the early squamous carcinogenesis of the esophagus.

Esophageal cancer is the eighth most common cancer and the sixth leading cause of cancer-related deaths worldwide, with an estimated 604,100 new cases and 544,076 deaths in 2020 (9). Despite recent advances in multidisciplinary treatment strategies, the outcomes of esophageal cancer treatment remain unsatisfactory (34). The survival of esophageal cancer patients after treatment depends largely on the clinical stage (cStage) of the tumor. The five-year survival rates of the esophageal cancer patients after surgery with clinical stage 0 and I were reported to be 91.7% and 77.5%, respectively, while that in esophageal cancer patients with advanced cStages declined to less than 60% (30). Therefore, early detection with appropriate care is mandatory to improve the outcome of the esophageal cancer treatment. To this end, endoscopy with narrow-band imaging (38) and endoscopic submucosal dissection (ESD) (22) have provided a new modality for the management of early-stage esophageal cancers.

Squamous cell carcinoma (SCC) and adenocarcinoma (AC) are the most common histological types of esophageal epithelial malignancy (21). Shibata *et al*. reported that the mean percentages of SCC and AC in Japan were 73.3% and less than 3%, respectively (24). Esophageal squamous cell carcinogenesis is known to progress in a stepwise manner from normal squamous epithelia to low-grade to high-grade intraepithelial neoplasia and finally to invasive SCC. Although the major genetic and epigenetic abnormalities or risk factors of the esophageal SCCs have been elucidated, the pathological features and the molecular characteristics of their precursor lesions remain areas for further research (21).

Macrophages differentiate into two different phenotypes depending on their microenvironment: inflammatory or tumor suppressive (M1) and tumor promotive (M2). Macrophages present in the tumor microenvironment, known as tumor-associated macrophages (TAMs), have been reported to show the M2-phenotype and frequently accelerate the tumor malignancy (28, 31). TAMs express specific molecules, such as the cluster of differentiation (CD)163 and CD204, which are used as markers for the M2-macrophages (15, 16). It was reported that TAMs expressing the M2-phenotype were associated with tumor aggressiveness of esophageal SCC (27). However, the roles of macrophages in the early squamous cell carcinogenesis of the esophagus remain obscure. The present study aimed to elucidate the morphological association of M2-skewed macrophages and intraepithelial neoplastic lesions of the esophagus using step-cut ESD and surgical specimens.

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MATERIALS AND METHODS

Tissue samples

A total of 38 carcinoma *in situ* (CIS) removed from 36 patients by ESD and 5 cases of invasive SCC of the esophagus resected by surgery at Kobe University Hospital (Kobe, Japan) were employed. The ESD cases consisted of 28 men and 8 women with an age range of 47 to 83 years and mean age of 66.1 years. The patients receiving esophagectomy consisted of 3 men and 2 women with an age range of 49 to 73 years and mean age of 63.0 years. None of them received adjuvant chemotherapy or radiotherapy before surgery. Informed consent was obtained from all patients, and the study was approved by the Kobe University Institutional Review Board.

All resected specimens were fixed in 10% buffered formalin. The whole specimens of 38 ESD samples and 5 cases of surgically resected esophageal squamous cell carcinoma were step-cut at 3-mm and 5-mm intervals, respectively. Four-μm thick sections were cut from the paraffin-embedded tissues and subjected to hematoxylin and eosin (H&E) staining and immunohistochemistry. Histological and clinicopathological evaluations were performed according to the Japanese Classification of Esophageal Cancer proposed by the Japan Esophageal Society (10, 11) along with the TNM classification of the Union for International Cancer Control (UICC) (29).

Immunohistochemistry

A modified version of the immunoglobulin enzyme bridge technique using a Linked Streptavidin-Biotin kit (DakoCytomation, Glostrup, Denmark) was applied to visualize the immunoreactivities of macrophage antigens in the tissue sections as described elsewhere (27). Specific mouse monoclonal antibodies to Ki-67 (MIB-1, 1:100; DakoCytomation), CD68 (Kp-1, 1:100; DakoCytomation), CD163 (10D6, 1:100; Novocastra, Newcastle upon Tyne, UK) and CD204 (SRA-E5, 1:50; Trans Genic Inc., Kobe, Japan) were used for the primary reaction. After gentle rinsing with 0.05 mol/L of Tris-HCl, the sections were incubated with biotinylated goat anti-mouse IgG and streptavidin conjugated to horseradish peroxidase. Chromogenic fixation was carried out by immersing the sections in a solution of 3,3'-diaminobenzidine. Sections were counterstained with Mayer's hematoxylin.

Macrophage count

CD68-, CD163- or CD204-positive round cells above the extrapolated line of the basement membrane were counted in each specimen. The macrophage count was performed 3 times separately. In the case of ESD samples, the number of macrophages per μm length of the CIS and adjacent non-neoplastic squamous epithelia in the representative section of each sample were calculated. The number of CD204- or CD163-positive cells per 5 mm unit length of the epithelial area was counted and plotted on each step-cut whole area of the resected esophagus to make a density map of intraepithelial macrophages.

Statistical analysis

The significance of differences in the mean macrophage count between the neoplastic and non-neoplastic squamous epithelia in ESD specimens was tested using the paired Student's *t*-test. Differences of the mean macrophage count among the unit area of squamous intraepithelial neoplasia, carcinoma in situ and non-neoplastic squamous epithelia within the resected esophagus were determined by the Mann-Whitney *U*-test. A *P*-value less than 0.05 was considered significant. The statistical analyses were conducted using EZR software (12).

RESULTS

Macrophages were frequently found in neoplastic squamous epithelia of the esophagus excised by ESD

Figure 1a demonstrates the representative results of immunohistochemistry of CIS lesion excised by ESD. H&E staining of a CIS specimen demonstrates that proliferative basal and parabasal-like cells replace the whole epithelial layer in the left which is demarcated with the non-neoplastic squamous epithelia in the right by so-called oblique-line. Ki-67 immunoreactive nuclei are irregularly distributed throughout the neoplastic epithelia, while they are limited to the para-basal part of the non-neoplastic epithelia. CD163- or CD204-positive macrophages are more frequently observed in the neoplastic than in the comparison with non-neoplastic squamous epithelia of the esophagus.

The number of CD68-positive macrophages per μm of CIS lesion ranged from 0.07 to 0.005 (mean, 0.024; SD, 0.0123) and that of non-neoplastic squamous epithelia ranged from 0.09 to 0.0004 (mean, 0.0025; SD, 0.00209). The number of CD163-positive macrophages per μm of CIS lesion ranged from 0.05 to 0.001 (mean, 0.016; SD, 0.0111) and that of non-neoplastic squamous epithelia ranged from 0.007 to 0 (mean, 0.0015; SD, 0.00157). The number of CD204-positive macrophages per μm of CIS lesion ranged from 0.03 to 0 (mean, 0.006; SD, 0.0074) and that of non-neoplastic squamous epithelia ranged from 0.002 to 0 (mean, 0.0003; SD, 0.00054). The mean numbers of CD68-, CD163- and CD204-positive macrophages per μm of CIS lesion were significantly higher than those of non-neoplastic squamous epithelia (each $P < 0.001$, Figure 1b).

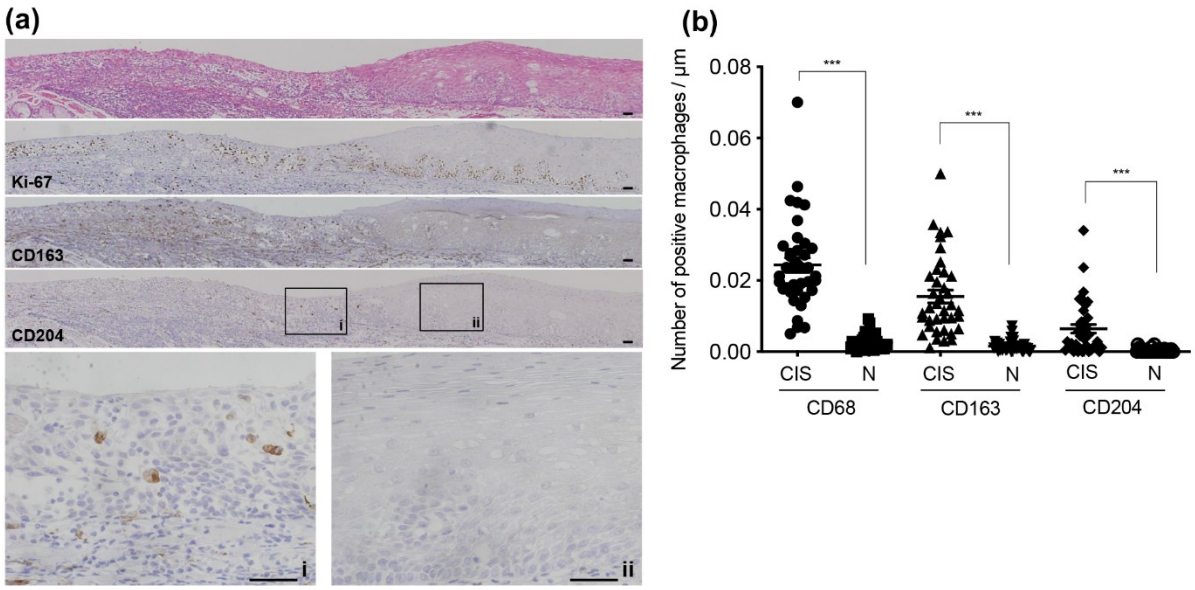

Figure 1. Frequent macrophage infiltration within the neoplastic squamous epithelia of the esophagus. (a) Hematoxylin and eosin specimen of a carcinoma *in situ* (CIS) excised by endoscopic submucosal dissection (top panel). Immunoreactivities of Ki-67, CD163 and CD204 are demonstrated in the following panels. i: Higher-power view of CD204 immunoreaction in the CIS (square, i). ii, Higher power view of CD204 immunoreaction in the non-neoplastic mucosa (square, ii). Bars, 100μm. (reproduced from Yokozaki, H., Koma, Y.-I., Shigeoka, M., Nishio, M. Cancer as a tissue: The significance of cancer-stromal interactions in the development, morphogenesis and progression of human upper digestive tract cancer. Pathol. Int., 2018; 68: 334–352. DOI: 10.1111/pin.12674) (37). (b) Numbers of intraepithelial CD68-, CD163- or CD204-positive macrophages per μm within 38 lesions of CIS excised by endoscopic submucosal dissection are compared with those of the corresponding non-neoplastic squamous epithelia (N). Student's *t*-test; ***, significant difference ($P < 0.001$).

Esophageal epithelial areas with high CD204- or CD163-positive macrophage density were morphologically neoplastic

To clarify the distribution of intraepithelial macrophages with CD204 or CD163 immunoreactivity within the whole squamous cell mucosal area, we investigated in 5 cases of surgically resected SCCs. Figures 2 to 6 show the mappings of the neoplastic lesions and the density of intraepithelial CD204-positive macrophages with representative histopathological findings of the neoplastic lesions outside the main SCC(s) of each case.

A total of 238 units of the mucosal area of the esophagus was observed for the CD204-positive intraepithelial macrophage density in case 1 (Figure 2). The maximum number of immunoreactive cells per unit area was 260. There were 12 intraepithelial neoplasia and 6 CIS independent of the invasive squamous cell carcinomas. The mean number of CD204-positive macrophages within the unit area of these intraepithelial neoplastic lesions was 10.1 ± 10.1 (SD), while that of the 20 units of the mucosal area of the non-neoplastic esophageal mucosa was 1.4 ± 1.6 (SD). There was a significant difference in the number of infiltrated CD204-positive macrophages between the neoplastic and non-neoplastic epithelia ($P = 0.011$). Figure 2c shows the representative images of the CIS indicated at the initial histopathological diagnosis. The mean numbers of intraepithelial CD204-positive macrophages in the units 29c, 32c and 46c were 20.0, 2.0 and 28.0, respectively (Supplemental Figure S1a). The distribution of CD163-positive macrophages within the same esophagus was almost parallel with that of CD204 (Supplemental Figures S2a and S3a).

A total of 172 units of the mucosal area of the esophagus was observed for the CD204-positive intraepithelial macrophage density in case 2 (Figure 3). The maximum number of immunoreactive cells per unit area was 434. There were 22 intraepithelial neoplasia and 19 CIS independent of the invasive squamous cell carcinomas. The mean number of CD204-positive macrophages within the unit area of these intraepithelial neoplastic lesions was 20.3 ± 25.8 (SD), while that of the 20 units of the mucosal area of the non-neoplastic esophageal mucosa was 101.4 ± 123.4 (SD). There was a significant difference in the number of infiltrated CD204-positive macrophages between the neoplastic and non-neoplastic epithelia ($P < 0.001$). Figure 3c shows the representative histological findings of the 3 intraepithelial neoplasia diagnosed at the initial histopathological examination. The mean numbers of intraepithelial CD204-positive macrophages in the units 14e, 27d and 33e were 8.0, 15.3 and 33.7, respectively (Supplemental Figure S1b). The distribution of CD163-positive macrophages within the same esophagus was almost parallel with that of CD204 (Supplemental Figures S2b and S3b).

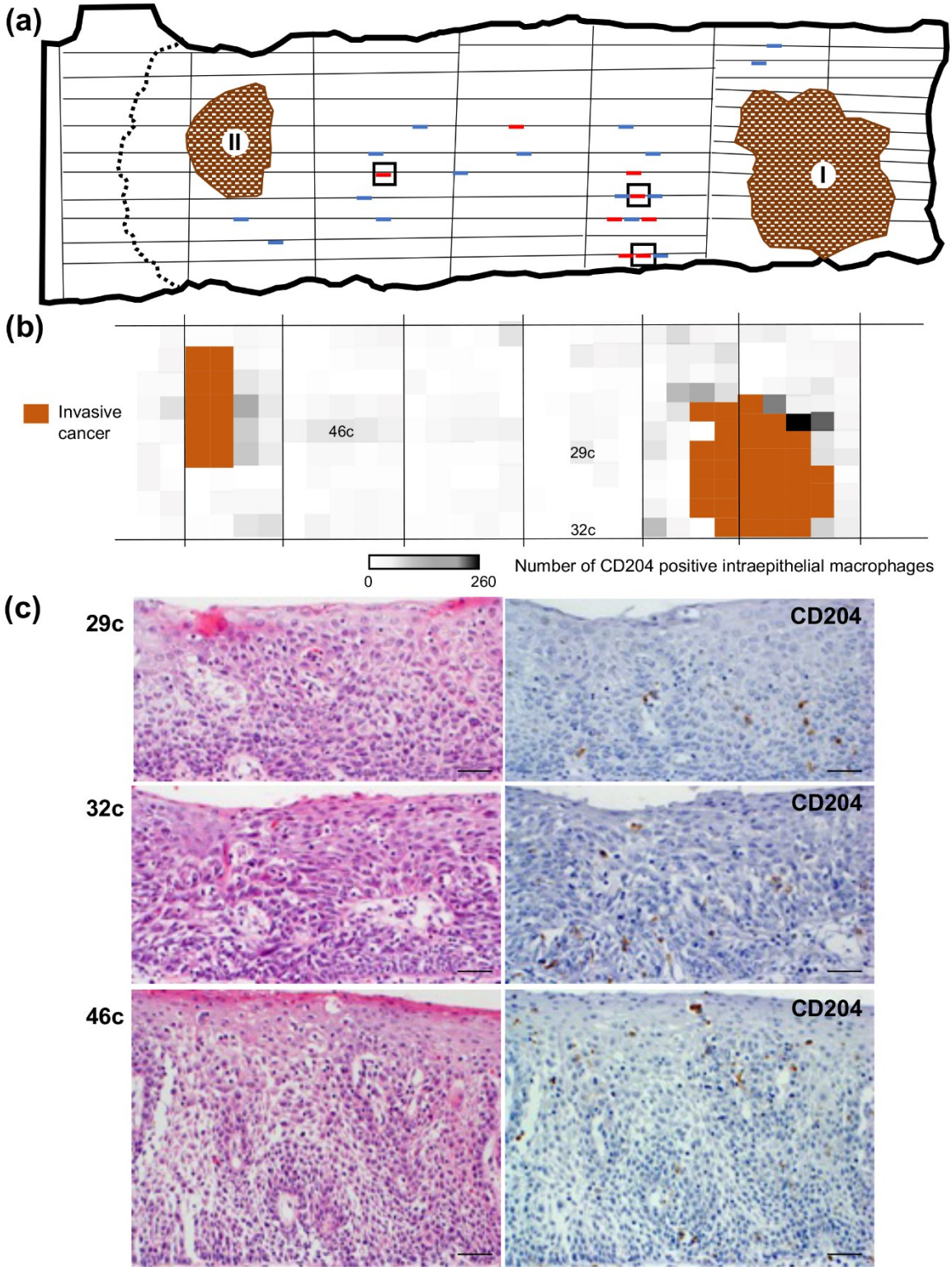

Figure 2. Intraepithelial macrophage density mapping of whole resected esophagus (case 1). (a) Mapping of the histopathological findings of the esophagus. There were two invasive cancers; a 30 x 30 mm plateau type elevated tumor with well-differentiated squamous cell carcinoma invading to the submucosa in the proximal side (I), and a 19 x 18 mm predominantly subepithelial type protruding lesion with moderately differentiated squamous cell carcinoma invading to the submucosa in the distal side (II). Scattered intraepithelial neoplasia (blue lines) and carcinoma *in situ* (red lines) were indicated by the step-cut histological examination. (b) Density mapping of CD204-positive intraepithelial macrophages. Number of CD204-positive cells per 5 mm unit length of epithelial area (0 to 260) is illustrated as white to black gradation. (c) Representative histopathological and immunohistochemical findings of the unit area with relatively high intraepithelial macrophage densities. Number of each panel law is corresponded to that in (b). Bars, 200 μm.

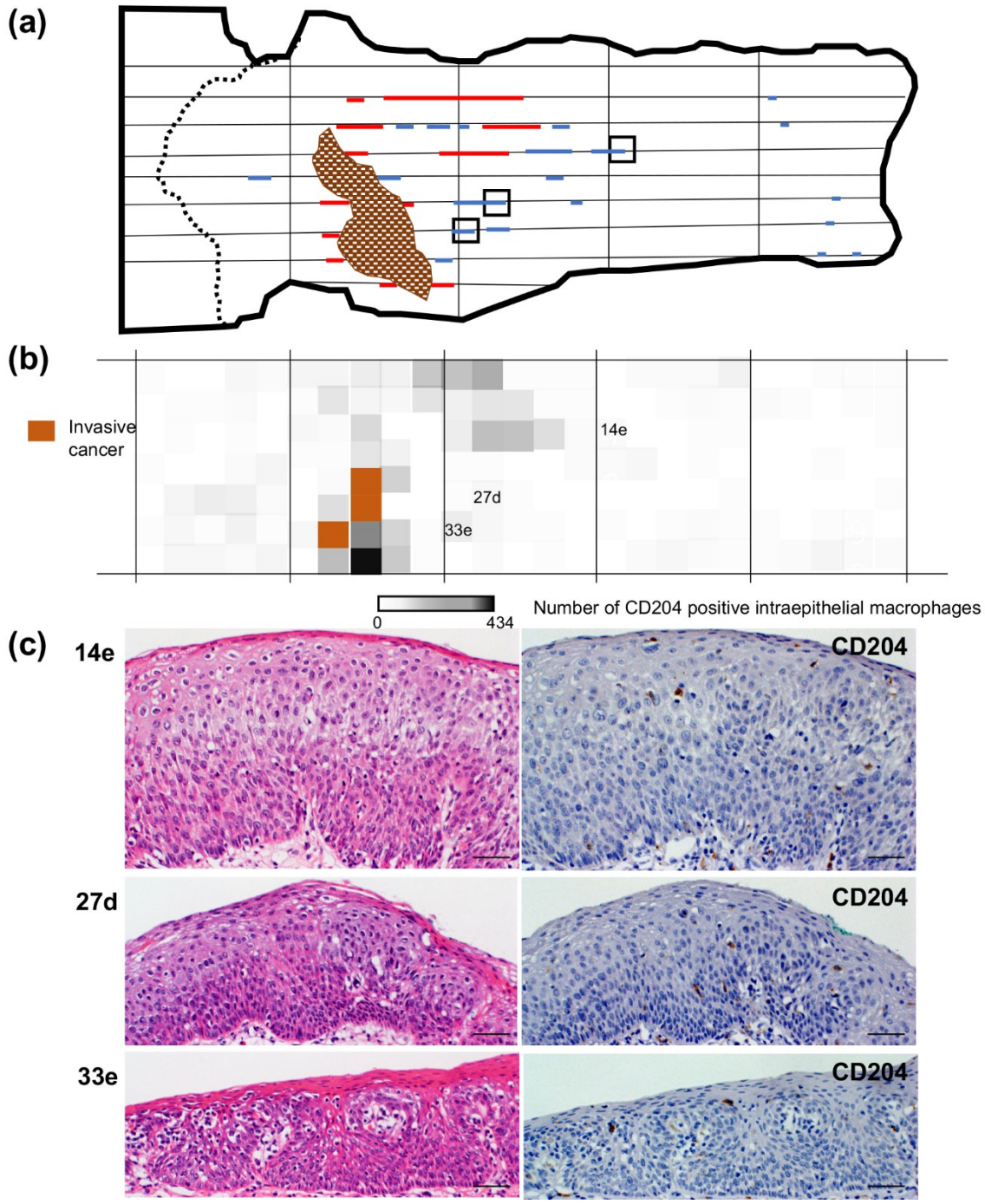

Figure 3. Intraepithelial macrophage density mapping of whole resected esophagus (case 2). (a) Mapping of the histopathological findings of the esophagus. There was an invasive cancer; a 45 x 30 mm slightly elevated type lesion with moderately differentiated squamous cell carcinoma invading to the submucosa. Scattered intraepithelial neoplasia (blue lines) and carcinoma *in situ* (red lines) were indicated by the step-cut histological examination. (b) Density mapping of CD204-positive intraepithelial macrophages. Number of CD204 positive cells per 5 mm unit length of epithelial area (0 to 434) is illustrated as white to black gradation. (c) Representative histopathological and immunohistochemical findings of the unit area with relatively high intraepithelial macrophage densities. Number of each panel law is corresponded to that in (b). Bars, 200 μm.

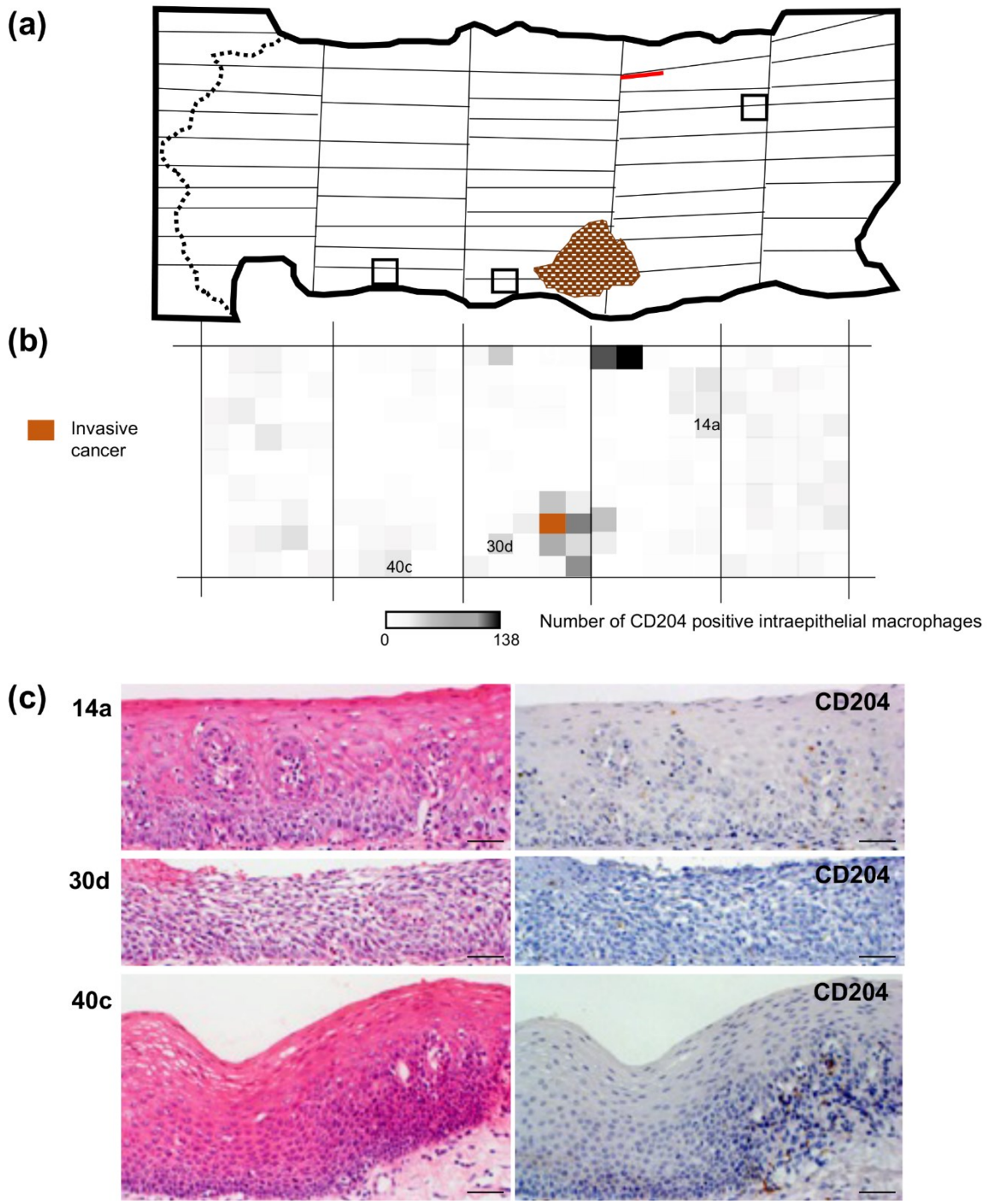

Figure 4. Intraepithelial macrophage density mapping of whole resected esophagus (case 3). (a) Mapping of the histopathological findings of the esophagus. There was an invasive cancer; a 25 x 19 mm superficial and flat plus slightly depressed type lesion with moderately differentiated squamous cell carcinoma invading to the muscularis mucosae. A carcinoma *in situ* (red lines) were indicated by the step-cut histological examination. (b) Density mapping of CD204-positive intraepithelial macrophages. Number of CD204-positive cells per 5 mm unit length of epithelial area (0 to 138) is illustrated as white to black gradation. (c) Representative histopathological and immunohistochemical findings of the unit area with relatively high intraepithelial macrophage densities. Number of each panel law is corresponded to that in (b). Bars, 200 μm.

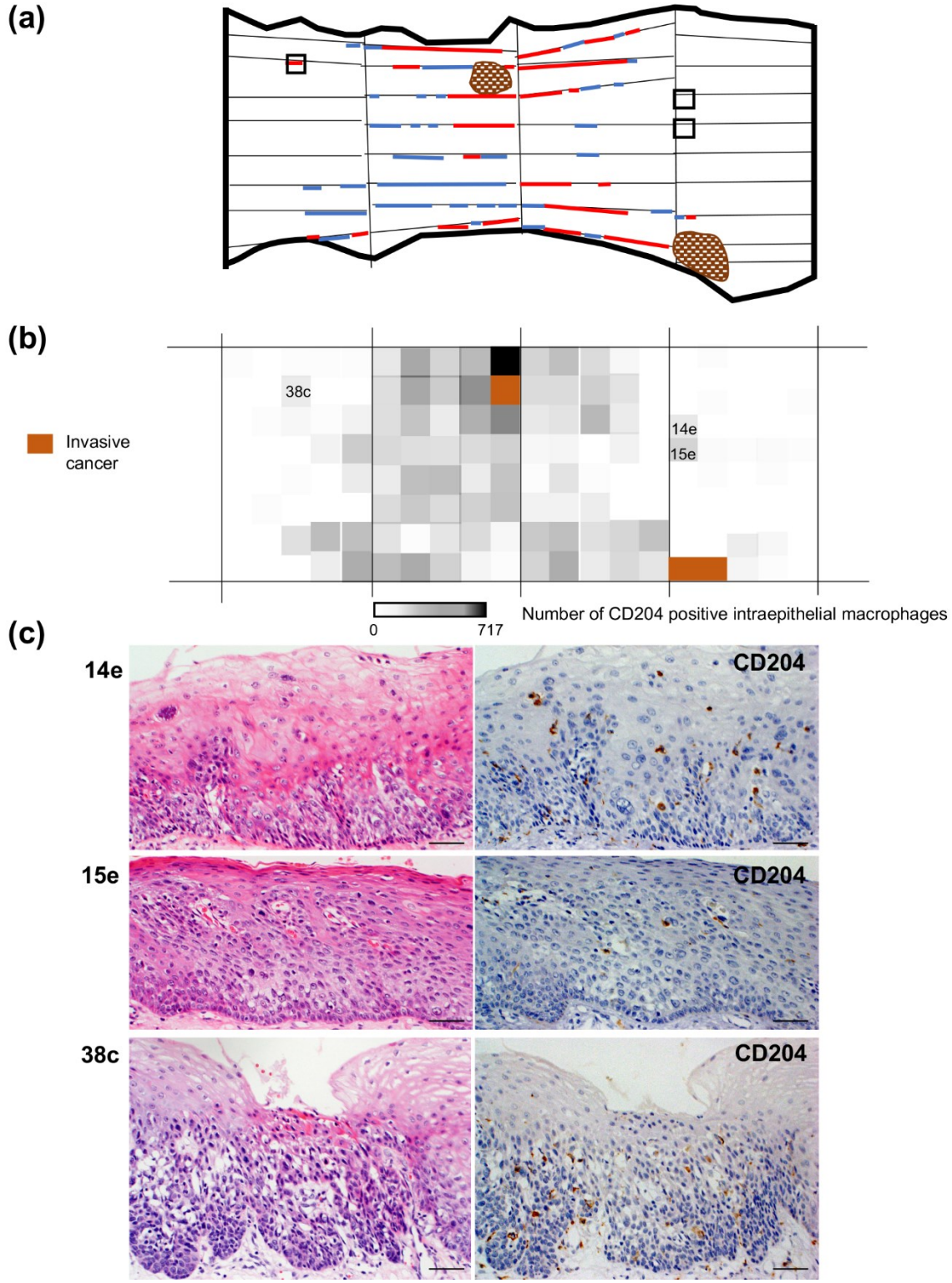

Figure 5. Intraepithelial macrophage density mapping of whole resected esophagus (case 4). (a) Mapping of the histopathological findings of the esophagus. There was an invasive cancer; a 70 x 40 mm flat plus slightly elevated type lesion with well-differentiated squamous cell carcinoma invading to the lamina propria mucosae. Scattered intraepithelial neoplasia (blue lines) and carcinoma *in situ* (red lines) were indicated by the step-cut histological examination. (b) Density mapping of CD204-positive intraepithelial macrophages. Number of CD204-positive cells per 5 mm unit length of epithelial area (0 to 717) is illustrated as white to black gradation. (c) Representative histopathological and immunohistochemical findings of the unit area with relatively high intraepithelial macrophage densities. Number of each panel law is corresponded to that in (b). Bars, 200 μm.

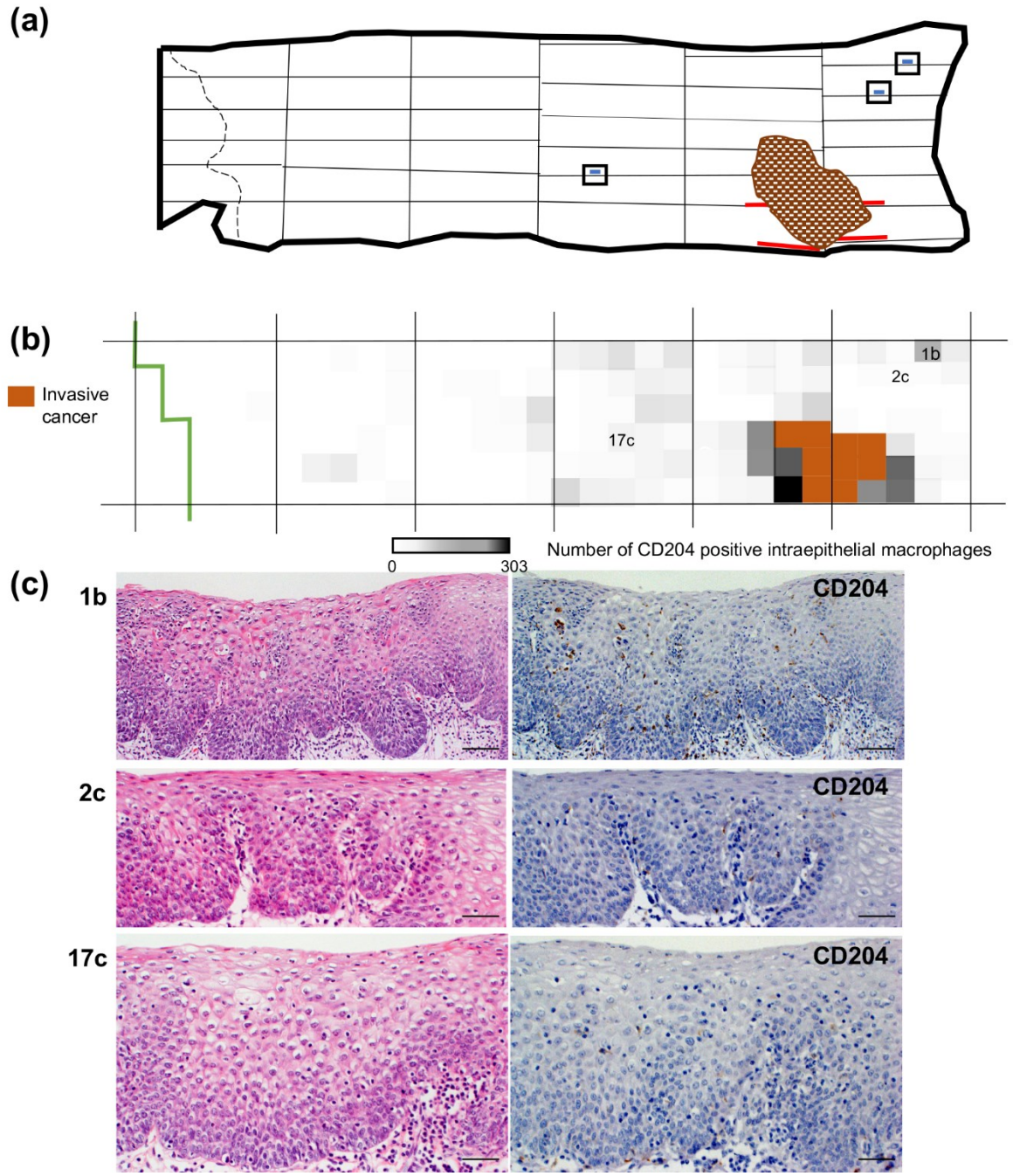

Figure 6. Intraepithelial macrophage density mapping of whole resected esophagus (case 5). (a) Mapping of the histopathological findings of the esophagus. There was an invasive cancer; a 33 x 20 mm slightly elevated plus flat type lesion with moderately differentiated squamous cell carcinoma invading to the submucosa. Three intraepithelial neoplasia (blue lines) were indicated by the step-cut histological examination. (b) Density mapping of CD204-positive intraepithelial macrophages. Number of CD204-positive cells per 5 mm unit length of epithelial area (0 to 303) is illustrated as white to black gradation. (c) Representative histopathological and immunohistochemical findings of the unit area with relatively high intraepithelial macrophage densities. Number of each panel law is corresponded to that in (b). Bars, 200 μm.

A total of 236 units of the mucosal area of the esophagus was observed for the CD204-positive intraepithelial macrophage density in case 3 (Figure 4). The maximum number of immunoreactive cells per unit area was 138. There were 2 CIS independent of the invasive squamous cell carcinomas. Although the mean number of CD204-positive macrophages within the unit area of these intraepithelial neoplastic lesions (115.7 ± 31.1 [SD]) was larger than that of the 20 units of the mucosal area of the non-neoplastic esophageal mucosa (0.9 ± 0.9 [SD]), the difference was not statistically significant ($P = 0.5$) because of the low number of CIS lesions. The representative histological findings of the units with higher numbers of macrophages than surrounding units are demonstrated in Figure 4c. Although they were not diagnosed as neoplastic lesions at the initial pathological diagnosis, each lesion shows definite neoplastic epithelial growth. The mean number of intraepithelial CD204-positive macrophages in the units 14a, 30a and 40c were 12.7, 22.7 and 12.3, respectively (Supplemental Figure S1c). The distribution of CD163-positive macrophages within the same esophagus was almost paralleled with that of CD204 (Supplemental Figures S2c and S3c).

A total of 165 units of the mucosal area of the esophagus was observed for the CD204-positive intraepithelial macrophage density in case 4 (Figure 5). The maximum number of immunoreactive cells per unit area was 717. There were 39 intraepithelial neoplasia and 37 CIS independent of the invasive squamous cell carcinomas. The mean number of CD204-positive macrophages within the unit area of these intraepithelial neoplastic lesions was 120.2 ± 100.7 (SD), while that of the 20 units of the mucosal area of the non-neoplastic esophageal mucosa was 1.0 ± 1.1 (SD). There was a significant difference in the number of infiltrated CD204-positive macrophages between the neoplastic and non-neoplastic epithelia ($P < 0.001$). Figure 5c depicts the representative histological findings of 2 units with higher numbers of macrophages than surrounding units (14e and 15e) and a unit with CIS (38e). Both units 14e and 15e demonstrate neoplastic growth within the basal part of the epithelia. The mean numbers of intraepithelial CD204-positive macrophages in the units 14e, 15e and 38e were 64.3, 119.3 and 241.7, respectively (Supplemental Figure S1d). The distribution of CD163-positive macrophages within the same esophagus was almost parallel with that of CD204 (Supplemental Figures S2a and S3a).

A total of 167 units of the mucosal area of the esophagus was observed for the CD204-positive intraepithelial macrophage density in case 5 (Figure 6, Supplemental Figure S1e). The maximum number of immunoreactive cells per unit area was 303. There were 3 intraepithelial neoplasia and 6 CIS independent of the invasive squamous cell carcinomas. The mean number of CD204-positive macrophages within the unit area of these intraepithelial neoplastic lesions was 136.6 ± 93.3 (SD), while that of the 20 units of mucosal area of the non-neoplastic esophageal mucosa was 0.9 ± 1.1 (SD). There was a significant difference in the number of infiltrated CD204-positive macrophages between the neoplastic and non-neoplastic epithelia ($P < 0.001$). The representative histological findings of the 3 intraepithelial neoplasia pointed out at the initial histopathological diagnosis are demonstrated in Figure 6c. The mean numbers of intraepithelial CD204-positive macrophages in the units 1b, 2c and 17c were 92.5, 2.0 and 14.0, respectively (Supplemental Figure S1b). The distribution of CD163-positive macrophages within the same esophagus was almost parallel with that of CD204 (Supplemental Figures S2e and S3e) in all units except the unit 37c (Supplemental Figure S4).

DISCUSSION

There is accumulating evidence of the clinicopathological significance of TAMs for the progression of SCCs arising not only in the esophagus but also in the other human organs has been accumulated. A high number of CD204-positive TAMs in the stroma was a significant prognostic factor of lung SCCs that was closely associated with an advanced pathological stage, T factor, N factor, vascular and pleural invasion (7). An increase in CD68-positive macrophages in the tumor stroma has been significantly associated with lymphatic metastasis of SCCs of the uterine cervix (2). A high number of CD163-positive TAMs in head and neck SCCs has been associated with advanced T stage, increased rates of nodal positivity, presence of vessel invasion, and poor patient outcome (3, 18). In addition, several investigators have investigated macrophage infiltration into the initial intraepithelial neoplastic growth of the squamous epithelia.

Hammes *et al.* counted CD68-positive macrophages in biopsy specimens of the normal uterine cervix, low-grade intraepithelial lesions, high-grade intraepithelial lesions, and invasive SCCs and found that the number of macrophages infiltrating the lesions was closely associated with the progression of cervical intraepithelial neoplasia independent of the background inflammation (6). Tongue leukoplakia tissues with high numbers of intraepithelial CD163-positive macrophages significantly associated with higher degrees of epithelial dysplasia, abnormal Ki-67 expression, and cytokeratin 13 loss in comparison with those with low CD163-positive macrophage infiltration (26). Pre-operative biopsy specimens of tongue leukoplakia progressed to invasive SCC have been shown to contain a significant number of CD163-positive intraepithelial macrophages (25). In another report, the number of CD163-positive macrophages infiltrating into early-stage SCCs of the esophagus was

significantly associated with the neo-angiogenic potential estimated by the increased microvessel density within the hot spots of the lesions (17).

Infiltration of a significant number of macrophages, especially macrophages expressing M2 markers, in the esophageal CIS lesions in comparison with the corresponding non-neoplastic squamous epithelia was also confirmed in the present study using histopathologically defined ESD specimens. Statistically significant associations of intraepithelial accumulation of M2-skewed macrophages with CIS and squamous intraepithelial neoplasia in the step-cut esophagectomy specimens were also demonstrated in 4 out of 5 esophageal cancer cases. A retrospective histopathological review of the mucosal units with a high number of intraepithelial CD204-positive macrophages in cases 3 and 4 or CD163-positive macrophages in case 5 also revealed intraepithelial neoplastic growth that was overlooked at the initial diagnosis of the esophagectomy specimens. These findings of the present study may suggest that macrophages have some biological roles in the early squamous carcinogenesis of the esophagus.

Wound-associated basal keratinocytes of mice were reported to secrete Ccl2 through activated Nrf2 and induced the production of EGF in macrophages to promote keratinocyte proliferation (33). It has been established that TAMs, especially those skewed to the M2-phenotype, promote growth and motility of cancer cells by secreting growth factors (20, 32) and chemokines (4, 8, 13). Macrophages activated by defined cytokines were reported to promote the growth and migration of an immortalized human esophageal squamous epithelial cell line with the induction of IL6 and p38MAPK signals by co-culture experiments (14). These reports and our present observations suggest that the macrophages found within the initial proliferative lesions of the squamous epithelia may play biological roles in their progression.

In terms of the triggers of macrophage recruitment into the pre-neoplastic squamous epithelia of the esophagus, several studies suggest that these triggers may consist of responses to the DNA damage signals elicited by the exposure to the carcinogen(s). Acetaldehyde associated with alcoholic beverages is carcinogenic to humans (Group 1) and confirmed the Group 1 classification of alcohol consumption and of ethanol in alcoholic beverages. (23) It has been well-established that the *aldehyde dehydrogenase 2* (*ALDH2*) variant allele confers higher relative risk of esophageal SCC (36). Amanuma *et al.* demonstrated that significantly elevated acetaldehyde-derived DNA damage signals represented by N^2-ethylidene-2'-deoxyguanosine were higher in the esophagus of *Aldh2*-knockout mice than in wild-type mice upon ethanol consumption (1). Another study found that infiltration of macrophages into the squamous epithelia of ethanol-treated *Aldh2*-knockuout mice was significantly elevated compared to that in wild-type animals (37). In addition, p38MAPK is known to be involved in the DNA damage stress responses during cancer development (5). Macrophages may receive the DNA damage signals from and recruited into the injured squamous epithelia of the esophagus establishing the cell-to-cell interactions to promote the pre-neoplastic growth and the accumulation of the replication stress that leads to the mutation of the driver gene(s) (37). In fact, intensive sequencing of micro-scale human esophageal samples demonstrated the progressive age-related expansion of clones carrying mutations of driver genes even in physiologically normal squamous epithelia, which was substantially accelerated by alcohol consumption and smoking (19, 35).

The limitation of the present study is that the results just demonstrate the close association of intraepithelial accumulation of M2-skewed macrophages with neoplastic epithelia of the human esophagus. Further molecular pathological analysis to elucidate the detailed biological roles of intraepithelial M2-macrophages in the development of esophageal SCC will be required. While detection of the intraepithelial M2-macrophages may assist in the histopathological assessment of esophageal mucosal lesions at risk.

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AUTHOR CONTRIBUTIONS

Y.I. and H.Y. conceived and performed the experiments; Y.I. and H.Y. wrote the manuscript.

CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

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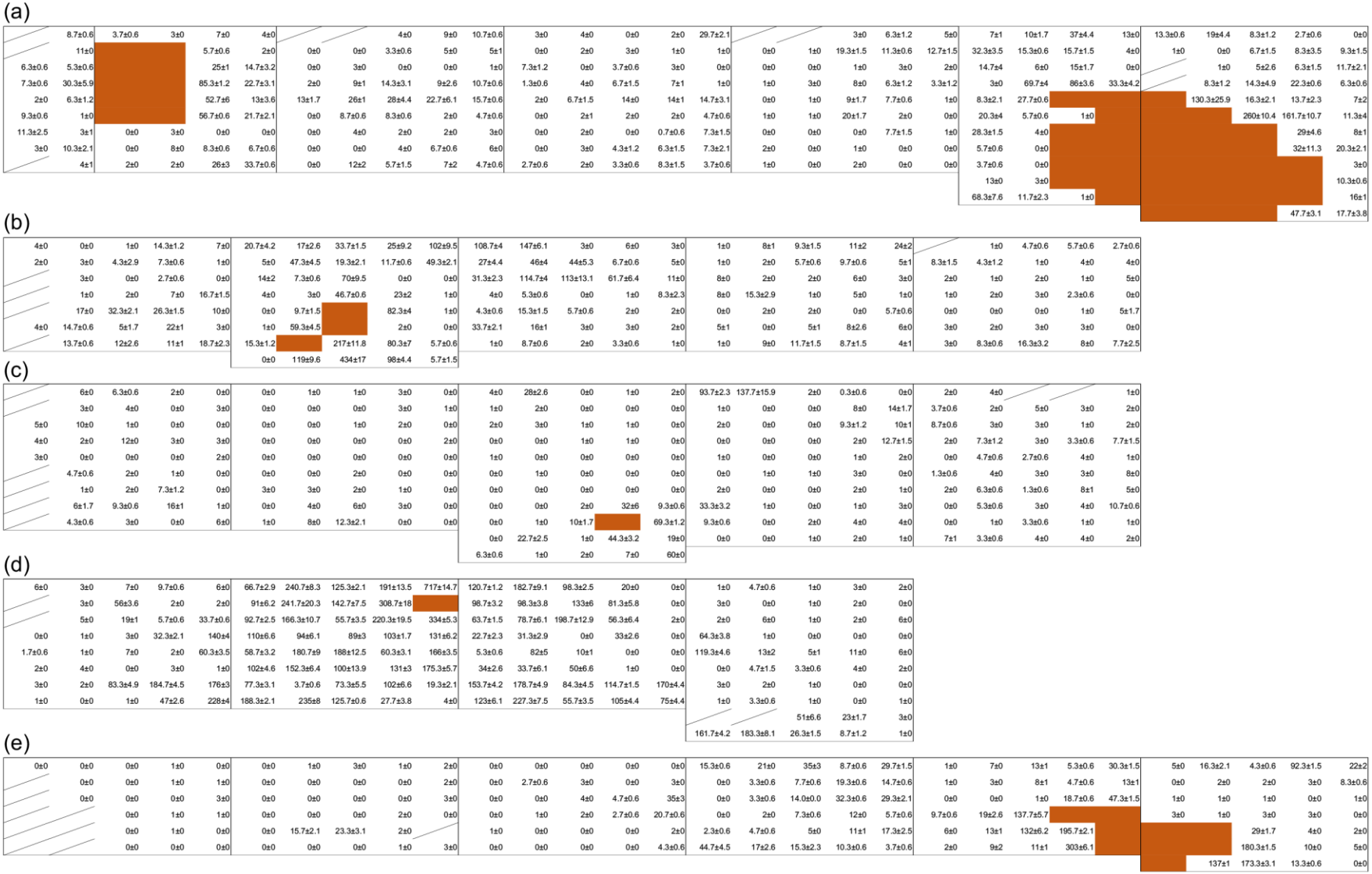
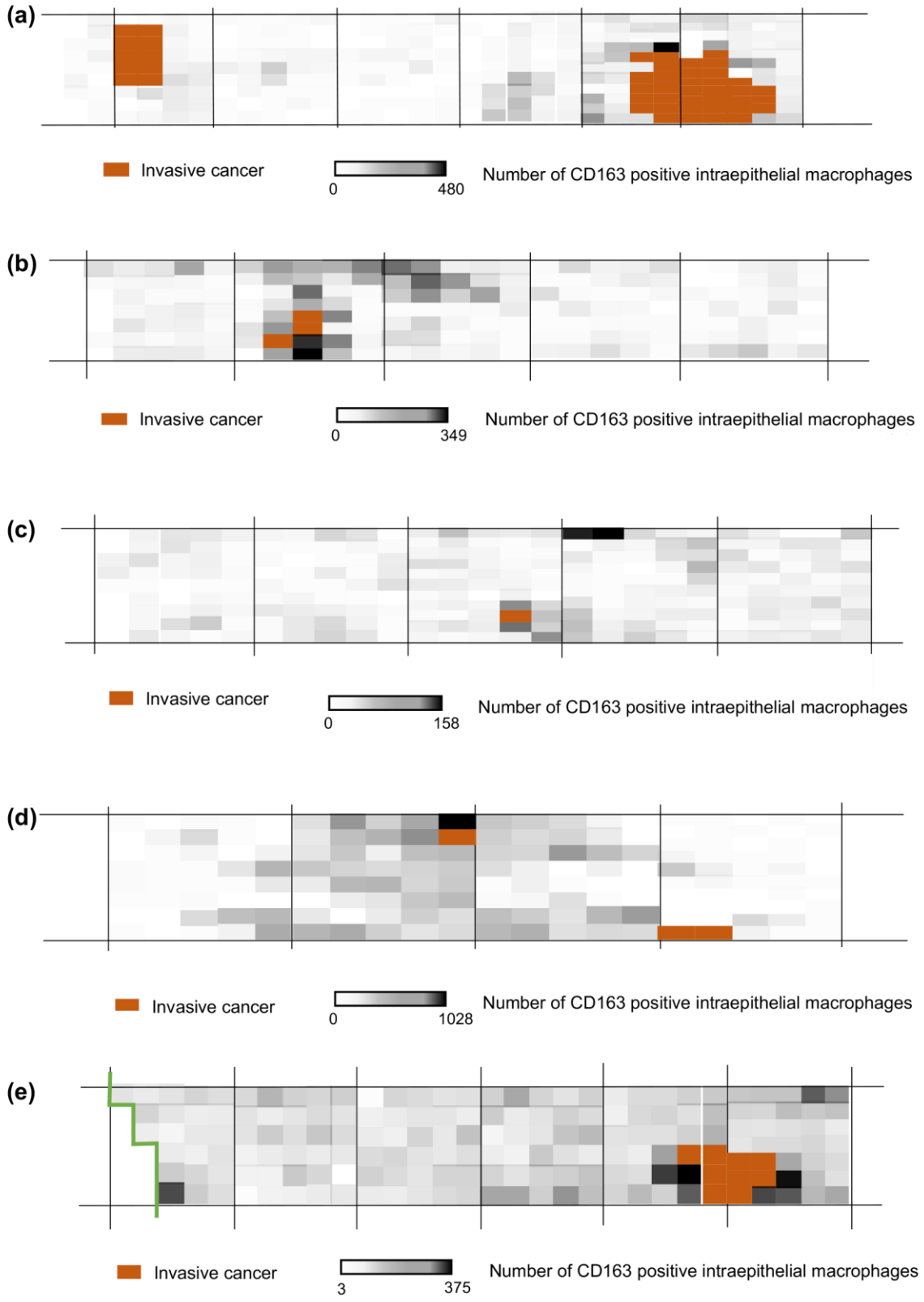
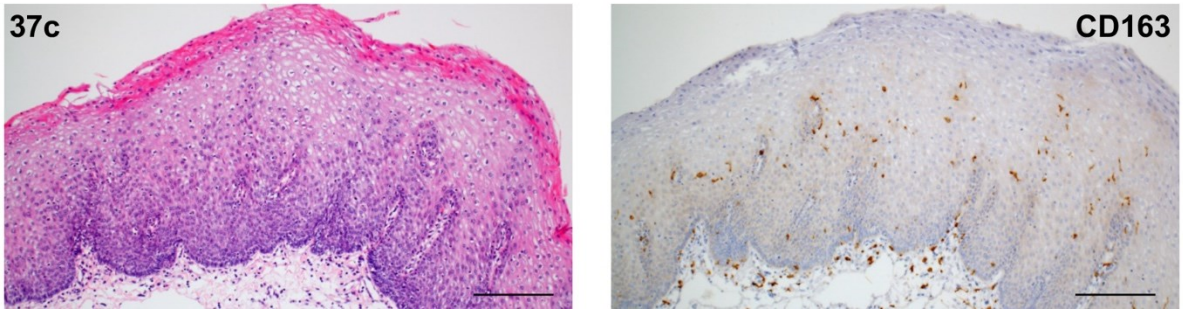


Supplemental Figure S1 Number of CD204-positive cells per 5 mm unit length of epithelial area of the resected esophagus of case 1 (a), case 2 (b), case 3 (c), case 4 (d) and case 5 (e). Mean number with SD of the triplicate counts per unit are described.



Supplemental Figure S2 Density mappings of CD163-positive intraepithelial macrophages of the resected esophagus of case 1 (a), case 2 (b), case 3 (c), case 4 (d) and case 5 (e). Number of CD163-positive cells per 5 mm unit length of epithelial area is plotted as white to black gradation.

Supplemental Figure S3 Number of CD163-positive cells per 5 mm unit length of epithelial area of the resected esophagus of case 1 (a), case 2 (b), case 3 (c), case 4 (d) and case 5 (e). Mean number with SD of the triplicate counts per unit are described.



Supplemental Figure S4 Histopathological findings of unit 37c of the case 5. Thickened squamous epithelia shows surface parakeratosis and neoplastic growth of the one third of the basal area with elongated vascular papillae (left). Scattered CD163-positive cells are observed within the epithelial area (right). The mean number of triplicate counts was 267.7 (Supplemental Figure 3e). Bars, 200 μm.