A Positive Correlation Between Neutrophils in Regional Lymph Nodes and Progression of Gastric Cancer

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Abstract. *Backgrond/Aim: Recent evidence indicates that inflammation is a hallmark of cancer. Tumor-associated neutrophils (TANs) contribute to tumor invasion. However, whether TANs and lymph node metastasis are related is unknown. Intranodal lymphatic vessel density (LVD) is significantly correlated with tumor progression, lymph node metastasis in gastric cancer. Herein, we investigated the effects of TANs in regional lymph nodes. Materials and Methods: We investigated the association of the density of TANs in regional lymph nodes with clinicopathological features by immunohistochemistry. Furthermore, we examined the prognostic value of TANs in lymph nodes. Results: The number of TANs in regional lymph nodes was positively correlated with intranodal LVD and micrometastases. Patients with higher cluster of differentiation 15+ TAN counts had significantly poorer prognosis than those with lower counts. Conclusion: Our results suggest that TANs in regional lymph nodes promote the invasion of lymph nodes by cancer cells via augmentation of lymphangiogenesis and thereby contribute to tumor progression.*

The immune system initially eradicates potentially tumorigenic cells. However, as a malignant lesion grows, such immune responses virtually vanish because of an immunosuppressive network that develops in the tumor microenvironment. The close link between inflammation and cancer is well-documented (1). Inflammatory cells are a pivotal component of the tumor microenvironment (2, 3), and many immunocomponent cells –including M2 macrophages, regulatory T-cells, myeloid-derived suppressor cells, and N2 neutrophils– have pro-tumorigenic functions (4, 5). Neutrophils are the most abundant sub-population of leukocytes in the circulation, and they are principally involved in the inflammatory response. Reportedly, Transforming Growth Factor-β within the tumor microenvironment induces a population of tumor-associated neutrophils (TANs) with a pro-tumor phenotype (6), and tumor-associated neutrophils (TANs) are associated with poor prognosis for patients with various types of solid tumors–including cervical cancer (7), melanoma (8), renal cell carcinoma (9), and gastric cancer (10). However, the extent of the pro-tumor activity of TANs in regional lymph nodes remains unclear. Additionally, lymphangiogenesis in regional lymph nodes is correlated with lymph node micrometastasis (MM) and progression of gastric cancer (11). In the present study, we aimed to investigate the correlation of lymph node MM and intranodal lymphangiogenesis with cluster of differentiation (CD) 15+ TANs in tumor growth and progression.

Materials and Methods

Patients and surgical specimens. Formalin-fixed, paraffin-embedded blocks of lymph nodes were collected from patients with advanced gastric cancer who underwent surgical treatment at the Osaka City University Hospital, Japan. A total of 1,596 lymph nodes from 52 consecutive patients treated with surgery in 2011 were used to estimate the number of TANs, and these patients were assigned to the estimation group. For survival analysis, data from 96 patients who underwent surgical resection in the same hospital during 2006 and 2007 were assigned to the survival group. Patients who received preoperative chemotherapy and radiotherapy were excluded from this study. All dissected lymph nodes were immunohistochemically-analyzed in the estimation group. For each primary tumor in the survival group, the seven lymph nodes adjacent to the tumor were analyzed because there were no significant differences between the average number of CD15+ TANs in all lymph nodes and that in the seven peritumonal lymph nodes in the estimation group.

Dissected primary tumors and lymph nodes were stained with hematoxylin and eosin (HE), and pathological stage was determined according to the seventh edition of the Union for International Cancer Coutrol (UICC) Tumor-Node-Metastasis (TNM) classification (12). Postoperative follow-up assessment was performed every three months for the first two years, every six months during the third and fourth years, and annually thereafter.

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A total of 1,596 lymph nodes were dissected from 52 patients. Of the 1,596 lymph node resected, 225 had metastasis based on HE staining. The remaining 1371 lymph nodes were subjected to immunohistochemistry (IHC) in order to detect occult metastasis. Each patient provided written informed consent.

Immunohistochemical staining. Mouse monoclonal antibody against CD15 (1:50 dilution; Abcam, Tokyo, Japan), mouse monoclonal antibody D2-40 (Dako, Kyoto, Japan), and monoclonal pan-cytokeratin antibody (AE1/AE3; Invitrogen, Camarillo, CA, USA) were used to label TANs, lymphatic vessels, and occult metastases, respectively. Specimens from paraffin-embedded blocks were cut into 4-μm thick sections. After incubation at 60°C for 10 min, samples were deparaffinized in xylene and rehydrated with a graded series of ethanolic solutions. Slides were then washed twice for 5 min in phosphate buffered saline (PBS). Endogenous peroxidase activity was blocked by incubating the sections for 15 min in absolute methanol containing 3% hydrogen peroxide. Samples were washed in PBS and then microwaved for 10 min for the purpose of antigen retrieval. Specimens were incubated in nonspecific staining blocking reagent (Dako) to prevent nonspecific labeling. Specimens were then incubated with primary antibodies at 4°C overnight, and then washed with PBS for 10 min. Sections were incubated with secondary antibody for 10 min at room temperature, then washed in PBS, and processed for labeling with 3-3'-diaminobenzidine for 5 min. Each specimen was counterstained with HE before mounting. All reactions were performed using appropriate positive and negative controls.

Evaluation of immunohistochemistry. Evaluation of the density of CD15+ TANs was performed as described previously (7). In brief, sections were scanned at low magnification (×100) in order to select five fields with the greatest number of CD15+ TANs ('hot spots'). The number of CD15+ TANs was then counted at ×200 magnification (examination area, 0.25 mm^2). The density of CD15+TANs was determined as the mean value of the cell counts in the five fields. Evaluation of lymphatic vessel density (LVD) and the classification of metastatic lymph nodes were previously reported (13).

Statistical analysis. The Mann–Whitney test was used to assess the association between the number of CD15+ TANs and clinicopathological features. The Kaplan–Meier method was used to produce the overall survival (OS) curves and a log-rank test was used to assess the significance of differences in survival. The day of surgery was taken for starting measurement of overall survival. A Cox proportional regression model was used for univariate and multivariate analyses of prognostic factors. *p*-Values of less than 0.05 were considered statistically significant. Each statistical analysis was performed using SPSSII (IBM Corporation, Armonk, New York, USA) software.

Results

Evaluation of CD15-positive TANs in regional lymph nodes. Using immunohistochemical analysis with a pan-cytokeratin antibody, we detected MM or isolated tumor cells (ITCs) in 189 out of the 1,596 lymph nodes that we analyzed. More specifically, MM was evident in 109 lymph nodes and ITCs were evident in 80 nodes. We excluded 225 metastatic lymph

nodes because we intended to more specifically investigate the relationship between TANs in lymph nodes and MM. Immunohistochemistry with the antibody CD15 was used to estimate the number of TANs in lymph nodes (Figure 1A and B). CD15+TANs were mainly located in the sub-capsular, cortical, and medullary sinuses of lymph nodes and were more abundant in regions surrounding MM (Figure 1C). For 1,415 lymph nodes examined, the mean number of TANs per lymph node was 49.6±27.7 (range=0-260), and the median value was 39.

We then calculated LVD to assess lymphangiogenesis in regional lymph nodes; we found that intranodal LVD in micrometastatic lymph nodes and those with ITCs were significantly higher than those of lymph nodes without metastasis (*p*<0.001) (Figure 2). Similarly, the mean number of CD15+ TANs in lymph nodes with micrometastasis or ITCS was significantly higher than that in those of lymph nodes without (*p*=0.0295, Figure 3A). To examine the correlation between intranodal LVD and the number of TANs in lymph nodes, we compared the density of CD15+ TANs in lymph nodes with high LVD with that in those with low LVD; each of 1415 lymph nodes were categorized as either a high-LVD (≥3.9) lymph node or a low-LVD (<3.9) lymph node based on the median LVD number, which is 3.9 and was determined in a previous study. The density of CD15+ TANs in high-LVD lymph nodes was significantly higher than that in low-LVD lymph nodes (*p*=0.0011) (Figure 3B). Thus, the density of CD15+ TANs increased in micrometastatic lymph nodes and was positively correlated to LVD.

Association of TANs in lymph nodes with clinicopathological factors. To evaluate the correlation between CD15+ TANs and clinicopathological features, we examined 11 patients with stage I/II and 41 patients with stage III/IV gastric cancer. Out of these 52 patients, 34 (67%) had lymph node metastases. The density of CD15+ TANs was higher in patients that had metastasis to lymph nodes; additionally, the average number of CD15+ TANs was higher in diffuse-type carcinoma than in intestinal-type carcinoma (Fig. 4). No correlation was found between the number of CD15+ TANs and depth of invasion, p-stage, lymphatic invasion, or venous invasion (Figure 4).

Impact of TANs in lymph nodes on survival of patients with gastric cancer. To investigate the prognostic value of CD15+ TAN counts in regional lymph nodes, we examined a survival group comprising of 96 patients; each of these patients was assigned to one of two groups: the high-TAN group (n=61 patients) or the low-TAN group (n=35 patients), based on the median number of TANs (Figure 5), which was 39 and was determined using data from the estimation group. The mean OS times for the high- and low-TAN groups were 50 months and 69 months, respectively. The survival rate of
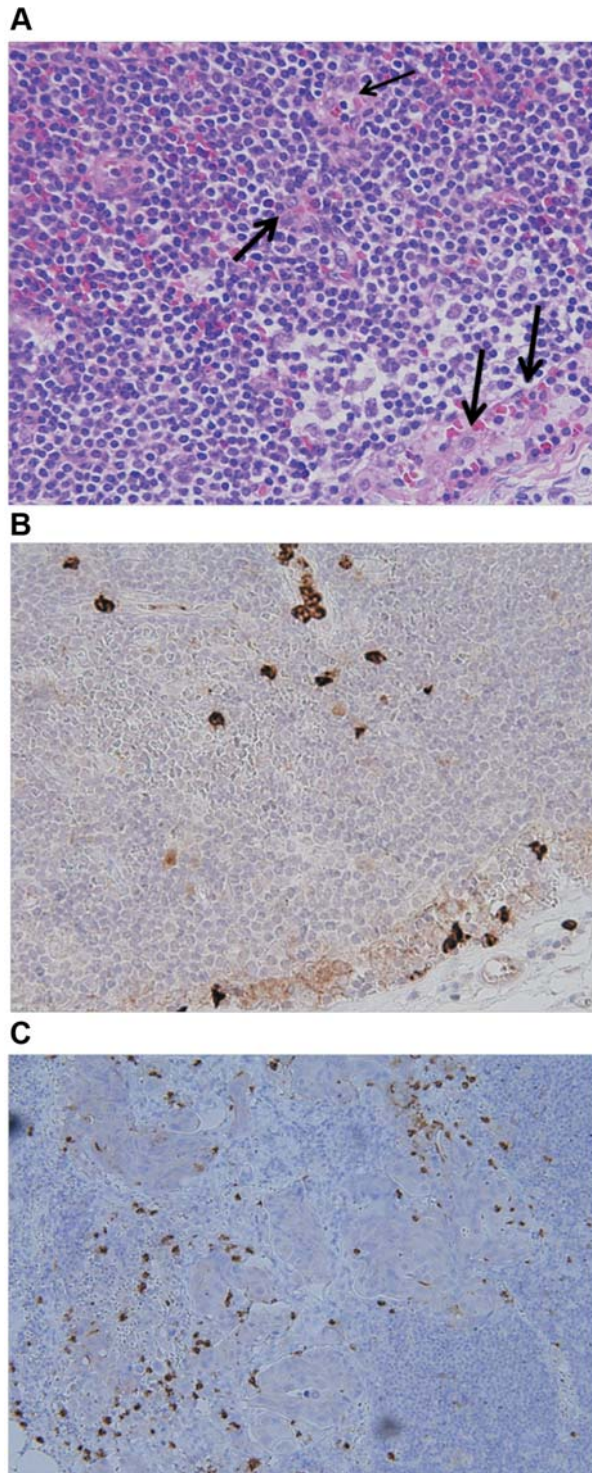
Figure 1. *AB: Hematoxylin and eosin stain and immunohistochemical staining of tumor-associated neutrophils (TANs) in lymph nodes (at ×400 magnification). TANs were identified as cells with lightly stained cytoplasm by HE staining (arrows). Cluster of differentiation(CD)15+ TANs were mainly detected in sub-capsular, cortical, and medullary sinuses of lymph nodes. C: CD15+ TANs mainly infiltrated to peritumoral region of micrometastases (×200 magnification).*
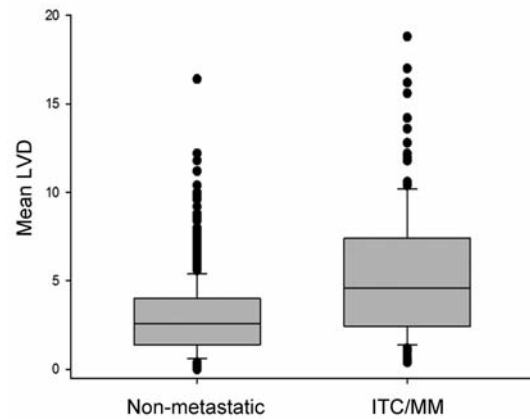

Figure 2. *Comparison of lymphatic vessel density (LVD) between micrometastatic and non-metastatic lymph nodes. Mean LVD of each lymph node were plotted (•). The box indicates the first and upper quartiles, and the bar in the box the median value. There were significant differences in LVD between micrometastatic lymph nodes/lymph nodes with isolated tumor cells (ITC/MM) and non-metastatic lymph nodes: 5.4±3.0 vs. 2.9±1.5, respectively, *p<0.001.*

the high-TAN group was significantly lower than that of the low-TAN group (Figure 6A, *p*=0.0039, log-rank test). Furthermore, the mean recurrence-free survival (RFS) times for the high- and low-TAN groups were 47 months and 69 months, respectively. The RFS rate of the high-TAN group was also significantly lower than that of the low-TAN group (Figure 6B, *p*=0.0011, log-rank test).

Univariate survival analysis showed that the p-stage, number of TANs, LVD, lymphatic invasion, and venous invasion each had prognostic value with regard to OS and RFS (Table I). Multivariate analysis showed that only p-stage was an independent prognostic factor of OS and RFS (*p*=0.031 and *p*=0.033, respectively, Table I).

Discussion

We found that the number of CD15+ TANs was i) significantly higher in lymph nodes with MM than in those without, and ii) significantly associated with high LVD in lymph nodes and poor prognosis. These results indicate that TANs appear to migrate to micrometastatic lymph nodes through the lymphatic system during the initial stage of metastasis and probably facilitate tumor progression by influencing the microenvironment in lymph nodes such that metastatic cancer cells will be more likely to proliferate.

Reportedly, increase of intranodal lymphangiogenesis is correlated with metastasis to distant lymph nodes and organs (14, 15). Accelerated intranodal lymphangiogenesis and ensuing expansion and dilation of the lymphatic network increase lymph flow and promote metastatic transformation of
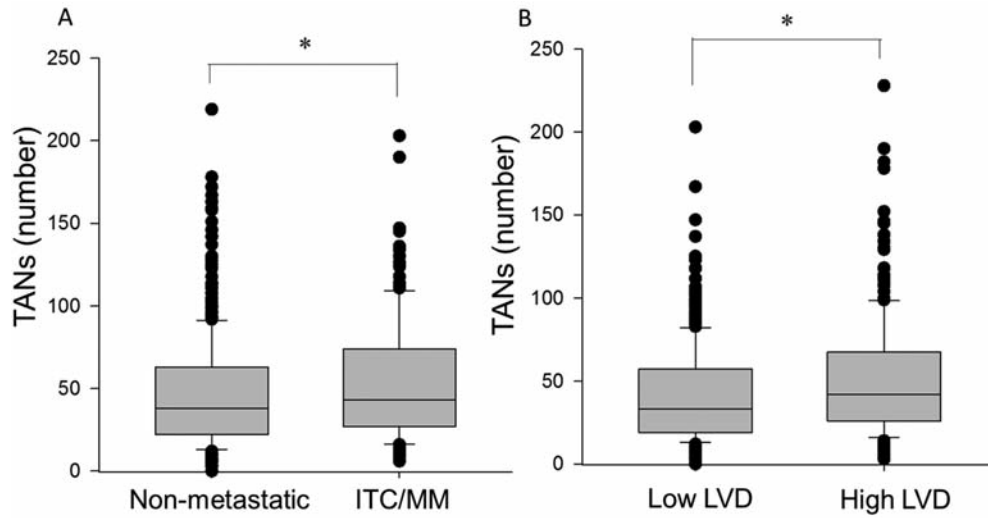
Figure 3. *Comparison of the number of Tumor-associated neutrophils (TANs) between micrometastatic and non-metastatic lymph nodes (A), and lymph nodes with low lymphatic vessel density (LVD) and those with high LVD (B). The mean number of TANs in each lymph node were plotted (•). The box indicates the first and upper quartiles, and the bar in the box the median value. The number of TANs was significantly increased in micrometastatic lymph nodes/lymph nodes with isolated tumor cells (ITC/MM) and lymph nodes with high LVD. *p<0.05.*
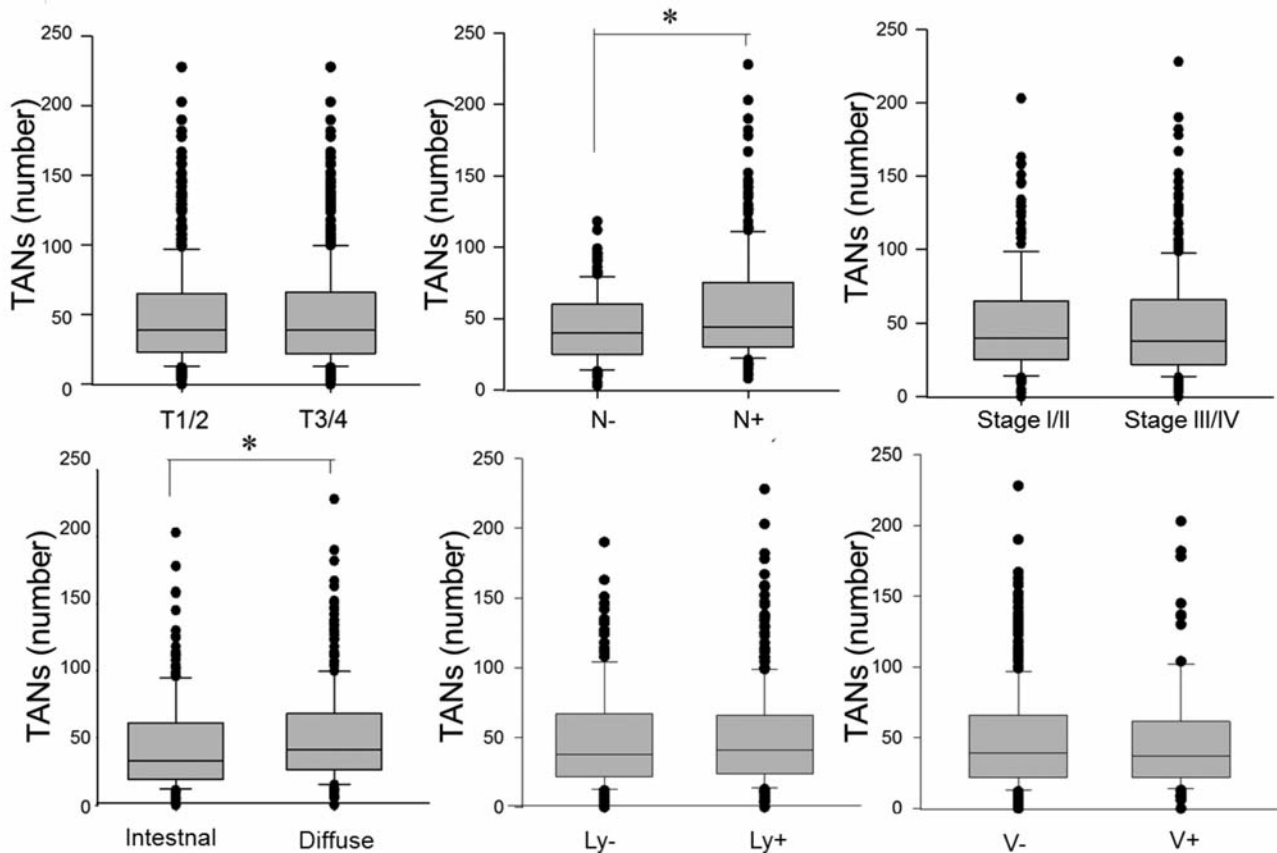

Figure 4. *Correlation between lymphatic vessel density (LVD) of lymph nodes and clinicopathological factors: pathological tumor invasion (pT) (A), pathological nodal metastasis (pN) (B), Union for International Cancer Coutrol (UICC) Tumor-Node-Metastasis (TNM) classification (pTNM) (C), histological type (D), lymphatic invasion (Ly) (E), and venous invasion (V) (F). The mean number of TAN in each lymph node were plotted (•). The box indicates the first and upper quartiles, and the bar in the box the median value. The density of cluster of differentiation 15+TANs was increased in patients with positive lymph nodes and diffuse-type carcinoma. +: With; −: without. *p<0.05.*
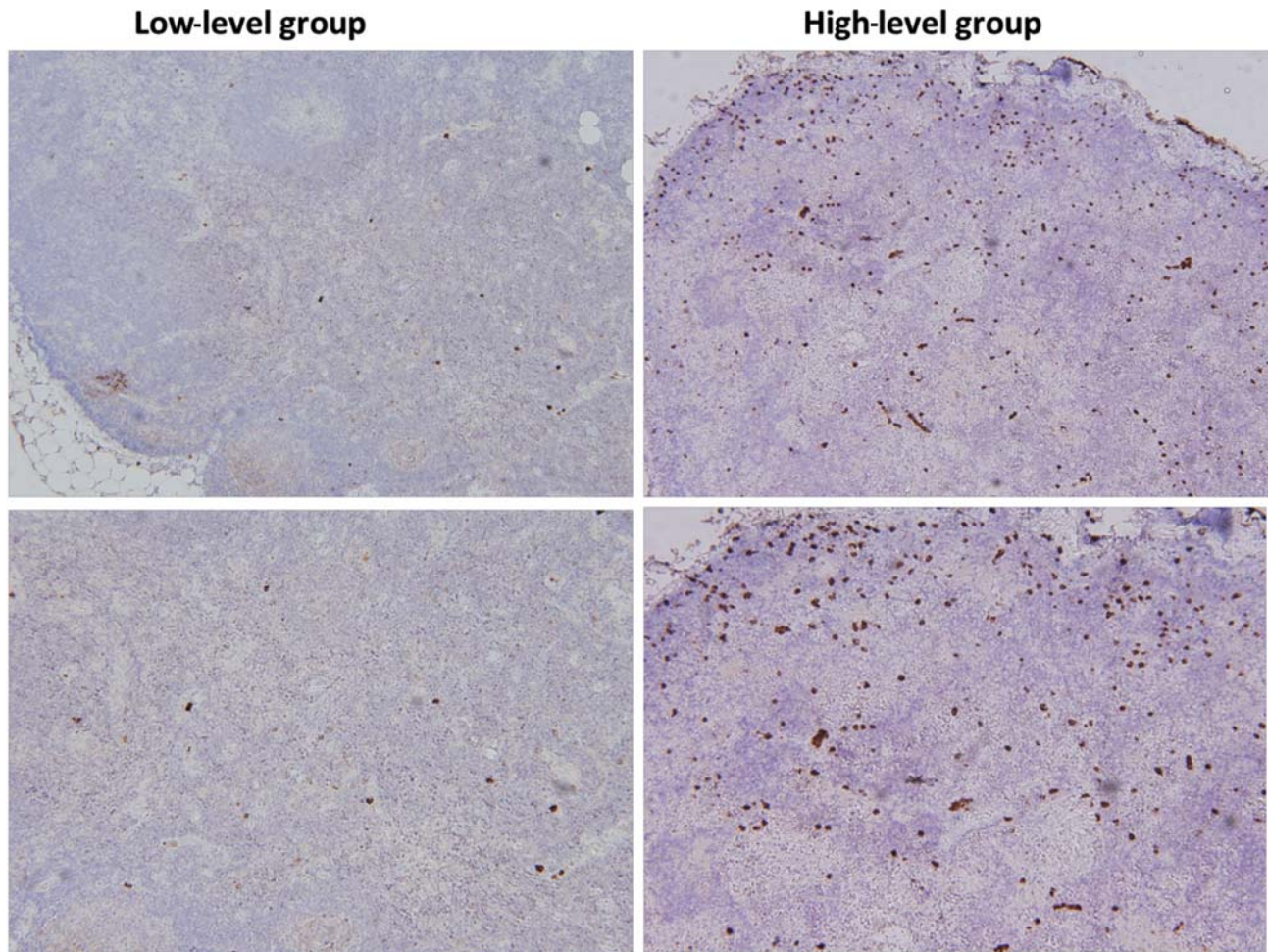
Figure 5. *Cluster of differentiation (CD) 15$^+$ Tumor-associated neutrophils (TANs) in regional lymph nodes. Low (A, B) and high (C, D) density of CD15$^+$ TANs in lymph nodes. A, C: ×100 magnification; B, D: ×200 magnification.*

cancer cells (16). Furthermore, lymphangiogenesis in sentinel lymph nodes is initiated prior to the arrival of cancer cells (17). Previously, we found that that increases of LVD are significantly correlated with MM in regional lymph nodes (12). Thus, it is probable that intranodal lymphangiogenesis is an initial stage that includes formation of tiny metastases, and lymph node MM is important as a preparatory phase of overt metastasis. Our findings indicate that TANs in lymph nodes play an important role in the formation of lymph node metastases.

TANs produce large quantities of arginase, which results in CD8$^+$ T-cell inactivation and inhibits CD8$^+$ T-cell-dependent anti-tumoral responses in tumorous mice (6). Moreover, the production of CC chemokine ligand (CCL) 17, which recruits T-regulatory cells (18) known to support tumor growth, was enhanced in TANs (19). Neutrophils also produce elastase which hydrolyses insulin receptor substrate-1, which leads to cell proliferation by enhancing the Platelet-Derived Growth Factor Receptor signaling (20). Matrix metalloprotease-9, which induces release of vascular endothelial growth factor (VEGF) from the extracellular matrix, was expressed only in neutrophils in a genetic mouse model of pancreatic cancer, and depletion of neutrophils inhibited VEGF-mediated angiogenesis (21). Queen *et al*. reported that breast cancer cells promote the release of oncostatin M by neutrophils and that cancer cells stimulated by oncostatin M subsequently increase VEGF production and cell invasiveness (22). Thus, neutrophils are believed to influence tumor progression. Our results also indicated that CD15$^+$ TANs in lymph nodes contribute to tumor progression by facilitating lymphangiogenesis and the consequent colonization of lymph nodes by cancer cells.
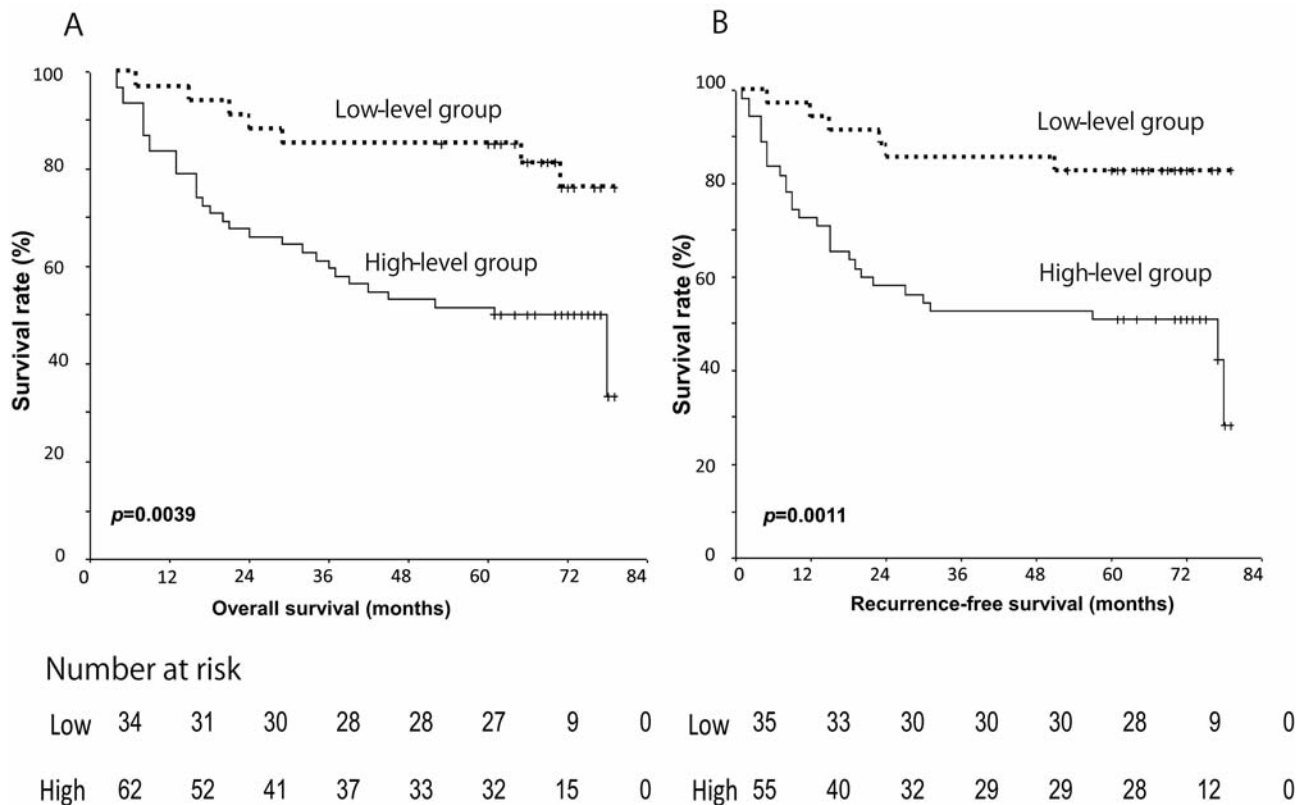
Figure 6. *Kaplan–Meier curves for patients with gastric cancer in the low-Tumor-associated neutrophils (TANs) and high-TAN groupc. A: The survival rate of patients in the high-TAN group was significantly lower than that of patients in the low-TAN group (p=0.0039, log-rank test). B: Recurrence-free survival times were significantly longer in the low-TAN group relative to those of patients in the high-TAN group (p=0.0011, log-rank test).*

The question of how TANs participate in lymphangiogenesis then arrises. As shown in many previous reports, macrophages are essential to inflammatory lymphangiogenesis. TANs might have the potential to recruit macrophages by secreting chemokines such as CCL2 and CCL7. On the other hand, TANs themselves contribute to inflammatory lymphangiogenesis by increasing the amounts of active VEGF-A and VEGF-D (23). We consider that neutrophils release chemokines and recruit macrophages, then macrophages promote lymphangiogenesis by increasing VEGF-C. Our finding, that TANs were increased in lymph nodes with high LVD, is consistent with this assumption.

In line with many studies of different types of cancers (7-10), we found that an elevated density of CD15^{+} TANs in regional lymph nodes was significantly correlated with poor prognosis. These findings indicate that neutrophils in lymph nodes polarized into pro-tumor N2 type, similar to M2 macrophage (6).

Thus, our findings strongly suggest that neutrophils could further cancer progression by changing the microenvironment such that it promotes proliferation and invasiveness of tumor cells.

However, there are several limitations to this study. The present study was performed on a small number of cases. Moreover, we did not directly observe lymphangiogenesis, migration, or proliferation of cancer cells induced by TANs in an experimental model of cancer. Therefore, further studies are required to characterize the tumor microenvironment during cancer progression.

In conclusion, we suggest that an increased number of neutrophils contributes to tumor progression and poor prognosis. Our findings indicate that monitoring TANs might have a predictive value with regard to lymph node metastasis and that innovative anti-inflammatory therapies might exploit this information.

Acknowledgements

Table I. *Univariate and multivariate analysis of prognostic factors of gastric cancer.*

	Overall survival				Progression-free survival			
	Univariate analysis		Multivaliate analysis		Univariate analysis		Multivaliate analysis	
Variable	Hazard ratio (95% CI)	*p*-Value	Hazard ratio (95% CI)	*p*-Value	Hazard ratio (95% CI)	*p*-Value	Hazard ratio (95% CI)	*p*-Value
pStage								
Stage I/II								
Stage III/IV	7.361 (3.681-14.718)	<0.001	2.907 (1.105-7.645)	0.031	7.311 (3.591-14.885)	<0.001	2.945 (1.094-7.927)	0.033
TANs								
Low								
High	3.122 (1.376-7.086)	0.002	1.157 (0.467-2.868)	0.752	3.861 (1.600-9.120)	0.003	1.713 (0.047-4.533)	0.477
LVD								
Low								
High	6.974 (2.473-19.670)	<0.001	2.354 (0.675-8.209)	0.179	5.104 (1.974-13.199)	0.001	1.539 (0.469-5.056)	0.358
Lymphatic invasion								
–								
+	4.678 (2.058-10.633)	<0.001	1.284 (0.442-3.732)	0.646	6.250 (2.417-16.165)	<0.001	1.753 (0.529-5.806)	0.358
Venous invasion								
–								
+	4.822 (2.540-9.1532)	<0.001	1.934 (0.8657-4.323)	0.108	4.419 (2.258-8.648)	<0.001	1.499 (0.654-3.430)	0.339
Histological type								
Intestinal								
Diffuse	1.583 (0.836-2.999)	0.159			1.547 (0.791-3.027)	0.202		

TANs: Tumor-assocated neutrophils; LVD: lymphatic vessel density.

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