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Diameter of involved nerves is a valuable prognostic factor for gastric cancer

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Summary. The prognostic role of perineural invasion (PNI) in gastric cancer remains unclear. We hypothesized that the diameter of the tumor-involved nerves might be a useful indicator for prognosis. By labeling nerves and cancer cells in 204 cases of gastric cancer with single or double immunochemistry, we found that 146 cases were PNI positive and that 58 were PNI negative. For each case with PNI, the maximum diameter of the involved nerve was measured microscopically. Then, we correlated this parameter with the patients' 5-year overall survival, and receiver operating curves were used to determine the cutoff value. We found that the optimal cutoff value for predicting 5-year survival was 65 μm (sensitivity 76.9%, specificity 70.0%). Next, all 204 patients were classified into two groups as follows: Group A, PNI-positive cases in which the largest involved nerves were ≥65 μm in diameter (110 cases); Group B, PNI-positive cases in which the largest involved nerves were <65 μm and all PNI-negative cases (94 cases). Compared with Group A, Group B had a better 5-year survival (74.5% vs 27.3%) and a better 5-year disease-free survival (63.8% vs 23.6%). Multivariate analysis suggested that a ≥65 μm maximum diameter of the involved nerves was an independent risk factor for both recurrence (P<0.001) and gastric cancer-related death (P<0.001) within 5 years. However, if all patients were classified simply based on whether PNI existed (regardless of the nerve size), this did not provide more information than traditional clinicopathological variables. In conclusion, the presence of cancer-involved nerves with a diameter ≥65 μm was a valuable prognostic factor for gastric cancer.

Key words: Gastric cancer, Perineural invasion, Nerve, Prognosis

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Introduction

Gastric cancer, a global health issue, remains one leading cause of cancer-related death worldwide. Although the prognosis of gastric cancer has improved in recent decades, its overall 5-year survival rate is only 27% (Siegel et al., 2012). Traditional clinicopathological variables, such as depth of tumor invasion (T), lymph node metastasis (N) and remote metastasis (M), are crucial for predicting the prognosis of gastric cancer. However, even for patients with the same TNM stage, the long-term outcome may differ markedly from patient to patient. Therefore, besides the TNM system, it is necessary to explore new pathological factors for accurate prognosis and treatment optimization for gastric cancer.

As a pathological entity, perineural invasion (PNI) is defined as tumor cell infiltration in, around, and through nerves (Batsakis, 1985). PNI may reflect a high invasive capability of cancer cells, and accumulating evidence suggests that PNI could serve as a valuable prognostic factor for head and neck cancer (Brandwein-Gensler et al., 2005; Mendenhall et al., 2007), prostate cancer (Lee et al., 2007), pancreatic cancer (Ozaki et al., 1999;

Chatterjee et al., 2012), as well as colorectal cancer (Liebig et al., 2009a). For head and neck cancer, the College of American Pathologists has determined that PNI status should be reported in pathologic analysis because of its significant prognostic value (College of American Pathologists, 2013). With regard to gastric cancer, previous studies have indicated that PNI is correlated with cancer progression (Tanaka et al., 1994a,b; Duraker et al., 2003; Tianhang et al., 2008), and as suggested by many studies, PNI is a prognostic factor independent of traditional clinicopathological variables (tumor size, depth of tumor invasion, lymph node metastasis, etc.) (Tianhang et al., 2008; Bilici et al., 2010). Although Duraker et al. (2003) demonstrated that PNI could not provide more information than these traditional variables, a recent meta-analysis including 24 studies indicated that PNI was an independent prognostic factor for gastric cancer (Deng et al., 2014).

To the best of our knowledge, most previous studies detected PNI in gastric cancer based on hematoxylin and eosin (H&E) staining, and only a few studies used immunohistochemical staining to label nerves to aid PNI determination. According to the study by Kurtz et al (2005) and our recent work (Zhou et al., 2014), H&E staining often leads to the misdiagnosis of PNI, and labeling nerves by immunochemical staining could significantly improve the detection of PNI. However, for diffuse gastric cancer, even when nerves are labeled, detecting PNI remains a great challenge and misdiagnosis also occurs because scattered small cancer cells are difficult to distinguish from inflammatory cells (Zhou et al., 2014). Thus, double immunochemical staining (labeling both nerves and cancer cells) is necessary for the determination of PNI in diffuse gastric cancer. Using double immunochemical staining, we previously found that PNI could not serve as an independent prognostic factor (Zhou et al., 2014). As a result, the prognostic role of PNI in gastric cancer remains to be elucidated.

To precisely clarify the prognostic significance of PNI in gastric cancer, employing a quantitative method to evaluate PNI lesions might be helpful. In our opinion, the diameter of the nerve involved in PNI lesions could be a useful parameter that corresponds to the invasive capabilities of cancer cells and patient outcome, and this parameter should be considered when using PNI to predict the prognosis of gastric cancer. To address this hypothesis, in the present study, we evaluated PNI status in gastric cancer with single and double immunochemical staining, and for each PNI-positive case, the diameter of the largest nerve (DOTLN) involved in the PNI lesion was measured. Then, the diameter was correlated with clinicopathological variables and patient prognosis.

Materials and methods

Patients and data

This study included 204 patients with gastric cancer who underwent curative gastrectomy in the 101 Hospital of the People's Liberation Army between January 2000 and December 2008. Patients with remote metastasis (M1) at diagnosis and those who had received chemotherapy before surgery were excluded from our study. Histological R0 resection was confirmed for each surgical specimen. Clinical data about sex, age, tumor location, tumor size, differentiation, Lauren classification, invasion depth of tumor, lymph node metastasis, and clinical stage were reviewed. Clinical staging was determined based on the 2010 seventh edition of the American Joint Commission on Cancer TNM staging system (Washington et al., 2010). Follow-up information for each patient was collected by registered telephone, mail, or outpatient service. Overall survival (OS) was defined as the time from the operation to death, while disease-free survival (DFS) was defined as the time from the operation to recurrence of the disease. The collection and use of clinical data and pathological material were approved by Institutional Review Board of the 101 Hospital of the People's Liberation Army.

Of the 204 patients, 156 were men and 48 were women. The median age was 60.8 years, ranging from 20 to 83 years. According to depth of tumor invasion, most cases (188/204) were advanced gastric cancer (T2-T4 stage), while 16 cases were in the early stage (T1 stage). The majority of the patients (131/204) had lymph node metastasis, of which 40 were classified as pN1, 46 as pN2, and 45 as pN3. All patients had no distant metastasis. The median follow-up time was 54 months (range, 3-120 months).

Immunochemical staining

All specimens were serially cut into 3-μm-thick sections for H&E staining and immunohistochemical staining. For each of the 204 cases, at least 3 tissue blocks that included the whole gastric wall and showed widespread cancer invasion were used. To assist the determination of PNI, immunohistochemical staining were performed, and S100 antibody was applied to label nerves for all cases. Briefly, antigen retrieval was carried out at 95°C for 15 minutes, and the sections were incubated with S100 antibody (monoclonal, rabbit anti-human, 1:100) for 16 hours at 4°C, followed by the horseradish peroxidase/diaminobenzidine system to visualize S100 (brown).

For 69 cases with diffuse gastric cancer (of the 204 cases in this study, 107 cases were diffuse gastric cancer), labeling nerves by S100 was not enough to help us evaluate PNI because it is not easy to recognize the scattered small cancer cells, which closely resemble inflammatory cells (Fig. 1). Therefore, double immunohistochemical staining was performed in serial sections of these cases. First, the nerves were labeled with S100 antibody (to label nerves) using the alkaline phosphatase-based system and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT, dark blue). Then, antigen retrieval was performed a

second time, and the sections were incubated with AE1/AE3 antibody (monoclonal, mouse anti-human, 1:100) for 2 hours at 25°C (to label cancer cells), followed by detection using the horseradish peroxidase/AEC system (red).

All reagents used in this study were from Maixin Biotech Company Limited (Fuzhou, China).

Evaluation of PNI and assay for the size of the nerves

According to previous studies, PNI was determined as positive when cancer cells were observed to have infiltrated into the perineurium or neural fasciculus intramurally. In the present study, PNI status was evaluated by experienced pathologists based on H&E staining and immunohistochemical staining. When a cased was determined to be PNI positive, the diameter of the nerve (perpendicular to the long axis of this nerve) in each PNI lesion was measured with an ocular micrometer (Fig. 2), and in accordance with a previous study (Chatterjee et al., 2012), we recorded the diameter of the largest nerve (DOTLN) involved by the tumor for each PNI-positive case.

Statistics

To evaluate the predictive value of DOTLN for 5-year overall survival (OS), receiver operating characteristic (ROC) curve analysis was employed to calculate the sensitivity and specificity, and the Youden index was estimated to determine the optimal cutoff value of DOTLN. After the cutoff value was determined, all patients were divided into two groups as follows: Group A, PNI-positive cases for which the DOTLN in each case was equal to or larger than the cutoff value; and Group B, all PNI-negative cases (DOTLN was defined as 0 in these cases) and PNI-positive cases for which the DOTLN was smaller than the cutoff value. Then, the clinicopathological variables were compared between Group A and Group B using the Chi-square test

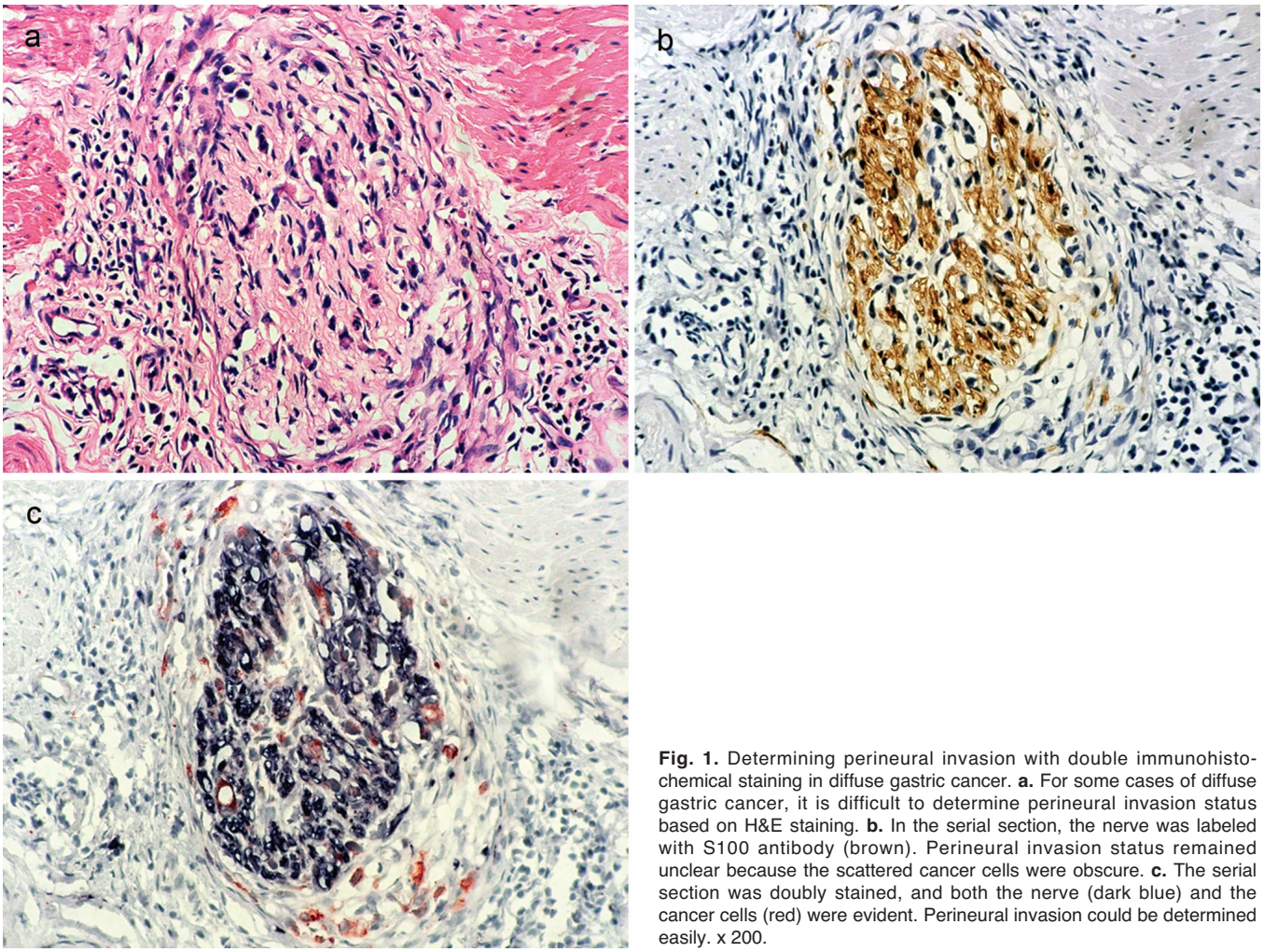

Fig. 1. Determining perineural invasion with double immunohistochemical staining in diffuse gastric cancer. **a.** For some cases of diffuse gastric cancer, it is difficult to determine perineural invasion status based on H&E staining. **b.** In the serial section, the nerve was labeled with S100 antibody (brown). Perineural invasion status remained unclear because the scattered cancer cells were obscure. **c.** The serial section was doubly stained, and both the nerve (dark blue) and the cancer cells (red) were evident. Perineural invasion could be determined easily. x 200.

and Fisher exact test. Five-year OS and 5-year DFS were evaluated using the Kaplan-Meier method and log-rank test. Cox proportional hazard regression analysis was employed to assess the prognostic significance of DOTLN and other clinicopathological variables, and hazard ratios (HRs) were calculated, including 95% confidence intervals (CIs). The results were considered significant at P<0.05.

Results

PNI status and the optimal cutoff value for DOTLN

Of the 204 patients, 146 (71.6%) were PNI positive and 58 were PNI negative. For PNI-positive patients, the diameter of the nerves in each PNI lesion was measured, and then, the DOTLN was determined for each PNI-positive patient. In the 146 PNI-positive patients, the DOTLN ranged from 20 μm to 645 μm. Using the 5-year OS as an endpoint, the area under the ROC curve for the DOTLN was 0.732 (Fig. 3). When the DOTLN was 65 μm, the Youden index was maximal (0.469), with a sensitivity of 76.9% and a specificity of 70.0% (the positive predictive value was 72.7%, and the negative predictive value was 74.5%). Thus, the optimal cutoff value for the DOTLN was determined to be 65 μm. According to this, all 204 patients were divided into two groups as follows: Group A, patients who were PNI positive with a DOTLN ≥65 μm; Group B, PNI-positive patients with a DOTLN <65 μm and all PNI-negative patients. As a result, there were 110 patients in Group A and 94 patients in Group B.

Differences in clinicopathological factors between Group A and Group B

Next, we compared clinicopathological variables between Group A and Group B (Table 1). In these two groups, there was no significant difference in age (P=0.694), sex (P=0.969) or tumor location (P=0.720), yet the tumor size was larger in Group A than in Group B (P<0.001). When classifying gastric cancer into the intestinal type and the diffuse type according to the Lauren classification, the diffuse type was more frequent in Group A than in Group B (64.5% vs 38.3%, P<0.001). Moreover, the gastric cancers in Group A showed poorer differentiation (P<0.001), deeper mural invasion (P<0.001), increased lymph node metastasis (P<0.001) and worse clinical stage (P<0.001), when compared with those in Group B. In addition, patients in Group A were more likely to show recurrence after surgery than those in Group B (P<0.001).

Prognostic value of our classification method (based on DOTLN)

We further assessed the prognosis of patients in

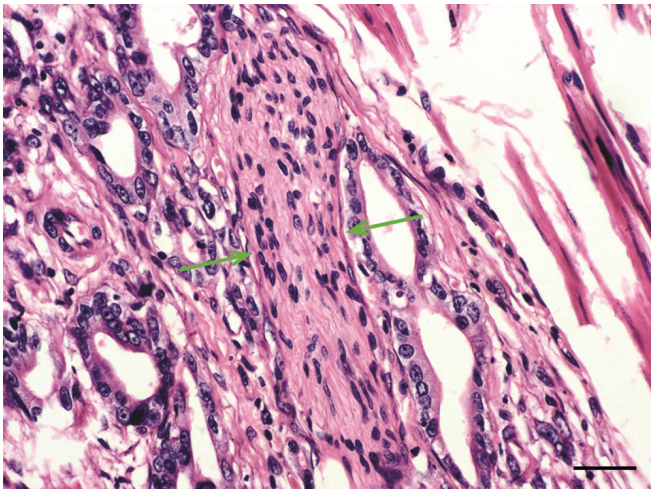

Fig. 2. Measurement of the diameter of the nerve involved in perineural invasion foci. The diameter of the nerve fiber was taken perpendicular to the long axis of this nerve (green arrows). Scale bar 50 μm.

Table 1. Difference of clinicopathological features between Group A and Group B.

Factors	Group A n (%)	Group B n (%)	P value
Gender			0.96
Male	84 (41.2)	72 (35.3)	
Female	26 (12.7)	22 (10.8)	
Age			0.69
<60 y	51 (25.0)	41 (20.1)	
≥60 y	59 (28.9)	53 (26.0)	
Location			0.72
Upper	42 (20.6)	43 (21.1)	
Middle	14 (6.9)	12 (5.9)	
Lower	43 (21.1)	31 (15.1)	
Diffuse	11 (5.4)	8 (3.9)	
Tumor Size			<0.001
≤3 cm	29 (14.2)	51 (25.0)	
≤6 cm	61 (29.9)	29 (14.2)	
>6 cm	20 (9.8)	14 (6.9)	
Tumor differentiation			<0.001
Well differentiated	3 (1.4)	23 (11.3)	
Moderately differentiated	34 (16.7)	37 (18.1)	
Poorly differentiated	73 (35.8)	34 (16.7)	
Histological type			<0.001
Intestinal	39 (19.1)	58 (28.4)	
Diffuse	71 (34.8)	36 (17.7)	
pT stage			<0.001
T1	0 (0)	16 (7.8)	
T2	5 (2.5)	32 (15.7)	
T3	35 (17.2)	28 (13.7)	
T4	70 (34.3)	18 (8.8)	
pN stage			<0.001
N0	17 (8.3)	56 (27.5)	
N1	24 (11.7)	16 (7.8)	
N2	33 (16.2)	13 (6.4)	
N3	36 (17.7)	9 (4.4)	
Clinical stage			<0.001
I	0 (0)	12 (5.9)	
II	32 (15.7)	59 (28.9)	
III	78 (38.2)	23 (11.3)	
Recurrence			<0.001
Presence	84 (41.2)	34 (16.7)	
Absence	26 (12.7)	60 (29.4)	

Group A and Group B. As shown in Fig. 4, patients in Group B had a higher 5-year OS than those in Group A (74.5% vs 27.3%, P<0.001), and Group B had a better 5-year DFS than Group A (63.8% vs 23.6%, P<0.001). Using a Cox multiple regression model, we found that our classification method of dividing gastric cancer patients into Group A and Group B was an independent factor for poor prognosis. Specifically, compared with group B, Group A had a higher likelihood of developing recurrence (P<0.001, HR 2.488, 95%CI 1.569-3.946) and of dying of gastric cancer within 5 years (P<0.001, HR 2.615, 95%CI 1.544-4.430). Other independent factors included depth of tumor invasion (for 5-year DFS: P=0.002, HR 1.730, 95%CI 1.223-2.449; for 5-year OS: P<0.001, HR 2.038, 95%CI 1.383-3.005), lymph node metastasis (for 5-year DFS: P<0.001, HR 2.018, 95%CI 1.445-2.817; for 5-year OS: P=0.001, HR 1.814, 95%CI 1.276-2.576) and age (for 5-year DFS: P=0.009, HR 0.600, 95%CI 0.410-0.879; for 5-year OS: P=0.006, HR 0.562, 95%CI 0.371-0.850).

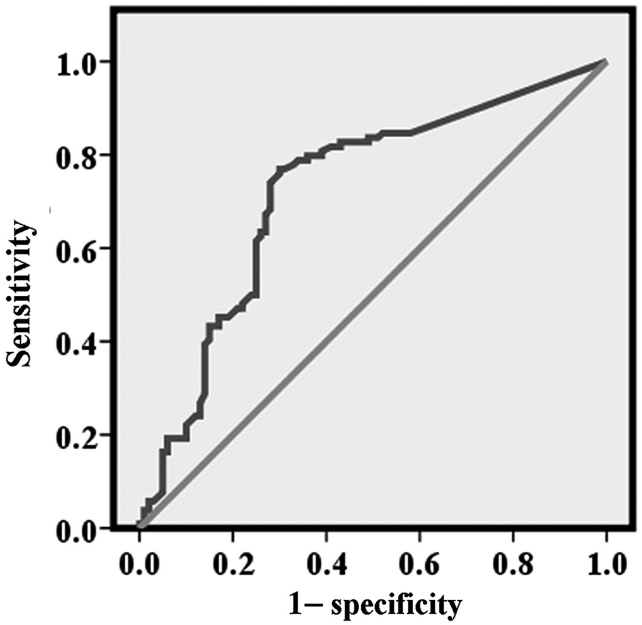

Fig. 3. Assessing the prognostic value of DOTLN in cancer. When 5-year OS was used as an endpoint, the area under the receiver operating characteristic curve was 0.732. Abbreviation: DOTLN, diameter of the largest nerve.

Prognostic value of the previous classification method (simply based on PNI status)

We also classified our cohort simply based on PNI status (PNI-positive or PNI negative), regardless of the DOLTN. As noted above, there were 146 PNI-positive patients and 56 PNI-negative patients in our cohort. We found that both 5-year OS and 5-year DFS were lower in the PNI-positive group than in the PNI-negative group (for OS, 39.7% vs 72.4%, P<0.001; for DFS 37.0% vs 70.7%, P<0.001). However, PNI was not an independent prognostic factor for 5-year OS (P=0.159, HR 1.562, 95%CI 0.840-2.904) or 5-year DFS (P=0.244, HR 1.382, 95%CI 0.802-2.381). These results were similar to our previous study (Zhou et al., 2014).

Discussion

In gastric cancer, the role of PNI in prognosis remains controversial. Most studies, if not all, have simply correlated gastric cancer prognosis with PNI

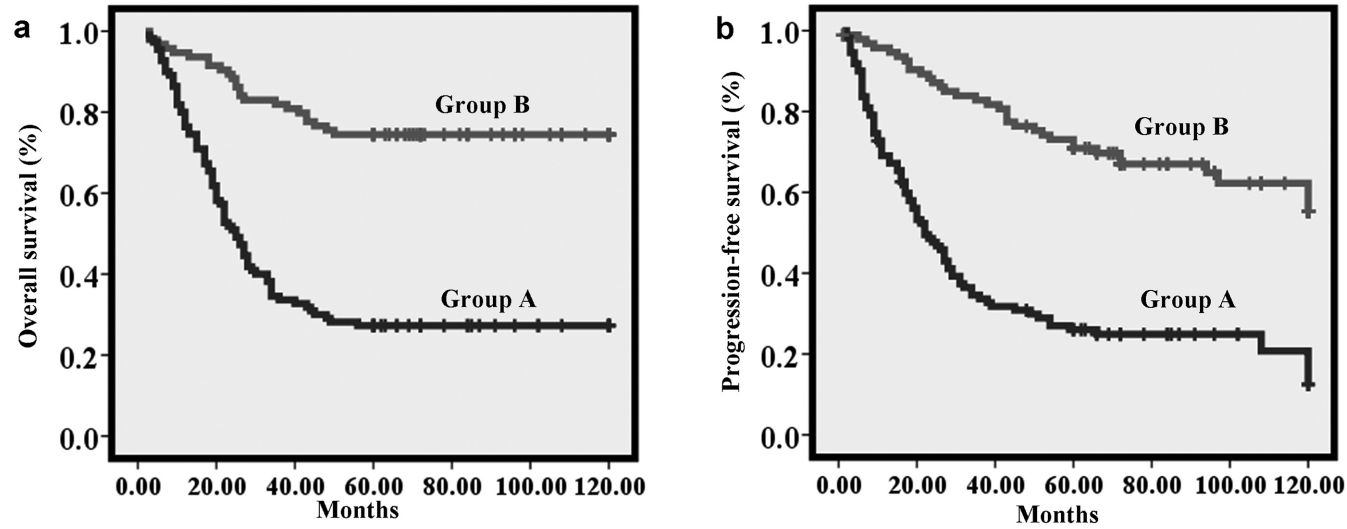

Fig. 4. Patients in Group B (n=94) exhibited a significantly better overall survival (log-rank test, P<0.001, **a**) and disease-free survival (log-rank test, P<0.001, **b**) than those in Group A (n=110).

status. In the present study, we proposed that more attention should be paid to the size of the nerves involved in PNI lesions. Our results suggested that the DOTLN was associated with the 5-year OS of gastric cancer, and the optimal cutoff value was set at 65 μm. Additionally, we found that patients with a DOTLN <65 μm and PNI-negative patients (Group B) had a better 5-year OS and DFS when compared with patients with a DOTLN ≥65 μm (Group A). Multivariate analysis further confirmed that our classification based on PNI status and DOTLN was an independent prognostic factor, providing more information than traditional clinicopathological variables. In addition, if the patients were classified simply based on PNI status, we found that PNI-positive patients had a poorer prognosis than PNI-negative patients. However, multivariate analysis indicated that this classification method was not an independent prognostic factor.

When the DOTLN was employed to predict 5-year OS, the sensitivity was 76.9% and the specificity was 70.0%. Thus, we believe that the DOTLN might be a valuable indicator in predicting patient outcome. The present study confirmed that a DOTLN ≥65 μm was an adverse prognostic factor for gastric cancer, independent of traditional clinicopathological variables. Therefore, when gastric cancer patients were determined as PNI positive, only reporting PNI status was not enough. Specifically, for patients with a DOTLN <65 μm, the 5-year OS rate was similar to that of PNI-negative patients, and PNI positivity should therefore not be emphasized in these patients. For patients with a DOTLN ≥65 μm, this indicator, together with TNM status, should be specified in pathologic reports. In addition, to evaluate the DOTLN in sections, an ocular micrometer should be provided with the microscope. If an ocular micrometer is not available, red blood cells (RBCs) can be used as a reference scale as the diameter of an RBC is 6.2-8.2 μm (Turgeon, 2004), and 65 μm corresponds to the size of approximately 8-10 RBCs.

When sections are observed with a low-power lens, nerves with a diameter of 65 μm (or a little larger than 65 μm) may be overlooked. Moreover, in some circumstance, nerves can be concealed in mucin pools generated by cancer cells (Liebig et al., 2009b). Thus, for the purpose of detecting PNI and measuring the diameter of the nerve, labeling nerves with immunohistochemistry can be helpful. However, in some diffuse gastric cancer cases, determining PNI is a tough task, even when nerves are labeled. This is because cancer cells are usually small with a high nucleus/plasma ratio, resembling lymphocytes closely in morphology, and this makes them difficult to recognize. In a previous study, we used double immunohistochemical staining to label both cancer cells and nerves. We found that this method could significantly improve the detection of PNI in diffuse gastric cancer when compared with single immunohistochemical staining (only labeling the nerves) (Zhou et al., 2014). In the present study, we also used double staining to evaluate PNI and DOTLN for diffuse gastric cancer, ensuring the reliability of the results.

Why is DOTLN valuable for predicting a patient's outcome? In our opinion, the presentation of PNI reflects a high invasive capability of cancer cells because the nerve fiber is not a low-resistance path for cancer cells but a dense structure surrounded by several layers of collagen and basement membrane (Liebig et al., 2009b). Thus, the presentation of PNI indicates the aggressiveness of the tumor and, consequently, poor outcome. Additionally, there are complicated interactions between the nerve and cancer cells. On the one hand, cancer cells promote the growth of the nerves, and it was previously reported that in prostate cancer, cancer cells caused axonogenesis in the tumor and an increased number of neurons in the prostatic ganglia by producing Semaphorin 4F (Ayala et al., 2008; Ding et al., 2013). Moreover, cancer-related neurogenesis is correlated with the recurrence of prostate cancer (Ayala et al., 2008). On the other hand, the nerve can promote carcinogenesis and tumor progression. A recent study found that in gastric cancer, a higher density of neurons and larger ganglia were correlated with carcinogenesis, and that neurons could activate Wnt signaling in gastric cancer stem cells, leading to tumor progression (Zhao et al., 2014). This study also found that the density of neurons in gastric cancer was correlated with clinical stage of the tumor. Thus, we believe that nerve growth may be significantly involved in gastric cancer progression, and the size of cancer-involved fiber is an indicator of cancer-related nerve growth. Consequently, when PNI positivity and the size of the cancer-involved nerve are combined as a prognostic factor (DOTLN), it becomes powerful for prognostic evaluation.

In summary, our present study indicated that a DOTLN ≥65 μm is a valuable prognostic factor, which suggests a significantly lower 5-year overall survival. Additionally, this factor is independent of traditional clinicopathological variables. Therefore, when a DOTLN ≥65 μm is present in gastric cancer, it should be specified in pathologic reports.

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Disclosure/conflict of interest. The authors declare no conflict of interest.

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