1 Stromal plasma cells expressing immunoglobulin G4 subclass in non-small cell lung

2 cancer

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19 **Key words**

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3 **Running head**

4 IgG4+ plasma cells in lung cancer

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Abstract

Inflammatory cell infiltration in tumor stroma may represent the interaction between the tumor and the immune system. The significance of immunoglobulin (Ig)G4+ plasmacytic infiltration, however, is poorly understood. Here, we analyzed the number of stromal IgG4+ plasma cells and the IgG4/IgG ratio of plasma cells in 294 primary non-small cell lung cancers (NSCLC) using tissue microarray (TMA) and conventional surgical specimens. In TMA, 35 (12%) cases of NSCLC revealed more than 20 IgG4+ plasma cells per high-power field. In surgical specimens, most (97%) of those IgG4+ plasma cell-enriched cases showed obliterative phlebitis or arteritis, one of the key morphologic features of IgG4-related disease, within or at the periphery of the tumor. Clinically, none of the patients showed symptoms associated with IgG4-related systemic diseases. In patients with stage I squamous cell carcinoma, IgG4-enriched stroma was significantly associated with a favorable prognosis (p = 0.04). In conclusion, considerable IgG4+ plasma cell infiltration can be seen in a minority of cases of NSCLC, and might contribute to prognostic modulation of NSCLC.

Introduction

  Despite a number of recent studies focused on the tumor microenvironment, the role

of plasma cells in cancer is not well understood [1]. The class and subclass of

immunoglobulin (Ig) produced by plasma cells are defined primarily by their heavy

chain constant domain sequences, which affect the function of the antibody [2]. IgG4,

the least abundant subclass in the IgG class, shows weak or negligible binding ability

to both C1q and Fcγ receptors compared with the other IgG subclasses, which leads

to a limited capacity to activate the classical complement pathway [2,3]. Thus, until

recently, it was believed that IgG4 plays only a limited role in immune activation [3]. As

a consequence of the current familiarity of IgG4-related diseases (IgG4-RD) to

pathologists and the widespread use of anti-IgG4 antibody for immunohistochemical

staining, cases of carcinoma accompanied by severe IgG4+ plasma cell infiltration in

patients with or without a medical history of IgG4-RD have been identified [4-9],

although the prevalence and significance of this phenomenon remain unclear.

  Differential diagnosis of IgG4-RD and cancers can be problematic, since IgG4-RD

often forms tumefactive lesions and peritumoral desmoplastic tissue can mimic

IgG4-RD when there is an abundance of IgG4+ plasma cells in a biopsy specimen.

Some morphologic findings such as obliterative phlebitis are considered to be rather

more specific to IgG4-RD than to cancer, but existing evidence supporting this idea

seems weak [10]. Serum IgG4 concentration is a helpful laboratory finding to

differentiate IgG4-RD and cancer, but it is not always elevated in patients with

IgG4-RD, while it can be elevated in cancer patients without IgG4-RD [4,8]. In addition,

clinicians must keep in mind the possibility of synchronous cancer and IgG4-RD [11].

In this study, we identified cases of lung cancer with stromal IgG4+ plasma cell

infiltration using tissue microarray (TMA). We analyzed the clinicopathologic

characteristics of these cases to investigate the relevance of lung cancer and IgG4+

plasma cells.

Materials and Methods

TMA

Paraffin-embedded tumor blocks from 363 cases of lung cancer surgically resected

from different patients in Kyoto University Hospital between 2001 and 2007 were

selected from the electronic pathology files of Kyoto University Hospital for

construction of TMA, with the patients' consent. TMAs were constructed by 2 of the

authors (A.Y. and S.S.) basically using the approach described by Kononen et al. [12].

Briefly, the most morphologically representative region of the tumor was selected on a

hematoxylin and eosin (H&E)-stained slide. Then, a tissue core of 2 mm in diameter

was punched out from each donor tumor block using a thin-walled stainless steel

1   needle for TMA construction (Azumaya, Tokyo, Japan), and arrayed in a recipient

2   paraffin block. Small cell carcinomas, recurrent carcinomas, carcinomas with

3   neoadjuvant chemotherapy and cases with multiple carcinomas (both synchronous

4   and metachronous) were excluded from this study, but were established in TMA for

5   another study (n=45).

6

7   **Immunohistochemistry**

8   Immunostains against IgG4 and IgG using mouse anti-human IgG4:HRP

9   (MCA2098P; dilution 1:200; AbD Serotec, Oxford, UK) and IgG (immunoglobulin G)

10   (polyclonal; dilution 1:2 of prediluted product; Ventana, Tucson, AZ, USA) were

11   performed on an autoimmunostainer (Ventana XT System Benchmark; Ventana

12   Medical Systems).

13

14   **Evaluation of immunohistochemistry**

15   In TMA sections, we regarded tumors infiltrated by more than 20 IgG4+ plasma

16   cells per high-power field (HPF) (x40 objective lens with field number 22) as IgG4+

17   cell-enriched cases, since this criterion is used for diagnosing IgG4-RD in lung biopsy

18   specimens [10]. To calculate the IgG4/IgG ratio in each TMA section, the total

19   numbers of IgG4+ and IgG+ plasma cells per core were counted manually under a

light microscope by M.F. and A.Y. For those IgG4+ cell-enriched cases with >25%

IgG4/IgG ratio, immunohistochemistry for IgG4 and IgG was performed on the original

surgical specimens to extract the cases fulfilling the quantitative criteria for IgG4-RD

(>50/HPF of plasma cells with IgG4/IgG ratio >40%) [10]. In each case, IgG4 and IgG

were counted in the region most highly stained for IgG4 in three HPFs to calculate the

IgG4/IgG ratio.

Histologic evaluation

Histologic types of lung cancers were determined according to the 2004 WHO

classification [13]. In adenocarcinomas, the predominant histologic subtype or variant

in each case was identified according to the new lung adenocarcinoma classification

proposed by the International Association for the Study of Lung Cancer, American

Thoracic Society and European Respiratory Society (IASLC/ATS/ERS classification)

[14], and it was also graded as well differentiated (G1), moderately differentiated (G2)

and poorly differentiated (G3) based on the 2004 WHO classification by three of the

authors (M.F., A.Y. and S.S.) [13]. Squamous cell carcinomas were not graded in

this study, since the stage of the disease and the performance status at diagnosis

remain the most powerful prognostic indicators, instead of the current grading system

mainly based on the degree of keratinization [13]. IgG4+ cell-enriched cases were

1   evaluated by two of the authors (M.F. and A.Y.) for the presence of obliterative

2   phlebitis/arteritis and increased number of eosinophils, which we defined as more

3   than 50 eosinophils per 10 HPFs.

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5   **Clinical information**

6   The electronic chart of Kyoto University Hospital, the electronic database of the

7   Department of Thoracic Surgery of Kyoto University Hospital and the electronic

8   pathology database of Kyoto University Hospital were used to search for patients'

9   prognosis and medical history.

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11   **Somatic *EGFR, KRAS* and *p53* mutations**

12   *EGFR*, *KRAS* and *p53* mutation analyses were carried out in selected cases by

13   methods described previously [15,16].

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15   **Statistical analysis**

16   GraphPad Prism (GraphPad Software, San Diego, CA, USA) and JMP Start

17   Statistics (Statistical Discovery Software SAS Institute, Cary, NC, USA) were used for

18   statistical analyses. Comparisons between two groups were performed with Fisher's

19   exact test or Mann-Whitney's test to analyze categorical variables and continuous

1   variables, respectively. The Kaplan-Meier method was used to evaluate patients'

2   prognosis and log-rank tests were used to compare the survival rates among groups.

3   Logistic regression analysis was performed to examine the interaction of the multiple

4   clinicopathological variables. Significance was defined as $p < 0.05$.

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Results

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8   TMA cores that were not completely enclosed on the sections or did not include

9   viable tumor on the sections were excluded from this study (n=24). As a result, 294

10   primary non-small cell lung carcinomas (NSCLC) were extracted. The median

11   follow-up period of patients was 1748 days (range: 7-3805).

12   The 294 lung cancers consisted of 233 adenocarcinomas (79.3%), 52 squamous

13   cell carcinomas (17.7%) and 9 other NSCLC (3.1%) (6 large cell carcinomas, 2

14   sarcomatoid carcinomas and 1 adenosquamous carcinoma). According to the 7th

15   UICC TNM classification [17], 215 cases (73.1%) were stage I, 37 cases (12.6%)

16   were stage II, 31 cases (10.5%) were stage III and 11 cases (3.7%) were stage IV.

17   Among the 233 adenocarcinomas, 60 cases (25.8%) were G1, 90 cases (38.6%)

18   were G2 and 83 cases (35.6%) were G3. The median numbers of IgG4+ and IgG+

19   plasma cells in the TMA core were 0 (range: 0-570) and 134 (range: 0-2178),

respectively. None of the 294 patients had IgG4-RD in their medical history and serum IgG4 concentration was not measured in any of the patients.

In adenocarcinomas, mutation analysis of *EGFR*, *KRAS* and *p53* was performed in 213 cases, 213 cases and 129 cases, respectively, and the mutation was confirmed in 102 cases (47.9%), 23 cases (10.8%) and 31 cases (24.0%), respectively.

Lung cancers with IgG4+ cell enrichment (n=35)

Out of 294 cases, 35 cases (11.9%) were IgG4+ cell-enriched, including 17 adenocarcinomas (7.3% of all adenocarcinomas), 17 squamous cell carcinomas (32.7% of all squamous cell carcinomas) and 1 adenosquamous carcinoma. Among the 17 adenocarcinomas, 15 cases were G3 and 2 were G2. G1 adenocarcinoma was not observed.

The median number of original tumor slides observed per case was 3 (range: 1-9) and Victoria blue H&E stain and/or Elastica van Gieson stain was performed in all but 1 case. Fibrosis was evident in cancer stroma of all 35 cases, but none of them showed storiform pattern fibrosis. Obliterative phlebitis/arteritis was found in 34 cases (97.1%) associated with desmoplasia. An increased number of eosinophils was seen in 3 cases (8.6%). In all 35 cases, pathological change for which IgG4-RD was suspected was absent in non-neoplastic lung tissue surrounding the cancers.

The result of comparison between IgG4+ cell-enriched cases and the cases with ≤20 IgG4+/HPF in the TMA core is shown in Table 1. We also performed multivariate logistic regression analysis to determine which factors, among those showing statistical significance in univariate analysis, were more significantly associated with >20/HPF IgG4+ plasma cell infiltration (Table 2). This analysis revealed that squamous cell carcinoma and G3 adenocarcinoma were associated with >20/HPF IgG4+ plasma cell infiltration with high statistical significance (p<0.0001 and p=0.0013, respectively).

No statistical difference in recurrence rate or death rate was observed between the two groups (Table 1). In stage I carcinoma, however, none of the 19 IgG4+ cell-enriched cases (10 G2-G3 adenocarcinomas, 8 squamous cell carcinomas and 1 adenosquamous carcinoma) was associated with patient death, and only 1 squamous cell carcinoma and 1 adenocarcinoma recurred. In contrast, among 27 stage I squamous cell carcinomas with ≤20 IgG4+ plasma cells/HPF, 10 patients (37.0%) died of disease and 11 cases (40.7%) recurred. Among 110 stage I G2-3 adenocarcinomas with ≤20 IgG4+ plasma cells/HPF, 13 patients (11.8%) died of disease and 29 cases (26.4%) recurred. A significant difference of overall patient survival was observed in stage I squamous cell carcinoma (p=0.0409), but this was not the case for disease-free survival rate (p=0.1053) (Figure 1). No significant

difference in overall survival or disease-free survival rate was observed in stage I

G2-3 adenocarcinoma (p=0.3280 and p=0.4407, respectively).

Lung cancers with >50 IgG4+ plasma cells/HPF and >40% IgG4/IgG plasma cells

in surgical specimens (n=6)

Among 35 IgG4+ cell-enriched cases in the TMA core, 12 cases were associated

with >25% IgG4/IgG ratio, and 6 of them showed >50 IgG4+ plasma cells/HPF and

>40% IgG4/IgG ratio in the surgical specimens. The clinicopathological features of

these 6 cases are summarized in Table 3. Histologic figures of selected cases are

shown in Figure 2.

In comparison of these 6 cases and the other 29 IgG4+ cell-enriched cases, no

statistically significant difference was obtained in any parameters shown in Table 1,

except the IgG4/IgG ratio in the TMA core (median: 0.28 vs. 0.12, p=0.0062).

Additionally, the numbers of cases with obliterative phlebitis/arteritis and increased

number of eosinophils in these 2 groups did not differ significantly (83.3% vs. 100%,

p=0.1714, and 16.7% vs. 6.9%, p=0.4417, respectively).

Discussion

IgG4+ plasma cell infiltration in cancer and its relevance to IgG4-RD have been a

matter of debate in the field of practical medicine [4-9]. Since chronic inflammation is

associated with a risk of cancer development, it could be hypothesized that IgG4-RD

is a source of carcinoma [11,18]. In our series, however, the idea that the patients with

IgG4+ cell-enriched tumor had lung IgG4-RD seems unlikely for two main reasons.

Firstly, none of the IgG4+ cell-enriched cases showed histology of IgG4-RD in the

non-neoplastic lung tissue, suggesting that the cancer is the factor causing the

inflammation and not the other way round. Secondly, although IgG4-RD is a systemic

disease and the affected patients commonly develop multi-visceral lesions or multiple

lesions in the same organ at the time of diagnosis or during the follow-up [6,19], none

of the patients in our series was clinically complicated by IgG4-RD at the time of

diagnosis or during the follow-up of cancer.

Considering the favorable prognosis observed in IgG4-enriched stage I squamous

cell carcinomas, IgG4 infiltration might cause prognostic modulation of NSCLC. At first,

this result seemed to be inconsistent with the notable anti-immune function of IgG4,

as seen in the transition of serum IgG4 level in immunotherapy [20]. Other previous

reports, however, show that the functional role of IgG4 varies in different

pathophysiological circumstances, and that IgG4 can be a pathogenic antibody in

certain immunologic disorders [21-25]. Our data may suggest that IgG4 reacts against

certain lung cancer antigens and damages tumor cells in spite of its poor complement-

and leucocyte-activating properties. By multivariate analysis, we found that squamous

cell carcinomas and high-grade adenocarcinomas are more likely to be accompanied

by intra-tumoral IgG4+ plasma cells; however, the reason for this tendency is unclear

at present.

 In stage I squamous cell carcinoma, intra-tumoral IgG4+ plasma cell infiltration may

be a better prognostic factor than conventional histological grade because the

histological grade of lung squamous cell carcinoma is not clearly related to its

prognosis [13]. IgG4+ plasma cells might have a cancer-inhibiting role in

adenocarcinomas as well, but our data did not show statistical significance. In

adenocarcinomas, high-grade histology or non-EGFR gene mutation may be a more

powerful prognostic factor than IgG4+ cell infiltration [15,16,26].

 A common histological finding of IgG4-RD is sclerosing fibrosis arranged at least

focally in a storiform pattern accompanied by marked IgG4+ plasma cell infiltration,

usually associated with obliterative phlebitis; however, there is variability in the

findings in certain organs [10]. In lung IgG4-RD, stroriform-type fibrosis is uncommon;

thus, histological findings tend to overlap with other pulmonary fibroinflammatory

conditions. In the pericancerous fibrous stroma of our IgG4+ cell-enriched cancers,

even obliterative phlebitis/arteritis, the histological finding of which is believed to be

quite specific to lung IgG4-RD, was observed in most of the cases. Pathologists

should be aware of this fact when diagnosing lung IgG4-RD on biopsy specimens.

   In conclusion, a non-negligible minority of cases of squamous cell carcinoma and high-grade adenocarcinoma of the lung showed IgG4+ plasma cell infiltration in the stroma. Obliterative vascular changes were commonly seen in those cases without clinical evidence of IgG4-RD. Since IgG4+ cell-enriched cases were associated with favorable prognosis in stage I squamous cell carcinomas, IgG4 infiltration may be part of the anti-cancer immune response.

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Table legends

Table 1. Comparison of lung cancers with and without >20 IgG4+ plasma cells/HPF in the tissue microarray (TMA) core (n=294).

Table 2. Logistic regression analysis for estimation of significant factors related to >20/HPF intra-tumoral IgG4+ plasma cell infiltration.

Table 3. Clinicopathological characteristics of 6 lung cancers that fulfilled the histological criteria of lung IgG4-RD (IgG4/IgG ratio >40% and >50/HPF IgG4+ plasma cells in surgical specimen).

Table 1

	Number of IgG4+ plasma cells/TMA core		p
	>20/HPF	≤20/HPF	
No. of cases	35 (11.9%)	259 (88.1%)	
Median of IgG4/IgG ratio in TMA core (range)	0.13 (0.021-0.47)	0 (0-0.75)	<0.0001*
Male	30 (85.7%)	144 (55.6%)	0.0005*
Median age (range)	72 (41-88)	67 (23-94)	0.1783
Never smokers	4 (11.4%)	107 (41.3%)	0.0004*
Preoperative history of cancer	7 (20.0%)	48 (18.5%)	0.8189
Preoperative history of asthma	4 (11.4%)	8 (3.1%)	0.0417*
Preoperative history of collagen vascular disease	1 (2.8%)	9 (3.5%)	1
Preoperative history of interstitial pneumonia	1 (2.9%)	15 (5.8%)	0.7032
Squamous cell carcinomas	17 (48.6%)	35 (13.5%)	<0.0001*
G3 adenocarcinomas	15 (42.9%)	68 (26.3%)	0.0468*
Gene mutation in adenocarcinomas	EGFR 3/14 (21.4%)	EGFR 99/199 (49.0%)	0.0524
	KRAS 1/14 (7.1%)	KRAS 22/199 (11.1%)	1
	p53 5/7 (71.4%)	p53 26/122 (21.3%)	0.0088*
Stage I cancers	19 (54.3%)	196 (75.7%)	0.0134*
Recurrence rate (Recurrence rate of stage I cancers)	34.3% (10.5%)	32.8% (20.9%)	0.8502 (0.3773)
Death rate (Death rate of stage I cancers)	20.0% (0%)	22.8% (12.2%)	0.8111 (0.1395)

* Statistically significant.

Table 2

Variable	Odds ratio	95% confidence interval	p
Sex (Male/Female)	1.8285	0.5631-6.9714	0.3392
Smoking history (Never/Current or Former)	0.6118	0.1400-2.2837	0.4829
Preoperative history of asthma (present/absent)	2.7665	0.6253-11.2757	0.1581
Squamous cell carcinoma	15.4307	4.5863-71.6024	<0.0001*
G3 adenocarcinoma	8.5662	2.5946-38.8643	0.0013*
Cancer stage (Stage I/Stage II-IV)	0.6655	0.2968-1.4987	0.3215

* Statistically significant.

Table 3

Case No.	1	2	3	4	5	6
Sex	Male	Male	Male	Male	Male	Male
Age at surgery	59	61	61	65	72	75
Smoking status	Current	Former	Former	Former	Never	Current
Past medical history	None	Hypopharyngeal squamous cell carcinoma	None	Asthma, Hepatitis C	Diabetes mellitus, Arrhythmia	Colon carcinoma, Renal carcinoma (histology not available)
Site	Right upper lobe	Left upper lobe	Right lower lobe	Right upper lobe	Left upper lobe	Left lower lobe
Stage	1B (T2aN0M0)	1A (T1aN0M0)	1A (T1bN0M0)	1B (T2aN0M0)	2A (T1bN1M0)	1A (T1aN0M0)
Histology	Adenosquamous carcinoma	Adenocarcinoma, solid predominant, G3	Squamous cell carcinoma	Squamous cell carcinoma	Adenocarcinoma, micropapillary predominant, G3	Squamous cell carcinoma
Obliterative phlebitis/ arteritis	Present	Present	Absent	Present	Present	Present
Eosinophilic infiltration >50/10 HPF	Absent	Absent	Absent	Present	Absent	Absent
IgG4/IgG ratio in TMA core (%)	206/820 (25.1)	570/1204 (47.3)	172/625 (27.5)	258/1012 (25.5)	216/648 (33.3)	348/1211 (28.7)
IgG4/IgG ratio in surgical specimen (%)	51.7/103.7 (49.9)	80.0/137.0 (58.4)	67.7/146.7 (46.1)	53.3/125.3 (42.5)	69.7/170.7 (40.8)	50.6/105.3 (48.1)
EGFR	Not done	Wild	Wild	Wild	Mutated	Wild
KRAS	Not done	Mutated	Wild	Wild	Wild	Wild
p53	Not done	Not done	Mutated	Wild	Not done	Wild
Recurrence of lung carcinoma	Absent	Absent	Absent	Absent	Present	Absent
Outcome	Alive	Alive	Alive	Alive	Died of disease	Alive
Follow-up period (days)	3480	1666	2747	2002	376	1468

Figure legends

Figure 1 Kaplan-Meier survival curves of 35 patients with stage I squamous cell

carcinomas separated by the number of IgG4+ plasma cells/HPF in the tissue

microarray core (>20/HPF vs. ≤20/HPF). (A) Overall survival curves (log-rank test,

p=0.0409), and (B) disease-free survival curves (log-rank test, p=0.1053).

Figure 2 Histology of lung cancers fulfilling the histological criteria for lung IgG4-RD,

(A)-(E) case 2 and (F)-(H) case 4.

(A) Case 2, solid predominant G3 adenocarcinoma (×20).

(B) Higher-magnification image of boxed area in (A) (×100).

(C) Intratumoral obliterative phlebitis (×100).

(D) >50/HPF IgG4+ plasma cells in the cancer stroma (×200).

(E) IgG immunostain at the same area as in (D), with IgG4/IgG ratio >40% (×200).

(F) Case 4, squamous cell carcinoma (×40).

(G), (H) Higher magnification of the areas demarcated by boxes in (F); obliterative

vasculitis and eosinophilic infiltration are shown, respectively (×200 and ×400).
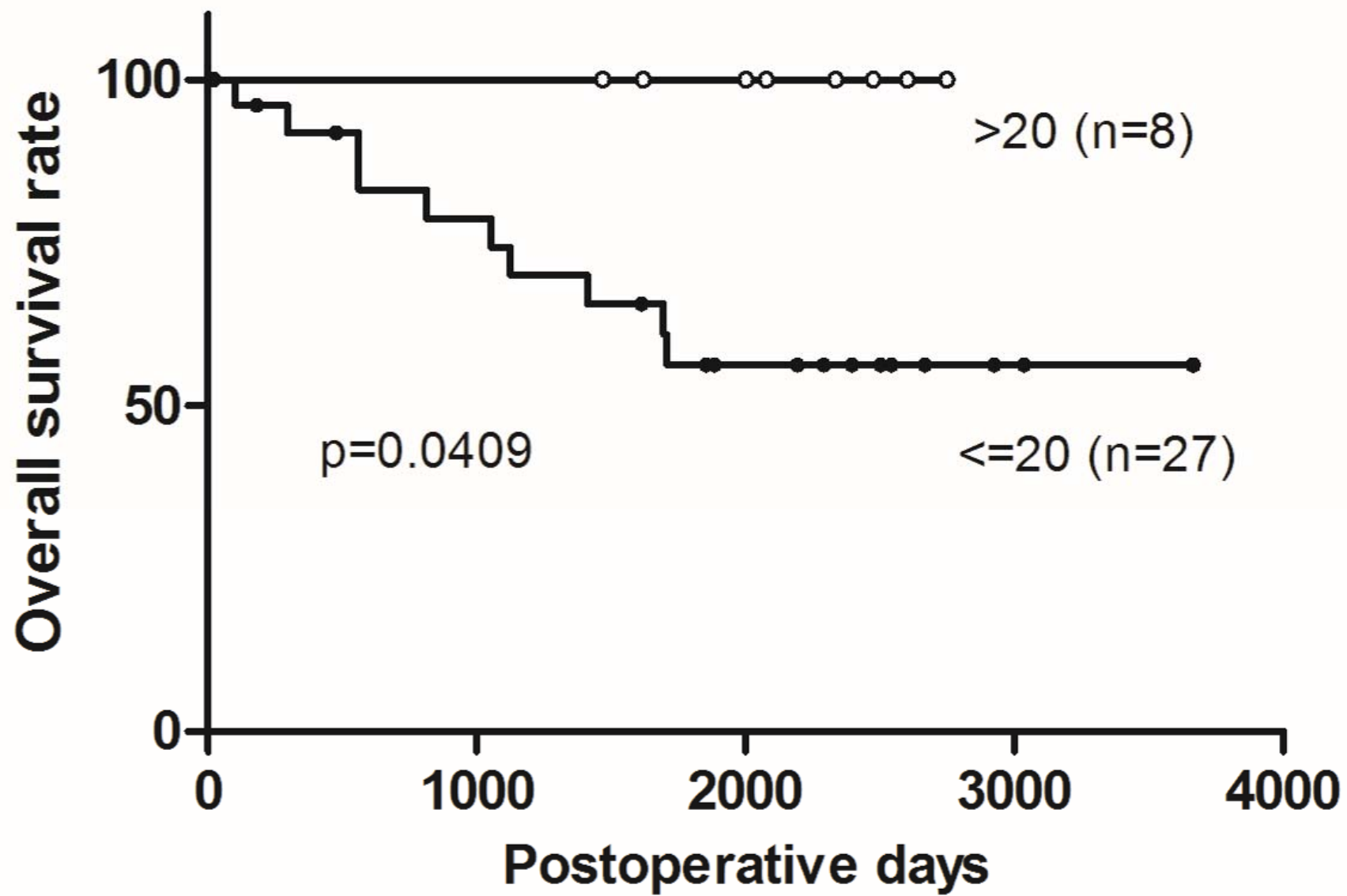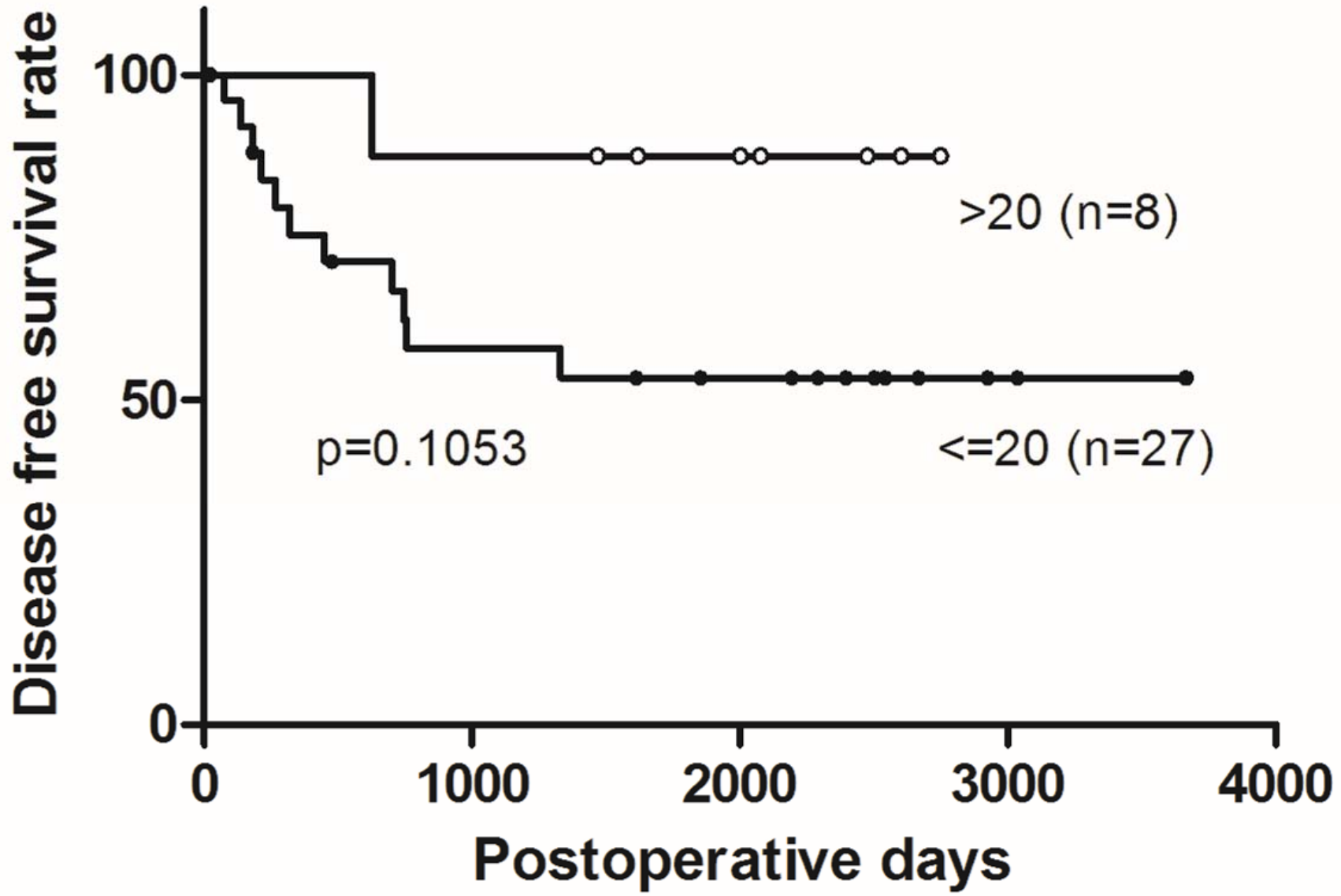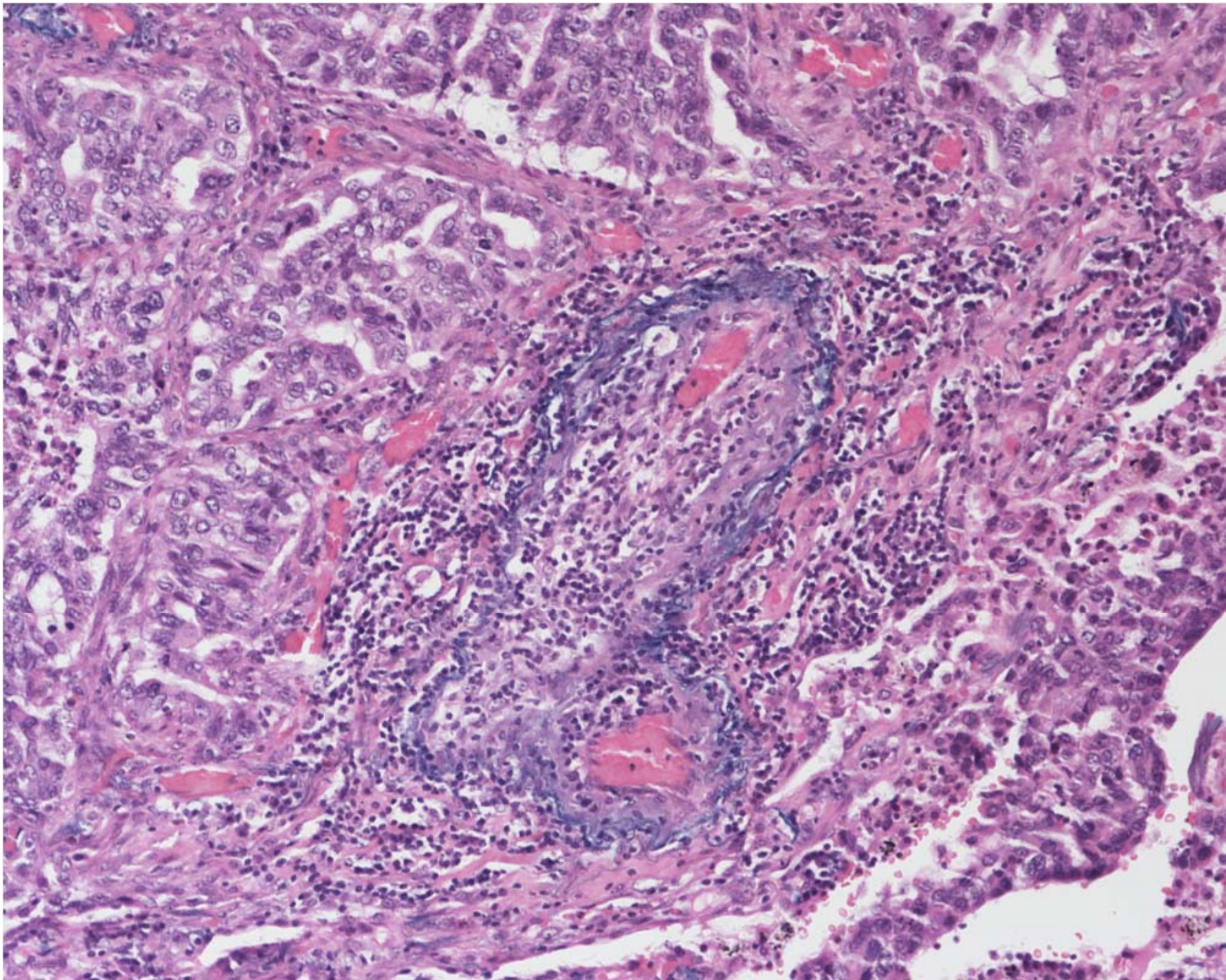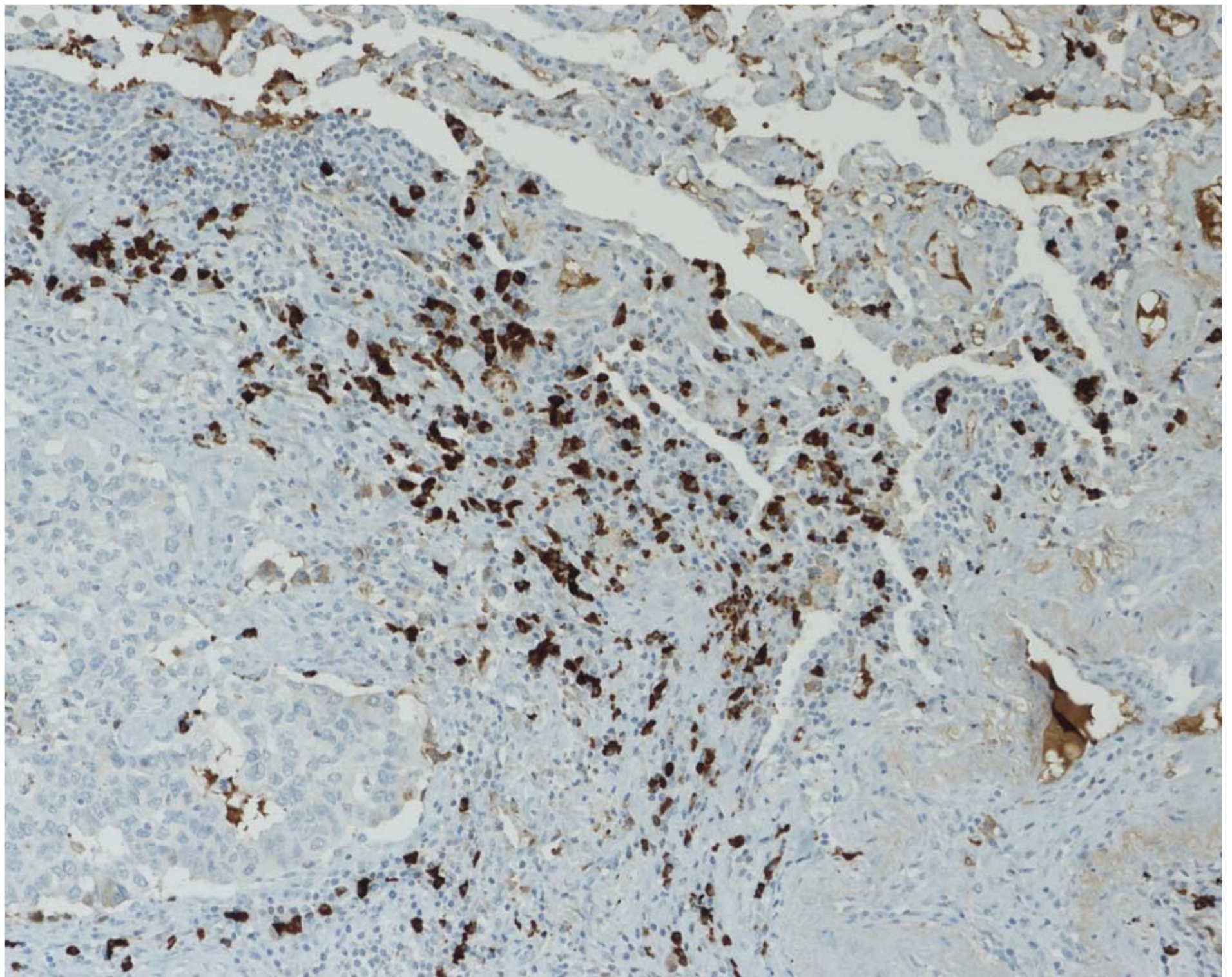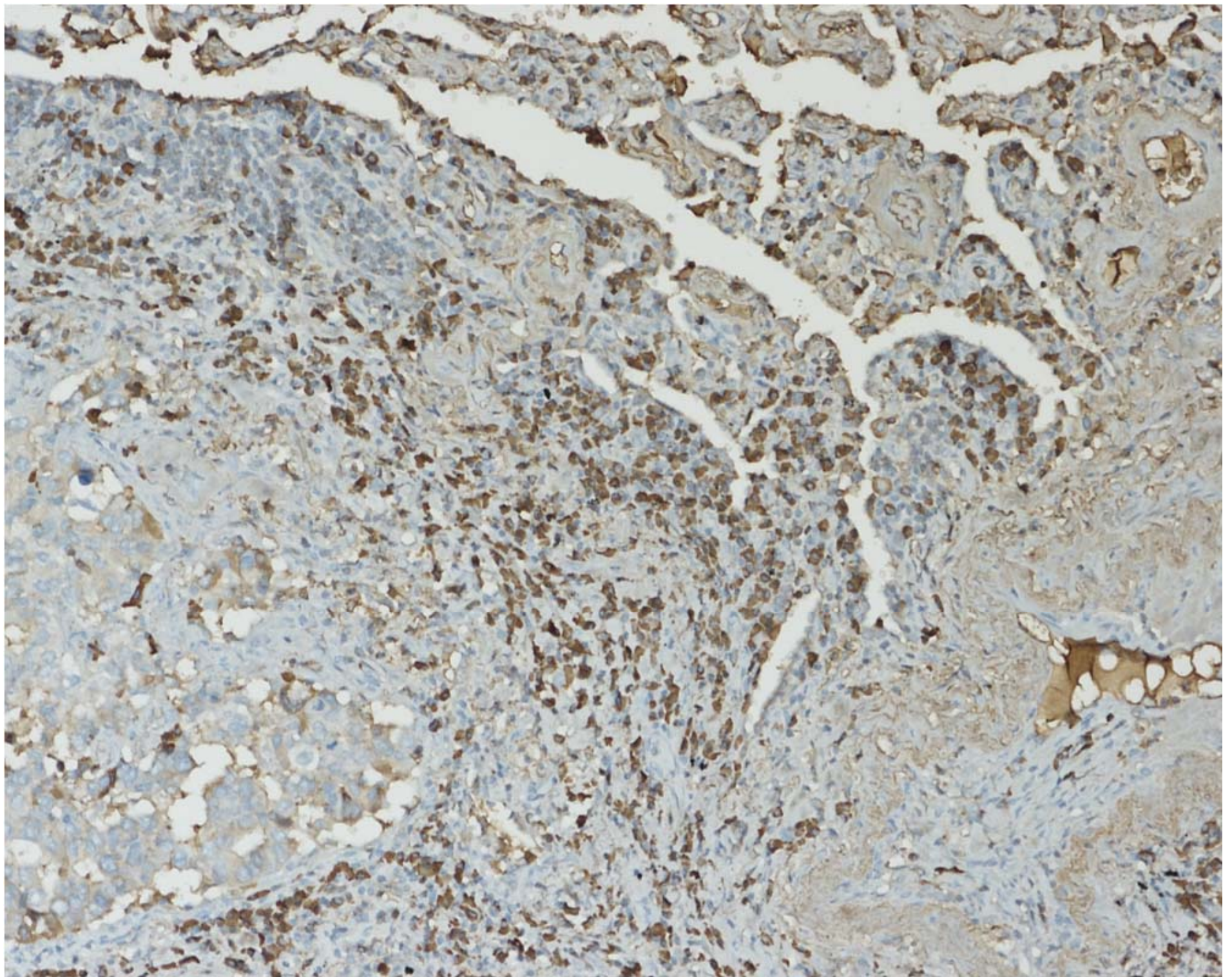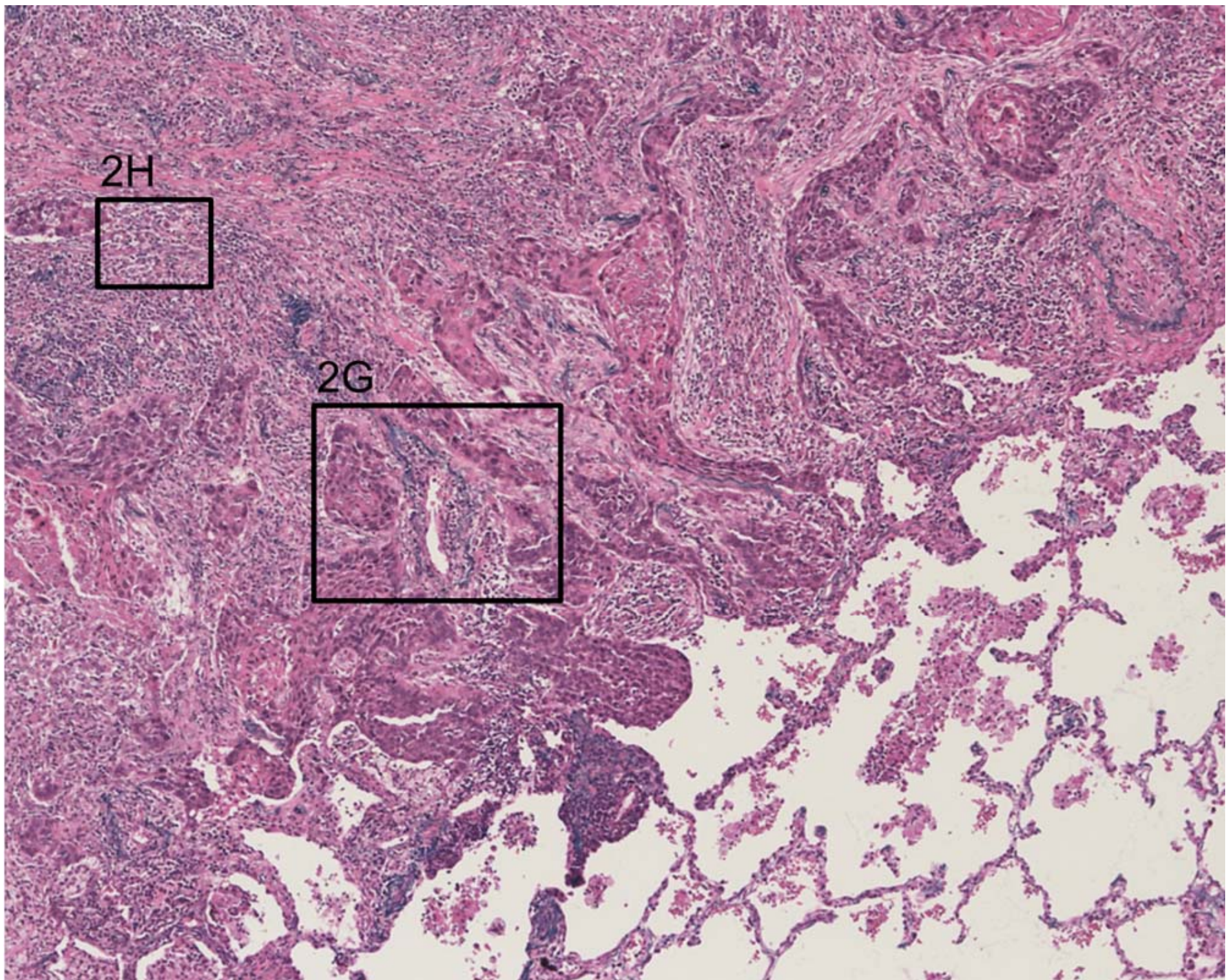
Figure 1(A)

Figure 1(B)
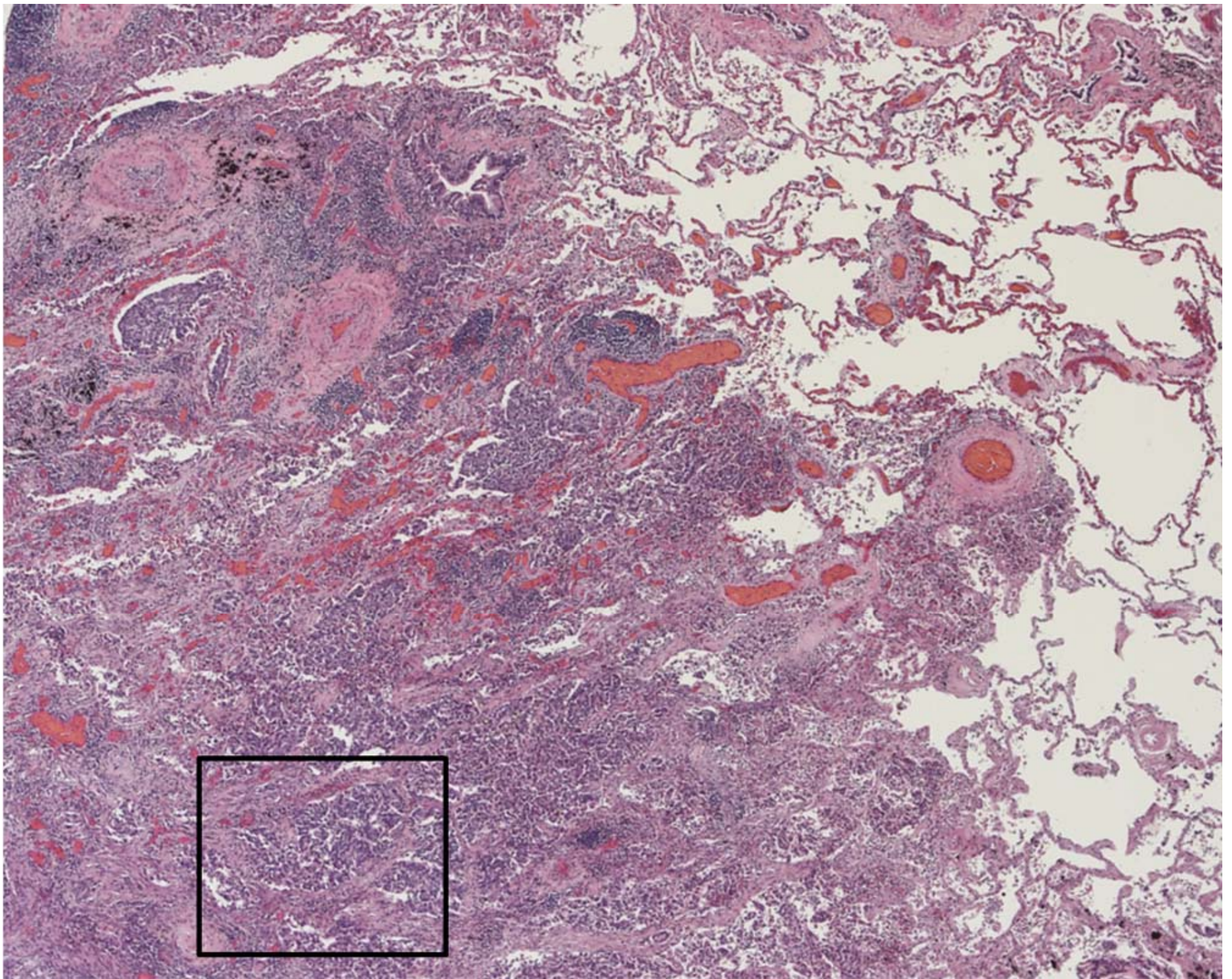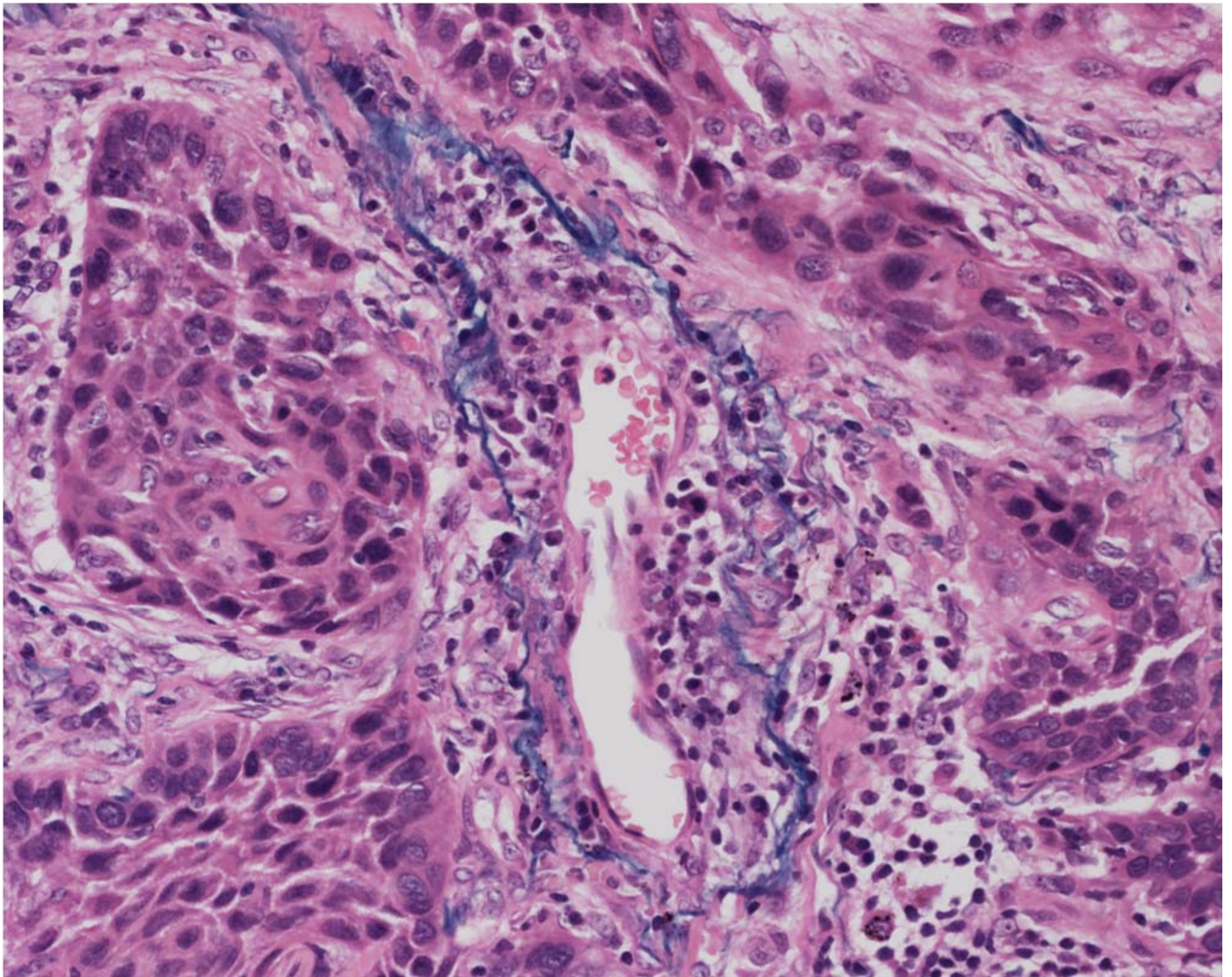
Figure 2(A)
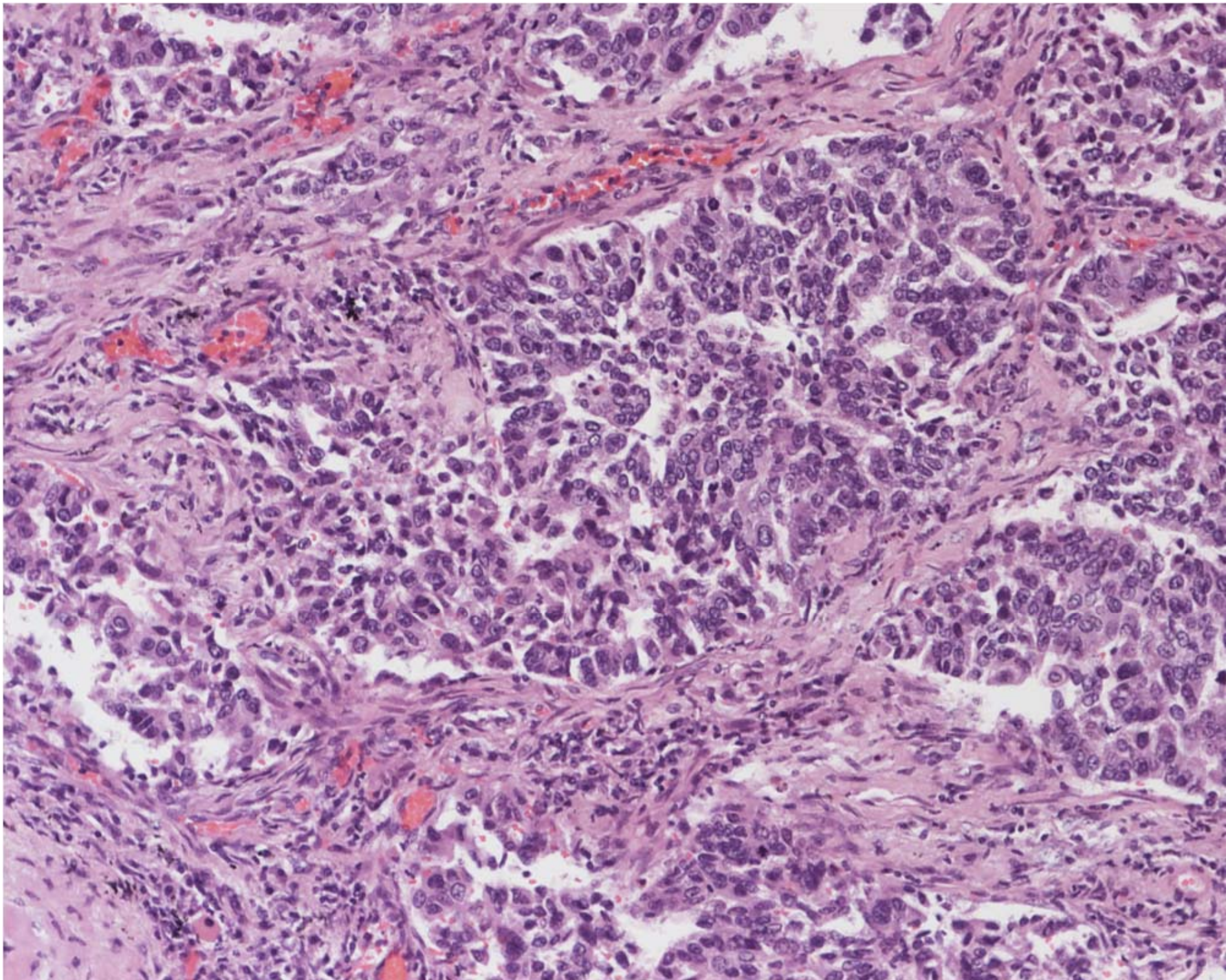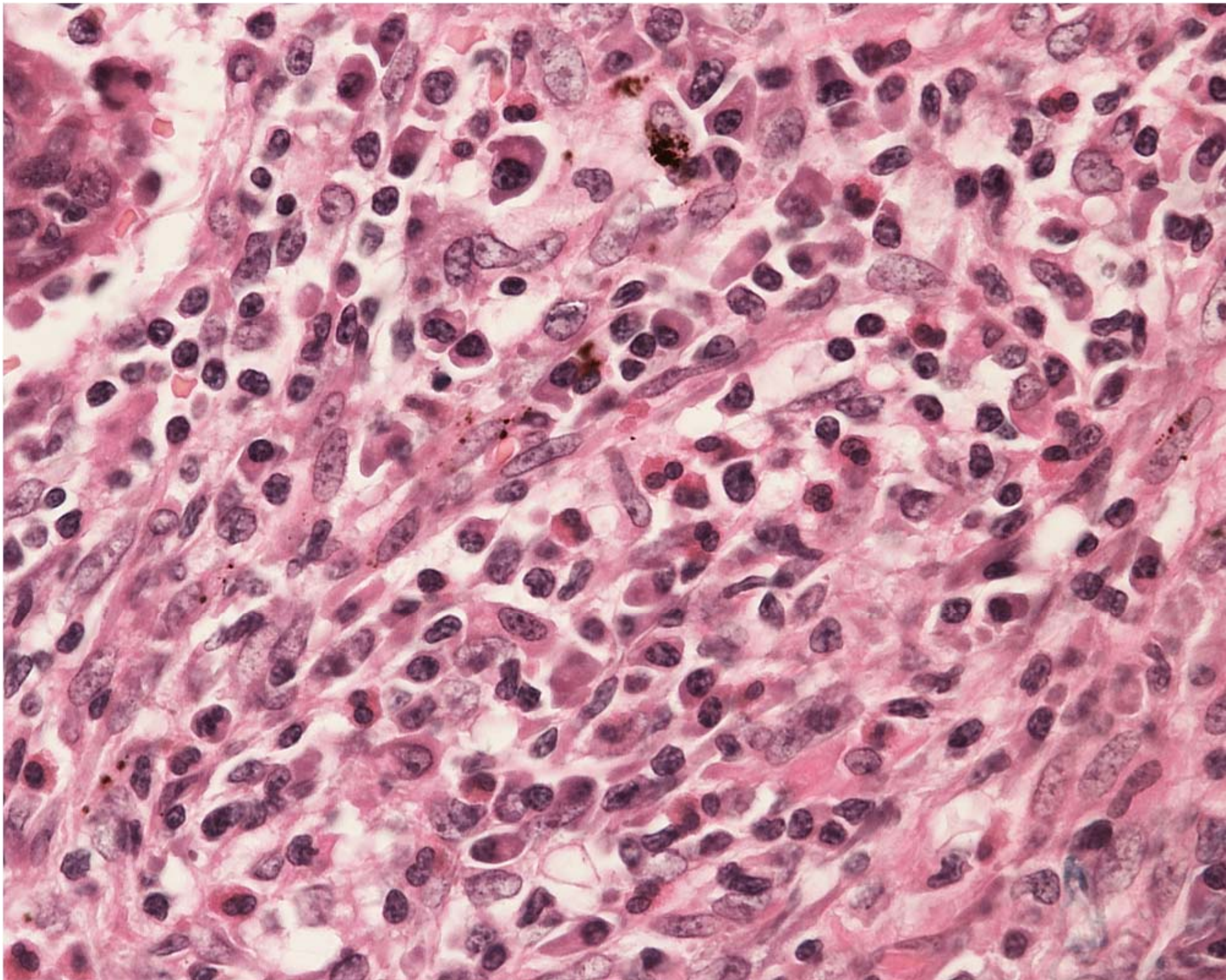
Figure 2(B)

Figure 2(C)

Figure 2(D)

Figure 2(E)

Figure 2(F)

Figure 2(G)

Figure 2(H)