Dual-Block Elastic Stain Significantly Increasing Visceral Pleural Invasion(VPI) Positivity and Analysis of Potential Predictors of VPI in Peripheral Non-Small Cell Lung Cancer

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Research

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Abstract

Background: Visceral pleural invasion (VPI) is a critical component in the staging of peripheral non–small cell lung carcinoma (NSCLC). Single tumor tissue block for elastic stain is conducive to identifying pleural invasion in routine pathologic examination. We aim to investigate whether dual-block elastic stain increase VPI positivity compared with single-block elastic stain, further analyze the potential predictors of VPI status.

Methods: Resected 8419 consecutive peripheral NSCLC cases including tumor size≤3cm 6008 patients were retrospectively reviewed. Total cases were divided into a cohort using one tumor tissue paraffin block (single-block group, n=5184) and a cohort using dual tumor tissue paraffin blocks (dual-block group, n=3235) for elastic stain. Each case was performed with Victoria-blue van Gieson staining to assess VPI status. The clinicopathologic features of patients were collected from the electronic medical record system.

Results: The overall incidence of VPI was 12.4% (1047/8419) in peripheral NSCLC patients. The VPI positivity detected by dual-block elastic stain was significantly higher than that by single-block elastic stain (17.7% (573/3235) v.s. 9.1% (474/5184), P□0.001). The presence of VPI in T1 ≤3cm patients detected by single and dual block elastic stain was 6.3% (235/3730) and 12.0% (273/2278), respectively (P□0.001). Therefore, 5.7% T1 patients (stage IA) are additionally upstaged to T2a (stage IB) by dual block elastic stain. But the incidence of VPI in pT2a patients had no significant difference between single-block group and dual-block group (16.8% vs 17.1%, P=0.916). The lymphovascular invasion, lymph node metastasis, poor differentiated carcinomas and the presence of STAS status could be well significant predictors of VPI (P□0.001). Area under the ROC curve of adenocarcinoma morphology was 0.263 for lepidic pattern, 0.544 for acinar and papillary pattern, and 0.720 for micropapillary and solid pattern in predicting invasion of pleura.

Conclusion: Our results indicated that using dual-block elastic stain identify more VPI positive T1 NSCLC patients who are upstaged to T2a and could benefit from optimal management after post-operation. The application of dual-block elastic stain is an efficient and practical method to detect VPI, especially for patients with high-risk prognostic factors.

Introduction

Lung cancer is the most commonly diagnosed cancer in the world with the highest morbidity and mortality[1]. Visceral pleural invasion (VPI) was a crucial factor of tumor upgrade from T1 to T2a in the 8th edition of the TNM classification system for pulmonary malignancies[2]. Since the existence of VPI affects pathological staging and postoperative treatment decision for patients with stage I non-small cell lung cancer (NSCLC), accurate VPI evaluation and rigorous T descriptions are of critical necessity.

The most detailed classification of VPI was proposed by Hammar[3]. Pleural invasion has been designated according to the degree of pleural invasion as no pleural invasion (PL0), invasion beyond the

elastic layer (PL1), invasion without involving adjacent structures (PL2), and invasion into parietal pleural and/or chest wall (PL3) in the eighth edition TNM classification system[2]. Though, VPI becomes controversial and even unrecognized when pleural structures are obscure as a result of inflammation processes and localized pleural adhesion. The visceral pleura consist of connective tissue and elastic fibers, as well as a rich network of capillaries and interconnecting lymphatic vessels. The presence or absence of elastic lamina penetration is important to differentiate the tumors from PL0 and PL1. Previous studies found that the application of elastic stains[4], including Verhoeff Van Gieson and Victoria Blue–Van Gieson, made the visceral pleural elastic layer noticeable, became a useful approach in assessing the status of VPI when visceral pleural elastic layer was disrupted by tumors.

Peripheral tumors are adequately sampled into several blocks for correct diagnosis of histologic subtypes and assessment of VPI status. Usually, pathologists evaluate VPI status through picking one paraffin block containing tumor cells abutting on visceral pleura for elastic stain on the basis of several hematoxylin and eosin (H&E) slides. But it may be difficult to choose which of the paraffin blocks is used for elastic stain on H&E slides, which could result in false negatives of VPI evaluation. In addition, the existence of factitious discrepancies makes it possible that a few tumor cells invading to the visceral pleural might be failing to diagnose and misdiagnosed[5 6]. Thus, developing efficient methods to assess exact status of VPI is a practical issue in pathological staging of peripheral NSCLC patients.

Previous studies showed that using dual paraffin blocks for assessing HER2 expression could reduce false-negative rate of HER2 due to heterogeneous staining of HER2 in gastric cancer (GC) patients[7]. GC patients with extra gained HER2 positivity by dual block HER2 assessment showed response to anti-HER2 treatment and were also eligible for the targeted treatment[8]. Thereby, evidence of the applying dual block for elastic stain improving the detection rate of VPI remain unclear. To cope with the false-negative rate of VPI and improve the accuracy of VPI evaluation, 8419 consecutive cases of peripheral NSCLC undergoing surgical resection were retrospectively reviewed including 6008 cases of tumor size ≤ 3 cm in stage I. We were the first to compare the positive rate of VPI between a cohort using one paraffin block and a cohort using dual paraffin blocks for elastic stain. The aim of the study is to explore whether using dual tumor tissue paraffin blocks for elastic stain increase the positive rate of VPI and further identify the high risks of VPI positivity in the early-stage peripheral NSCLC patients.

Material And Methods

Study cohort

This study was approved by Shanghai Pulmonary Hospital ethical committee (Shanghai, China). Prior written informed consent was collected from all patients. Consecutive cases of primary peripheral NSCLC underwent complete resection at Shanghai Pulmonary Hospital from January 2018 to December 2019 were retrospectively reviewed. Cases were excluded from further study by the following criteria: (1) involvement of visceral pleural surface or parietal pleura (PL2 and PL3); (2) patients who presented with neuroendocrine tumors. Furthermore, patients in this study who had received therapy and recurrent lung

cancer were ineligible for inclusion. A total of 8419 cases were collected including 6008 patients with tumor size ≤ 3 cm in stage I. Those patients were divided into a cohort using one tumor tissue paraffin block (single-block group, n = 5184) and a cohort using dual tumor tissue paraffin blocks (dual-block group, n = 3235) for elastic stain. Clinicopathological characteristics, including sex, age, tumor size, lymphovascular invasion, lymph nodes status, the degree of tumor differentiation, STAS status and predominant growth pattern of non-mucinous adenocarcinoma (lepidic, acinar, papillary, micropapillary, and solid), were collected.

To analyze histological differentiation of adenocarcinoma for predicting VPI, lepidic growth, pattern, acinar and papillary growth pattern, solid and micropapillary growth pattern were defined as well, moderate and poor differentiation, respectively.

Histopathological Evaluation

All the tumor specimens (H&E staining) with their pleurae (stained by Victoria Blue–Van Gieson) were reviewed independently by two thoracic pathologists. A discussion panel involving the third observer was introduced for cases with discrepant assessment of VPI status. Tumors classification and pathological staging were in accordance with International Union Against Cancer and American Joint Committee on Cancer[9]. Representative block per case, which was abutting on the visceral pleura, was selected for evaluating VPI status. Pleural invasion was classified according to the eighth edition TNM staging system as following: PL0, without pleural involvement or reaching the visceral pleural connective tissue beneath the elastic layer; PL1, tumor invades beyond the visceral pleural elastic layer but is not exposed on the surface[2].

Statistical analysis

Statistics were collected using standard methods and analyses were performed using IBM SPSS 20.0 software (IBM Co., Chicago, Illinois, United States). The relationship between several clinicopathological parameters were evaluated using the $\chi 2$ test and Fisher's exact test. Receiver operating characteristic (ROC) curve analysis was used to identify the histopathological factors that significantly predicted the extent of pleural infiltration. P values less than 0.05 were considered statistically significant.

Results

Detection rate of VPI between single-block and dual-block groups

The destruction of elastic layers of pleura was questionable on the block of the H&E slides from the same tumor in some cases. There was controversial about which one of blocks with pleural involvement or not (Fig. 1A, 1B and 2A, 2B). With the application of dual blocks for elastic stain, tumor cells invasion beyond

elastic layers was visualized and the status of pleural invasion was clearly assessed (Fig. 1C, 1D and 2C, 2D). Generally, the detection rate of VPI was 9.1% (474/5182), 17.7% (573/3235) in single-block and dual-block group, respectively, which showed significantly difference between two groups ($P < 0.001$). In dual-block group, 410 cases showed one tissue block with VPI positivity and 163 cases with two-blocks VPI positivity. Comparing with single-block group, the VPI-positive rate increased by 8.6% by dual-block elastic stain as shown Table 1.

Table 1
Visceral pleural involvement confirmed by single and dual-block ES in peripheral 8419 NSCLC patients

Variables	VPI (%)	Non-VPI (%)	Total	*P* value
Single-block ES	474 (9.1)	4710 (90.9)	5184	
Dual-block ES	573 (17.7)	2662 (82.3)	3235	0.001*
one block positive	410 (12.7)			
Two blocks positive	163 (5.0)			
VPI, visceral pleural invasion; ES, elastic stain.				
*VPI positivity between Single-block ES group and dual-block ES group.				

The role of dual-block elastic stain in evaluating VPI status for staging of NSCLC patients with Lymph Node Negative and Tumor size ≤ 4 cm.

Cases of 6008 (T1, tumor size ≤ 3cm) and 804 (T2a tumor size, 3cm to ≤ 4cm) NSCLC patients without lymph node metastasis were retrospectively reviewed. For comparing the different VPI positivity in 6008 NSCLC patients (T ≤ 3cm), the incidence of VPI were 6.3% (235/3730) in single-block group and 12.0% (273/2278) in dual-block group, respectively. The application of dual-block elastic stain had significant correlation with higher detection rate of pleural involvement than that in single-block elastic stain (P0.001). In single-block group, 235 of 3730 (T ≤ 3cm, stage IA) cases with VPI positivity were upstaged to T2a (stage IB). Compared with single-block group, 5.7% patients with tumor size ≤ 3cm (stage IA) were additionally upstaged to T2a (stage IB) in dual-block group. In VPI-positivity patients of dual-block group, 202 cases (74.0%) were VPI positive in one block and 71 cases (26.0%) were VPI positive in both two blocks (Table 2). However, similar incidence of VPI were not found between single-block group (16.8%, 82/488) and dual-block group (17.1%, 54/316) for elastic stain in NSCLC patients with pT2a (P = 0.916) (Table 3). In addition, the VPI was significantly more frequent (16.8% versus 8.5%, P0.001) in T2a (> 3 to ≤ 4 cm) tumors than that in T1 (≤ 3 cm) tumors (Table 4).

Table 2
VPI positivity between single-block and dual-block group of 6008 NSCLC patients with tumor size ≤ 3cm.

Variables	VPI (%)	Non-VPI (%)	Total	P value
Single-block ES	235 (6.3)	3495 (93.7)	3730	
Dual-block ES	273 (12.0)	2005 (88.0)	2278	□0.001*
one block positive	202 (8.9)			
two blocks positive	71 (3.1)			
VPI, visceral pleural invasion; ES, elastic stain.				
*VPI positivity between Single-block ES group and dual-block ES group.				

Table 3
VPI positivity between single-block and dual-block group of 804 NSCLC patients with tumor size(> 3 to ≤ 4cm)

Variables	VPI (%)	Non-VPI (%)	Total	*P* value
Single-block ES	82 (16.8)	406 (83.2)	488	0.916*
Dual-block ES	54 (17.1)	262 (82.9)	316	
one block positive	34 (10.8)			
two blocks positive	20 (6.3)			
VPI, visceral pleural invasion; ES, elastic stain.				
*VPI positivity between Single-block ES group and dual-block ES group.				

Table 4
VPI positivity difference between T1(≤ 3cm) and T2a (□3cm to ≤ 4cm) NSCLC patients.

Variables	VPI (%)	Non-VPI (%)	Total	*P* value
T1 (≤ 3cm)	508 (8.5%)	5500 (91.5%)	6008	□0.001
T2a (□3cm to ≤ 4cm)	136 (16.9%)	668 (83.1%)	804	

Correlation between clinicopathological features and visceral pleural involvement in peripheral NSCLC Patients

The clinical and pathological characteristics of NSCLC patients are listed in Table 5. There were 4514 female patients and 3905 male patients, with a median age of 64 years (range: 25–89 years). Overall, VPI occurred in 1047 of 8419 cases (12.4%). The percentage of male patients with VPI was higher than that of female patients (P□0.001). However, the presence of VPI had no difference in terms of age (P = 0.364). Furthermore, VPI positivity was associated with tumor size larger than 3 cm (P□0.001), lymphovascular

invasion (P□0.001), lymph nodes involvement (P□0.001), and the presence of STAS status also played a significant role in the incidence of VPI (P□0.001).

Table 5
Correlation between VPI and clinical-pathologic characteristics of 8419 peripheral NSCLC patients

Characteristics	Patients(n)	VPI		Non-VPI		*P* value
		number	%	number	%	
Total	8419	1047	12.4	7372	87.6	
Sex						
Female	4514	491	10.9	4023	89.1	
Male	3905	556	14.2	3349	85.8	< 0.001
Age (year)						
< 60	2439	291	11.9	2148	88.1	
≥ 60	5980	756	12.6	5224	87.4	0.364
Tumor size(cm)						
≤ 3cm	6667	718	10.8	5949	89.2	
> 3cm	1752	329	18.8	1423	81.2	< 0.001
Lymph nodes						
Negative	6932	646	9.3	6286	90.7	
Positive	1212	360	29.7	852	70.3	< 0.001
NA	275	26	9.5	249	90.5	
STAS status						

VPI, visceral pleural invasion. NA, no answer (patients did not undergo lungs and hilar lymph nodes dissection). STAS, tumor spread through air space;

visceral pleural invasion. NA, not available (patients did not undergo systemic hilar and mediastinal lymph nodes dissection). STAS, tumor spread through air space;

*26 lymphoepithelioma-like carcinoma, 23 mixed carcinoma and 7 cases with two different primary tumors were excluded;

** include 210 invasive mucinous adenocarcinoma /collioid carcinoma, 3 enteric adenocarcinoma and 1 low-grade fetal adenocarcinoma;

***compared with invasive adenocarcinoma;

****include 67 large cell carcinoma, 78 sarcomatoid carcinoma, 65 adenosquamous carcinoma, and 1 poorly differentiated carcinoma with SMARC4-deficient.

Characteristics	Patients(n)	VPI		Non-VPI		*P* value
		number	%	number	%	
Negative	6788	652	9.6	6136	90.4	
Positive	1631	396	24.3	1235	75.7	< 0.001
Lymphovascular invasion						
Negative	7974	897	11.2	7077	88.8	
Positive	445	150	33.7	295	66.3	< 0.001
Pathologic subtype*						
Invasive adenocarcinoma**	7481	901	12.0	6580	88.0	
non-mucinous adenocarcinoma	7270	901	12.4	6369	87.6	
mucinous adenocarcinoma	210	0	0.0	210	100	< 0.001
squamous cell carcinoma	672	78	11.6	594	88.4	0.725
Others***	211	54	25.6	157	74.4	< 0.001

VPI, visceral pleural invasion. NA, no answer (patients did not undergo lungs and hilar lymph nodes dissection). STAS, tumor spread through air space;

visceral pleural invasion. NA, not available (patients did not undergo systemic hilar and mediastinal lymph nodes dissection). STAS, tumor spread through air space;

*26 lymphoepithelioma-like carcinoma, 23 mixed carcinoma and 7 cases with two different primary tumors were excluded;

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***compared with invasive adenocarcinoma;

****include 67 large cell carcinoma, 78 sarcomatoid carcinoma, 65 adenosquamous carcinoma, and 1 poorly differentiated carcinoma with SMARC4-deficient.

In addition, the incidence of VPI in patients with poor differentiated histologic subtypes, including 67 cases of large cell carcinoma, 78 cases of sarcomatoid carcinoma, 65 cases of adenosquamous carcinoma, and 1 poorly differentiated carcinoma with SMARCA4-deficient, was 25.6% (54/210), which was much higher than that in adenocarcinoma (12.0%, 901/7481) and squamous cell carcinoma patients (11.6%, 78/672), respectively (P□0.001). However, the percentage of VPI had no difference between

adenocarcinoma and squamous cell carcinoma (P = 0.725). Remarkably, none of invasive mucinous adenocarcinoma cases (0/210) present pleural involvement, irrespective of tumor size in our study (Fig. 3).

Correlation between growth pattern of peripheral invasive adenocarcinoma and VPI status

As shown in Fig. 4, The ROC analysis of the histogram parameters between VPI presence and VPI absence showed that AUC of well, moderate and poor differentiation were 0.263 (95% confidence interval [CI]:0.246–0.280), 0.544 (95% CI: 0.518–0.570), and 0.720 (95% CI:0.698–0.743), respectively, highlighting that lepidic histology was a protective factor of VPI, while micropapillary and solid histology were risk factors of VPI, and the combination of micropapillary and solid growth pattern was the most beneficial parameter for predicting the extent of VPI (P□0.001). Micropapillary and solid predominant histology were significant factors for predicting pleural involvement (AUC = 0.720, cut-off value of 3.5 × 10 − 3 mm2/sec, sensitivity 74.5%, specificity 69.6%) (as shown in Table 6).

Table 6
Univariate analysis of correlation between different growth pattern of adenocarcinoma and the presence of VPI

histological comparison	AUC	95% confidence interval		*P* value
		lower-bound	upper-bound	
Lepidic	0.263	0.246	0.28	< 0.0001
acinar and papillary	0.544	0.518	0.57	< 0.0001
micropapillary and solid	0.720	0.698	0.743	< 0.0001

Discussion

Visceral pleural invasion is a risk factor of upstaging the T classification from T1 to T2a and has been known to be an adverse prognostic factor in stage I NSCLC. Elastic stain is recommended to assess VPI status which could not be clearly evaluated by H&E stains. This study for the first time revealed a significant impact of dual-block elastic stains on evaluating the VPI status compared with single-block elastic stain in large cohort of peripheral NSCLC patients. Our results indicated that higher detection rate of VPI was found by dual-block than single-block elastic stain in NSCLC patients with T1 (≤ 3cm), and provided a simple and practical approach for precise T classification of peripherally located NSCLC patients.

The total incidence of VPI was encountered in 12.4% of NSCLC cases in our study, which was identical with reported data between 11.5% and 26.8%[10–12]. In T1 (≤ 3cm) patients, VPI positivity was much higher in dual-block elastic staining group than that in single-block elastic staining group (12.0% v.s. 6.3%, P□0.0001). These results showed that dual blocks for elastic stain can greatly improve the detection rate of VPI in T1 tumors (≤ 3cm). In the VPI-positive group determined by dual-block elastic stain, 202

cases (74.0%) were positive in only one block, whereas 71 cases (26.0%) were positive in both two blocks, which indicated that a certain proportion of VPI-positive cases might be missed diagnosed because of being performed single-block elastic stain. Given that pleural infiltration plays an important role in clinicopathological staging and treatment decision[13], we recommend to perform dual-block elastic stain to assess VPI status in routine practice.

Inevitably, the assessment of VPI became difficult because of reactive fibrosis, inflammation, and even though the specialists would fail to detect the invasion of pleural infiltration by H&E stains. Previous study reported that VPI status in 10% of cases was indeterminate by H&E staining[14]. In that case, elastic stain can make it much easier for the pathologists to identify the extent of pleural invasion[4]. However, peripherally located NSCLC tumors need to be generously sampled into several blocks containing visceral pleura. In some cases, it is possible that VPI might be failing to diagnose due to the application of single-block elastic stain on the base of H&E-stained slides. Our results revealed that performing dual-block elastic stain could improve the detection of pleural invasion. Dual-block elastic stain results changed pathologic stages in 5.7% resected NSCLC cases which were upstaged from IA to IB. These patients may be potential beneficiaries of dual-block elastic stain when VPI had been missed diagnosed by single-block elastic stain[15 16].

An intriguing finding in current study was that no difference was observed in the occurrence of VPI demonstrated by single-block and dual-block elastic stain (17.1% v.s. 16.8%) in 804 pT2a patients (3cm to ≤ 4cm). We also observed that the frequency of VPI was variable. According to T1/2a subsets, VPI presence was 8.5 % in T1 tumors (≤ 3cm) and 16.9% in T2a tumors (3cm to ≤ 4cm), respectively. Previous research indicated that the VPI was more frequent when the size of the tumor increased[17 18]. It significantly correlated with a tumor size larger than 3 cm. For T ≤ 3cm tumor, more patients with VPI presence were detected by dual-block than single-block elastic stain. Therefore, dual-block elastic stain is significantly efficient method to evalutate VPI status for NSCLC patients with T ≤ 3cm tumor adjacent pleura. In addition, the VPI was frequently found in patients with lymphovascular invasion, lymph nodes involvement[19], poor differentiation and the presence of STAS status, which were consistent with the previous studies[20–23].

Studies showed that the degree of differentiation is one of the predominant prognostic factors in lung cancer[24]. The occurrence of VPI in poorly differentiated histologic NSCLC subtypes was much higher than that in adenocarcinoma and squamous cell carcinoma. Conversely, none of mucinous invasive adenocarcinoma was VPI present. In addition to subtypes, several morphologic patterns presented in invasive adenocarcinoma. The ROC analysis of different histomorphology showed that the micropapillary and solid predominant patterns had a stronger relationship with the incidence of VPI compared to lepidic growth patterns. Micropapillary united solid morphology achieved the highest AUC at 0.720, while the AUC of lepidic pattern was 0.263, thereby micropapillary and solid predominant patterns can be efficient predictors of VPI with high sensitivity and specificity[21]. These results were reasonable for poorly differentiated carcinomas may have a greater ability for migration and invasion and hence correlate with a more frequent occurrence of VPI.

In the current study, retrospective nature of our study is the major limitation. Some NSCLC cases with lymph node metastasis were enrolled to evaluate the risk factors of VPI presence. However, the proportion was relatively small. The lack of follow-up information about the prognosis of the patients with T1 tumors (≤ 3cm) and VPI presence upstaged to T2a category was another limitation, although T1 tumors with VPI presence had worse prognosis than that with VPI negativity, which had been verified by previous studies[18 25]. Prospective studies in which VPI status predicts the prognosis of T1 tumors are robustly warranted.

In conclusion, our study demonstrates that the use of dual-block elastic stain is simple and efficient approach, and it could provide a definite and accurate assessment of pleural invasion status in patients with peripheral NSCLC. The presence of VPI detected by dual-block elastic stain could more accurately differentiate the upstaging NSCLC patients from stage IA to IB, who benefit from optimal management after post-operation.

Abbreviations

NSCLC
Non-small cell lung cancer; VPI:Visceral pleural invasion; AUC:Area under curve; ROC:Receiver operating characteristic; H&E:Hematoxylin-eosin; STAS:Spread through air space;

Declarations

Ethical approval and consent to participate

The study was approved by the ethics committee of Shanghai Pulmonary Hospital (No. L20-333Y). Additional patient consent for this retrospective study was not required.

Consent for publication

Not applicable.

Availability of data and materials

The raw data are available upon request.

Competing interests

The authors declare that they have no conflicts of interests.

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Authors' contributions

CY W and LK H designed the study and interpreted the data. SL L and Y H wrote the manuscript. LP Z and ZW D collected and analyzed the clinical data. W W and W Z performed the experiments. All authors read and approved the final manuscript.

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Figures

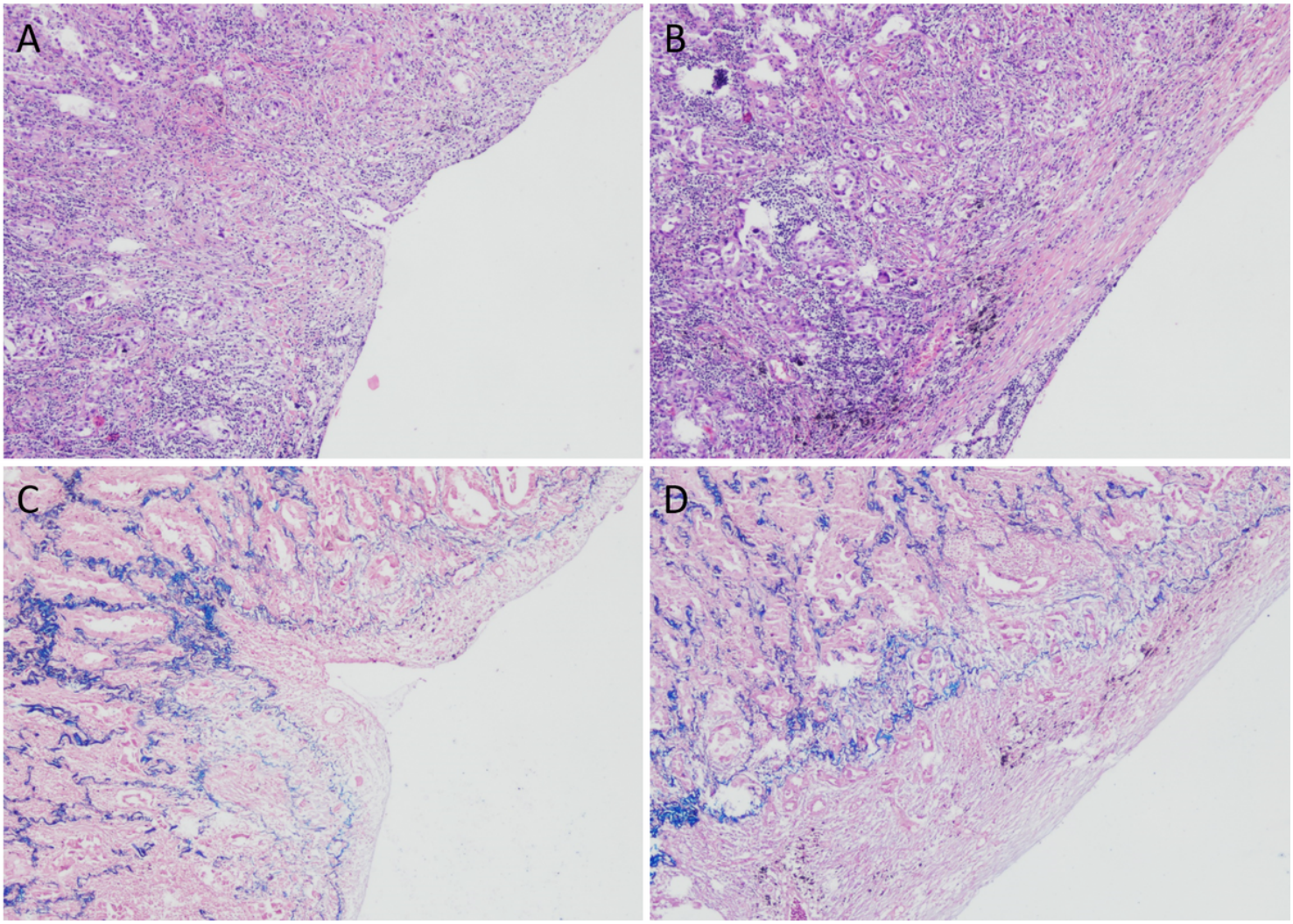

Figure 1

Dual-block for elastic staining to evaluate VPI status of peripheral adenocaricinoma It is difficult to determine the extent to which tumor extends into the visceral pleura on dual H&E sections from the same case (A and B); Tumor cells unequivocally reach out beneath elastic layer on elastic staining (C); Tumor cells obviously invade beyond the visceral pleural elastic layer on elastic staining (D).

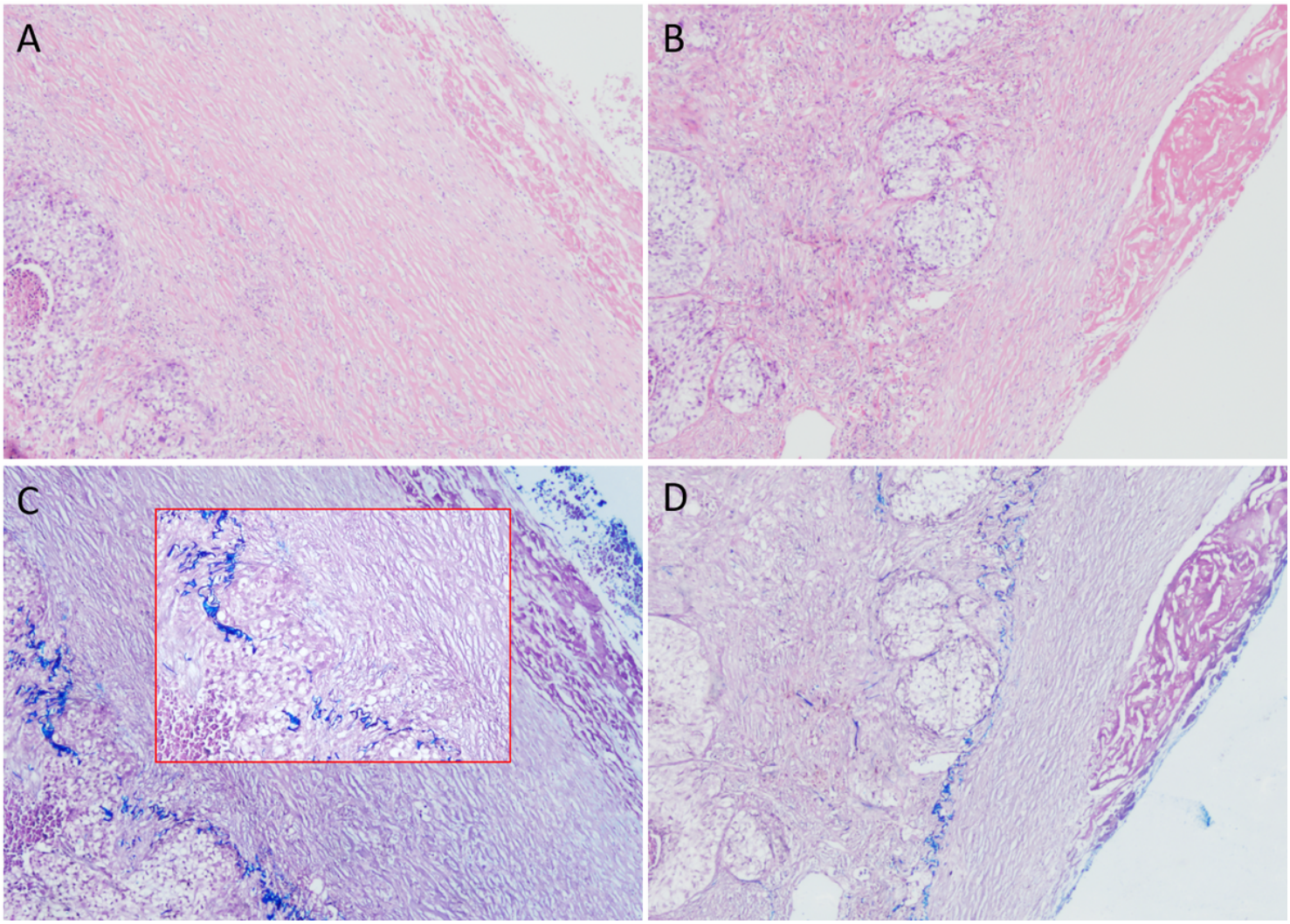

Figure 2

Dual-block for elastic staining to evaluate VPI status of peripheral squamous cell carcinoma tumor cells appear a certain distance from the visceral pleural surface on dual H&E sections from the same case (A and B); Tumor cells invade beyond the visceral pleural elastic layer on elastic staining, which is breached by a large nest of tumor cells (red square area, at medium magnification) (C). Tumor cells extend to but not traverse visceral pleural elastic layer on elastic staining (D)

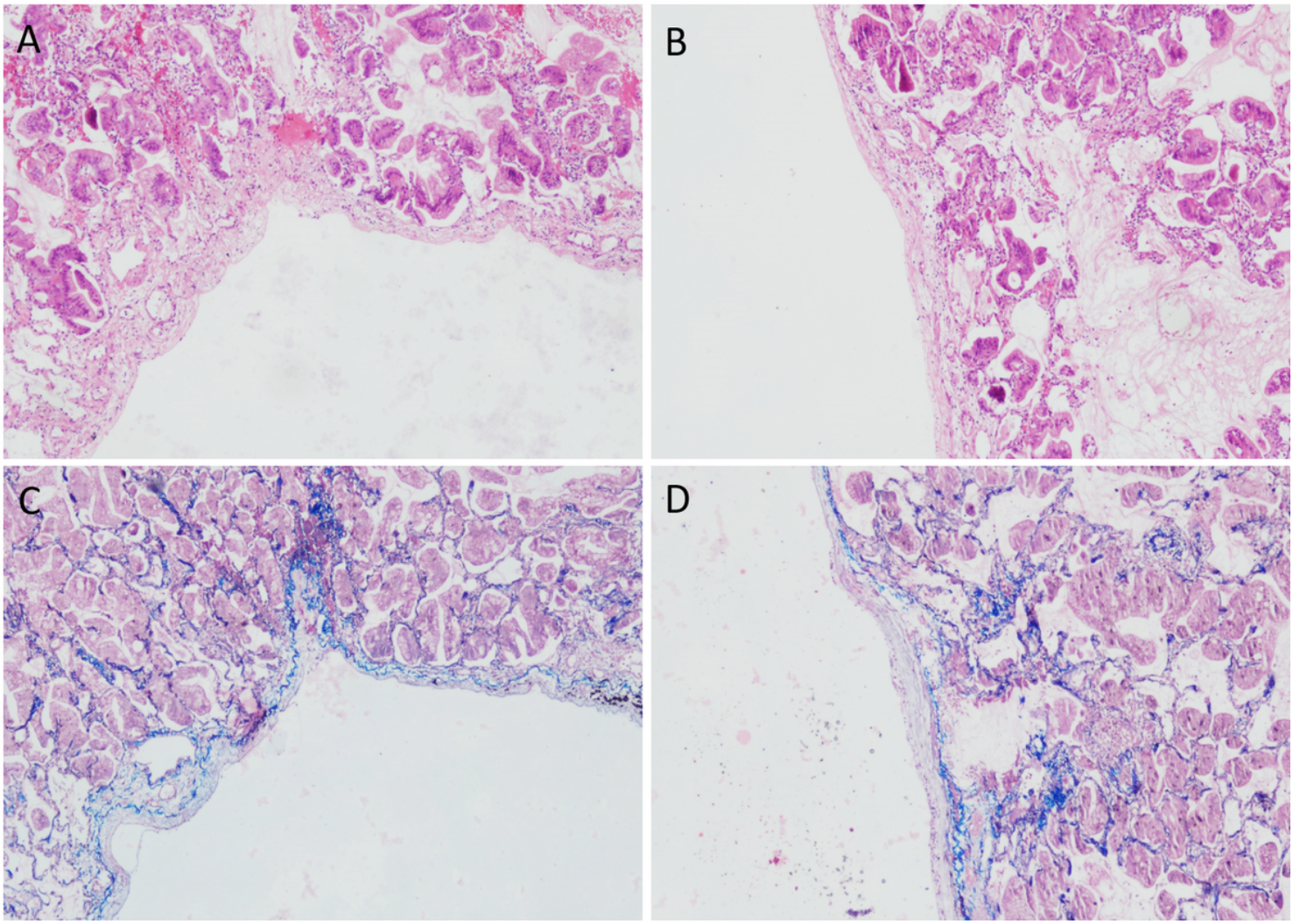

Figure 3

Dual-block for elastic staining to evaluate VPI status of peripheral invasive mucinous adenocarcinoma Dual H&E sections from the same case show tumor cells abutting on visceral pleural elastic layer (A and B); Tumor cells unequivocally do not penetrate the visceral pleural elastic layer on elastic staining (C and D).

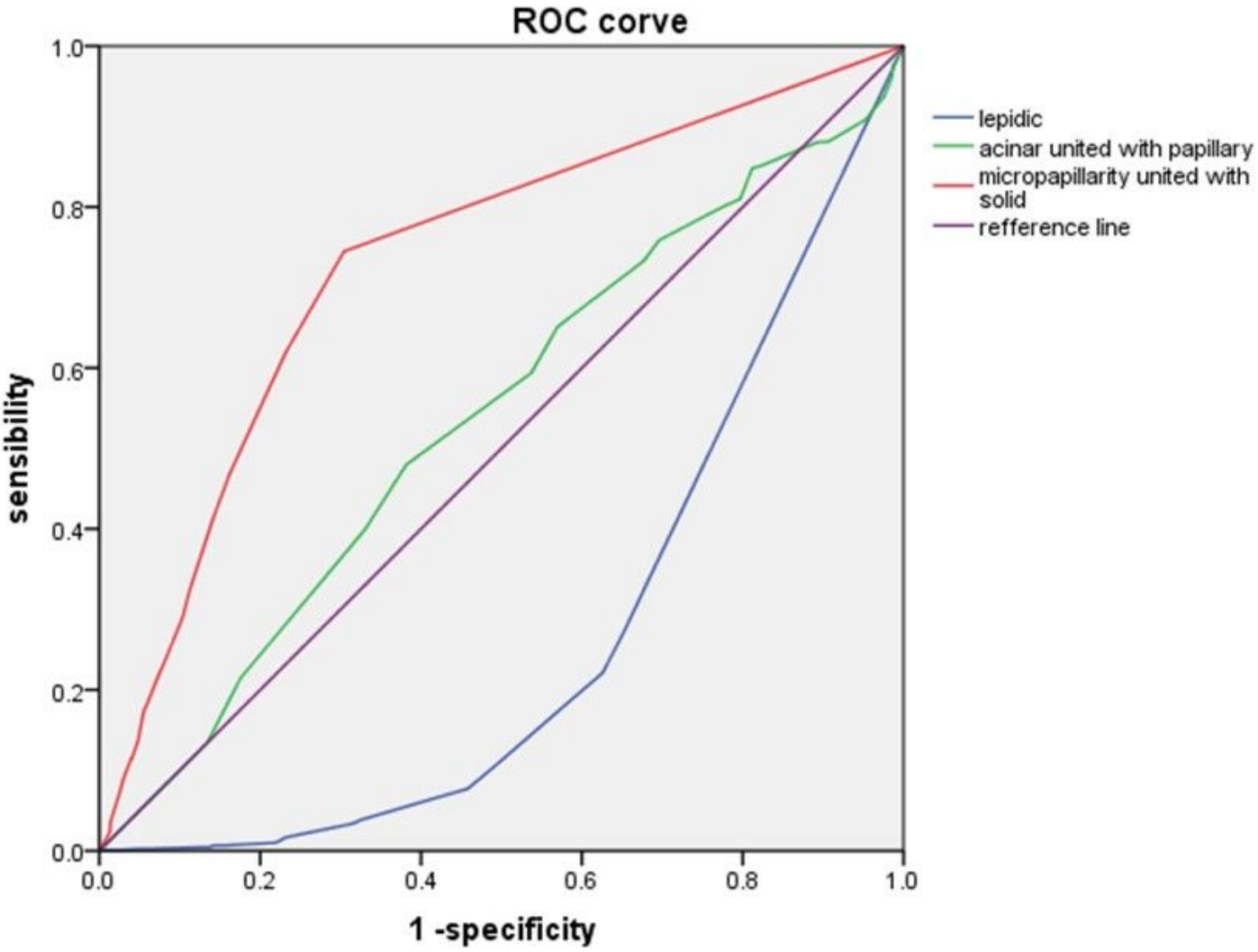

Figure 4

ROC curve of different growth pattern of invasive adenocarcinoma in predicting pleural invasion