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Clinical Significance of Subcellular Localization of Maspin in Patients with Pathological Stage IA Lung Adenocarcinoma

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Abstract. *Background/Aim: Maspin is a tumor-suppressor protein and its prognostic value in lung adenocarcinoma has been reported. However, little is known about the clinical impact of subcellular localization of maspin in early-stage lung adenocarcinoma. We aimed to evaluate the clinical significance of subcellular localization of maspin in patients with pathological stage (p-stage) IA lung adenocarcinoma categorized by the new eighth edition TNM classification. Patients and Methods: We immunohistochemically analyzed 181 tissue samples from p-stage IA1 (n=37), IA2 (n=92) and IA3 (n=52) lung adenocarcinomas using antibody for maspin. Results: The 181 cases fell into five predominant subtypes: lepidic (n=32), acinar (n=97), papillary (n=30), solid (n=20) and micropapillary (n=2). The frequencies of maspin staining were: cytoplasmic-only in 24.9%; pancellular (nuclear and cytoplasmic) in 8.8%; nuclear-only in 0.6%; no staining in 65.7%. Cytoplasmic-only staining significantly correlated with high pathological T-classification (p=0.039), lymphatic invasion (p=0.002) and poorer tumor differentiation (p=0.002). The patients were followed-up for 12-151 months (median=74 months), and the cytoplasmic-only staining significantly correlated with shorter disease-free survival (DFS) (p=0.034) and disease-specific survival (DSS) (p=0.036) by log-rank tests. In Cox's multivariate analysis, lymphatic invasion had the most significant effect on shorter DFS and DSS. Conclusion: The expression of maspin in the cytoplasm alone could be useful for predicting unfavorable prognoses in patients with p-stage IA lung adenocarcinoma.*

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Adenocarcinoma is the most frequent histological subtype of lung cancer accounting for >40% of all lung cancers (1). With the development of imaging technology, smaller and earlier-stage lung adenocarcinomas are now being detected. However, the 5-year recurrence rate of adenocarcinoma was reported to range from 16% to 20% even in patients with pathological stage (p-stage) I disease (2-4). The eighth edition of the tumor-node-metastasis (TNM) classification of lung cancer was recently published, and stage IA was divided into stages IA1, IA2 and IA3 according to the greatest dimension of the invasive component (5). The 5-year survival rates of patients with stage IA1, IA2 and IA3 cancer were reported to be 92%, 83% and 77%, respectively (6).

Maspin, a non-inhibitory member of the serine protease inhibitor (serpin) superfamily, was detected originally as a tumor-suppressor protein expressed in normal breast epithelial cells but whose expression is reduced in or absent from breast carcinoma (7). Maspin has been shown to inhibit both tumor growth and metastasis in multiple animal models and cancer cell lines, and has shown pro-apoptotic, anti-metastatic and anti-angiogenic properties, exerting an inhibitory effect on cancer cell motility, invasiveness and metastasis ability (8). Although the exact biochemical pathways leading to these biological endpoints are incompletely characterized, several studies have reported an association between maspin expression and clinicopathological factors in many types of cancer, including lung cancer. However, there are conflicting results regarding whether maspin expression is a favorable or unfavorable indicator in patients with lung cancer (9-15). We also reported that the cytoplasmic-only expression of maspin was a poor prognostic indicator in patients with lung adenocarcinoma measuring <3 cm (13). However, that study included adenocarcinoma *in situ* (AIS) and minimally invasive adenocarcinoma (MIA), for which the 5-year disease-free survival (DFS) rate and 5-year disease-specific survival (DSS) rate are both 100% (16). To our knowledge,

there is no report validating the clinical impact of maspin expression in p-stage IA lung adenocarcinoma as categorized by the new TNM classification. The aim of our study was to clarify the prognostic value of maspin expression with focus on its subcellular localization in patients with p-stage IA lung adenocarcinoma.

Patients and Methods

Patients and tumor specimens. From January 2005 to December 2012 at the Tottori University Hospital (Tottori, Japan), 229 consecutive patients underwent curative surgical resection of lung cancer diagnosed as p-stage IA lung adenocarcinoma according to the eighth edition of the TNM classification (5). Eight cases were excluded for the following reasons: in two cases, a history of neoadjuvant chemotherapy was noted; in three cases, there was a history of radiation therapy; and in the remaining three cases, death occurred within 30 days after surgery (due to pulmonary embolism, adult respiratory distress syndrome and acute renal failure). Nine cases of invasive mucinous adenocarcinoma and 31 cases of MIA were also excluded. Thus, 181 cases with p-stage IA (IA1 in 37 cases, IA2 in 92 and IA3 in 52) were included in this study. The median follow-up time was 74 months (range=12-151 months). The patients' clinicopathological data were obtained from their hospital medical records.

Histopathological evaluation. The pathological diagnosis was performed using hematoxylin and eosin (H&E)-stained sections according to the criteria of the current World Health Organization (WHO) classification of lung cancer (1), and all H&E slides were reviewed by T.O. and Y.U. without knowledge of any of the clinical data. The other histopathological factors, such as tumor differentiation and tumor size were diagnosed histopathologically. The size of the invasive component was evaluated by Elastica van Gieson staining. Lymphatic invasion was evaluated by immunohistochemistry using an antibody for podoplanin. The tumor stage was determined based on the eighth edition of the TNM classification of lung cancer (5). All cases were classified into five predominant subtypes, namely lepidic, acinar, papillary, solid or micropapillary, according to the WHO classification of lung adenocarcinoma (1). Written informed consent for their data to be used was obtained from all patients, and the present study was approved by the Ethics Committee of the Faculty of Medicine, Tottori University (approval no.: 1706A059; June 14, 2017).

Immunohistochemistry. All specimens were fixed in 10% neutrally buffered formalin and embedded in paraffin. After sections (4-μm thick) were deparaffinized and endogenous peroxidase activity was blocked, they were pretreated in citrate buffer (0.01M, pH 6.0) in a microwave oven for 15 min. We then performed the immunohistochemical examination using a monoclonal antibody to human maspin (clone EAW24, diluted 1:150; Leica Biosystems, Newcastle upon Tyne, UK) as described elsewhere (15).

Evaluation of immunohistochemical findings. We assessed the cells as being positive for maspin expression at any location when strong staining was identified. Strong staining was defined as a staining intensity equal to that of the nuclei of basal cells of the bronchus, and that staining intensity served as an internal positive control when present (17). Tumors with >10% positive cells were considered maspin-positive. Alveolar epithelial cells served as an internal negative control (17). All slides were evaluated by T.O. and Y.U., who were blinded to the patient clinicopathological data.

Table I. *Clinicopathological characteristics of 181 patients with pathological stage IA lung adenocarcinoma*.

Variable	Value
Age (mean±SD, years)	69.7±9.5
Gender, n (%)	
Male	87 (48.1)
Female	94 (51.9)
Smoking history, n (%)	
Ever smoker	82 (45.3)
Never smoker	99 (54.7)
Surgical procedure, n (%)	
Segmentectomy	28 (15.5)
Lobectomy	153 (84.5)
Tumor size (mean±SD), mm	21.9±7.9
Size of invasive component (mean±SD), mm	16.9±6.7
Pathological tumor status, n (%)	
pT1a	37 (20.4)
pT1b	92 (50.8)
pT1c	52 (28.7)
Lymphatic invasion, n (%)	
Present	39 (21.5)
Absent	142 (78.5)
Tumor differentiation, n (%)	
Well	32 (17.7)
Moderate	127 (70.2)
Poor	22 (12.2)
Histological subtype, n (%)	
Lepidic	32 (17.7)
Acinar	97 (53.6)
Papillary	30 (16.6)
Solid	20 (11.0)
Micropapillary	2 (1.1)

SD, Standard deviation.

Statistical analysis. All statistical analyses were performed using SPSS ver. 23 software (IBM SPSS Statistics, IBM, Armonk, NY, USA). We evaluated the association between maspin status and clinicopathological factors by performing non-parametric tests. The Chi-square test was used when there were two categorical variables of interest and the Kruskal–Wallis test was used when there were three or more variables. For the survival analysis, we used two different endpoints to calculate the DFS and DSS rates: cancer relapse (local recurrence or distant recurrence) and cancer-related death, respectively. DFS was defined as the period from the date of initial surgery to the date of clinical or pathological cancer relapse. DSS was defined as the period from the date of initial surgery to the date of cancer-related death. The cases of the patients who died from a cause unrelated to their lung cancer or for whom the cause of death was not entirely clear were censored for the purposes of survival analyses. Survival curves were computed according to the Kaplan–Meier method, and differences in the DFS and DSS were analyzed using the log-rank test. We used the Cox hazard regression

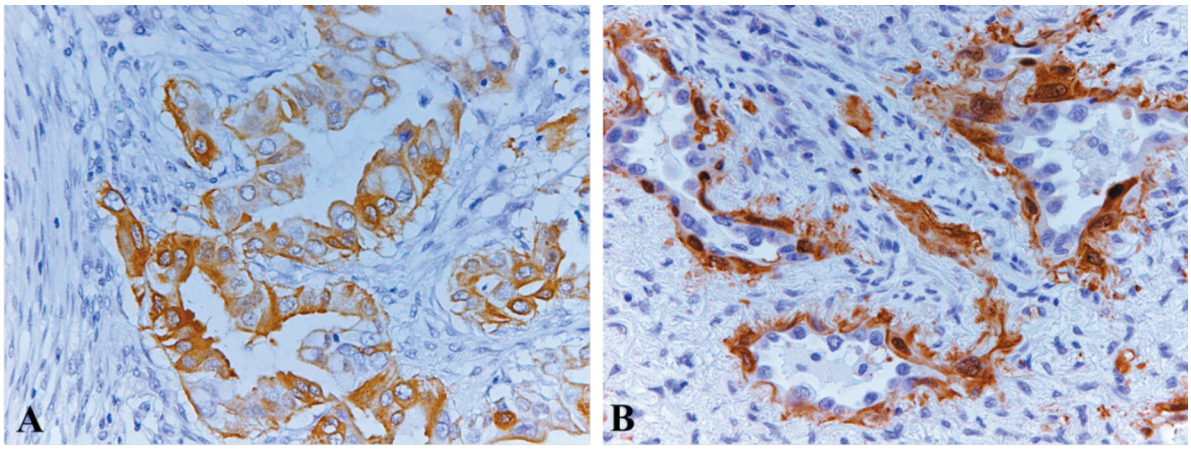

Figure 1. *Immunohistochemical staining of maspin in lung adenocarcinoma. A: Strong cytoplasmic-only expression of maspin. B: Pancellular (nuclear and cytoplasmic) expression of maspin.*

model to evaluate the effects of various factors on the DFS and DSS in order to determine the independent prognostic value of maspin status. All tests of significance were two-sided, and *p*-values of less than 0.05 were considered significant in all tests.

Results

Immunohistochemistry. Representative immunohistochemical staining patterns of maspin expression are shown in Figure 1. In normal lung tissue, the nuclei of basal cells of the bronchus stained strongly, whereas alveolar epithelial cells were not stained. The subcellular localization of maspin expression was classified into four categories: cytoplasmic-only in 45 cases (24.9%), pancellular (combined nuclear and cytoplasmic) in 16 cases (8.8%), nuclear-only in one case (0.6%) and no staining in 119 cases (65.7%). No cases of membrane-only staining were observed. Strong staining intensity was observed in 62 cases (cytoplasmic-only in 45 cases, pancellular in 16 cases and nuclear-only in one case), weak staining in 18 cases and no staining in 101 cases.

Clinicopathological characteristics and correlation with maspin expression. The clinicopathological characteristics of the 181 patients with adenocarcinoma are summarized in Table I. A total of 181 cases with invasive adenocarcinoma were classified into five subtypes: lepidic-predominant in 32 patients (17.7%), acinar-predominant in 97 patients (53.6%), papillary-predominant in 30 patients (16.6%), solid-predominant in 20 patients (11.0%) and micropapillary-predominant in two patients (1.1%). The correlations between the clinicopathological characteristics and maspin immunostaining are summarized in Table II. The pancellular expression of maspin was not correlated with any clinicopathological factor, whereas cytoplasmic-only expression of maspin was significantly correlated with a greater frequency of high pathological T-classification (p=0.039), lymphatic invasion (p=0.002), and poor tumor differentiation (p=0.002).

Survival analysis. Fourteen and 13 patients experienced locoregional and distant recurrence, respectively. Eleven patients died of lung cancer progression and 23 died of other causes, including other cancer-related death, pneumonia, interstitial pneumonia and cerebral hemorrhage. The survival curves for the patients are shown in Figures 2 and 3. The 5-year DFS rates of the group with cytoplasmic-only maspin staining *versus* the other categories were 67.9% *versus* 81.0%, and the corresponding 5-year DSS rates were 92.5% *versus* 97.9%, respectively. The log-rank test showed that the group with cytoplasmic-only staining had significantly shorter DFS and DSS values compared to the other groups (p=0.034 and p=0.036, respectively). The 5-year DFS rates of the groups with pancellular and nuclear-only staining combined and of the no-staining group were 75.0% and 81.5%, respectively, and the corresponding 5-year DSS rates were 93.3% and 98.8%. The log-rank test showed that the cytoplasmic-only staining group had significantly shorter DFS and DSS values compared to the no-staining group (p=0.041 and p=0.037, respectively) (Figure 3). Univariate analyses showed significant correlations between shorter DFS and cytoplasmic-only staining (p=0.037), higher age (p=0.029), higher pathological T-classification (p=0.029), and the presence of lymphatic invasion (p<0.001) (Table III). Cytoplasmic-only staining (p=0.048) and the presence of lymphatic invasion (p=0.008) were also significantly correlated with shorter DSS. According to the multivariate analysis using the Cox hazard regression model, the presence of lymphatic invasion had the most significant effect on DFS (p<0.001) and DSS (p=0.008) (Table IV).

Table II. *Association between subcellular localization of maspin and clinicopathological characteristics.*

Factor		Cytoplasmic-only expression, n			Pancellular expression, n		
	Total (N=181)	Positive (N=45)	Negative (N=136)	*p*-Value	Positive (N=16)	Negative (N=165)	*p*-Value
Age							
<70 Years	75	15	60	0.203	8	67	0.466
≥70 Years	106	30	76		8	98	
Gender							
Male	87	25	62	0.246	5	82	0.159
Female	94	20	74		11	83	
Smoking history							
Ever smoker	82	26	56	0.052	5	77	0.237
Never smoker	99	19	80		11	88	
Surgical procedure							
Segmentectomy	28	9	19	0.332	2	26	0.731
Lobectomy	153	36	117		14	139	
Pathological tumor status							
pT1a	37	5	32	0.039	4	33	0.325
pT1b	92	21	71		10	82	
pT1c	52	19	33		2	50	
Lymphatic invasion							
Present	39	17	22	0.002	3	36	0.776
Absent	142	28	114		13	129	
Tumor differentiation							
Well	32	5	27	0.002	5	27	0.139
Moderate	127	28	99		11	116	
Poor	22	12	10		0	22	
Histological subtype							
Lepidic	32	5	27		5	27	
Acinar	97	20	77		7	90	
Papillary	30	8	22		4	26	
Solid	20	11	9		0	20	
Micropapillary	2	1	1		0	2	

Discussion

Several studies investigated the prognostic value of maspin expression in patients with non-small cell lung carcinoma (NSCLC) (9-12) or adenocarcinoma only (13-15), but there are conflicting results regarding whether maspin expression is a favorable or unfavorable indicator. Hirai *et al*. reported that cytoplasmic maspin expression was a significant poor prognostic factor in 132 patients with NSCLC including 94 adenocarcinomas (9). Lonardo *et al*. reported that the nuclear-only expression of maspin may be useful to stratify subtypes of lung adenocarcinoma with favorable clinicopathological features (13). By contrast, the authors of the largest series (352 NSCLCs) reported that the cytoplasmic or nuclear expression of maspin was not correlated with tumor-specific survival (12). Factors contributing to the potential complexity may include the differences in the study populations (especially TNM stage), the histological types investigated, the antibodies used, criteria for positivity, including cut-offs, and whether subcellular localization was examined.

To our knowledge, only two prior studies have investigated the prognostic value of maspin expression in patients with early-stage lung adenocarcinoma (14, 15). Frey *et al*. reported that the nuclear-only expression of maspin was the only predictor of improved survival in 46 patients with stage I lung adenocarcinomas (14). We also demonstrated that the cytoplasmic-only expression of maspin was an independent poor prognostic indicator in 110 patients with lung adenocarcinoma measuring <3 cm (15). However, our previous study included AIS and MIA cases, for which the 5-year DFS rate and 5-year DSS rate are both 100% (16), and it also included patients with stage IB (n=8), IIA (n=3), IIB (n=2) and IIIA (n=9) adenocarcinomas. In the present study, we thus attempted to more strictly stratify patients with early-stage lung adenocarcinoma by using the new edition of the TNM classification of lung cancer. The present patient series thus included patients with p-stage IA lung

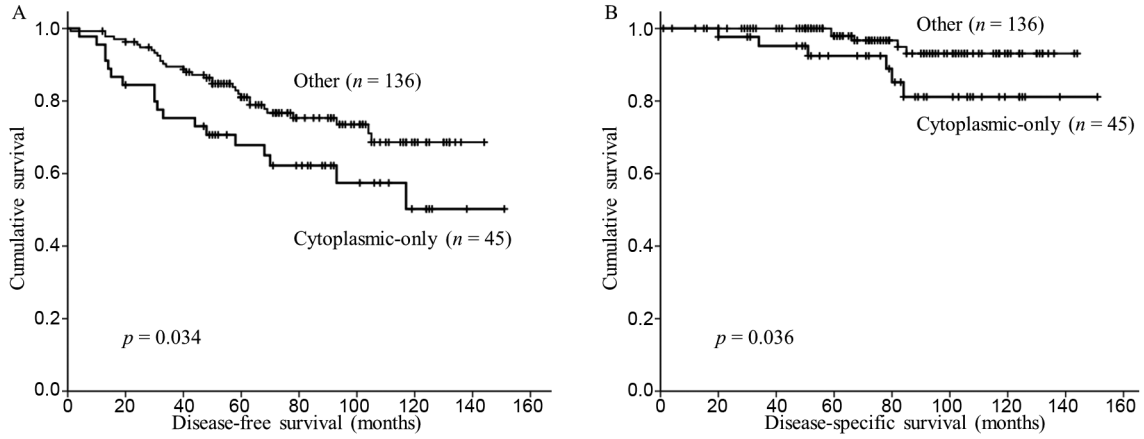

Figure 2. *Kaplan–Meier survival curves for disease-free (A) and disease-specific (B) survival of 181 patients according to maspin expression status (cytoplasmic-only staining versus all other categories).*

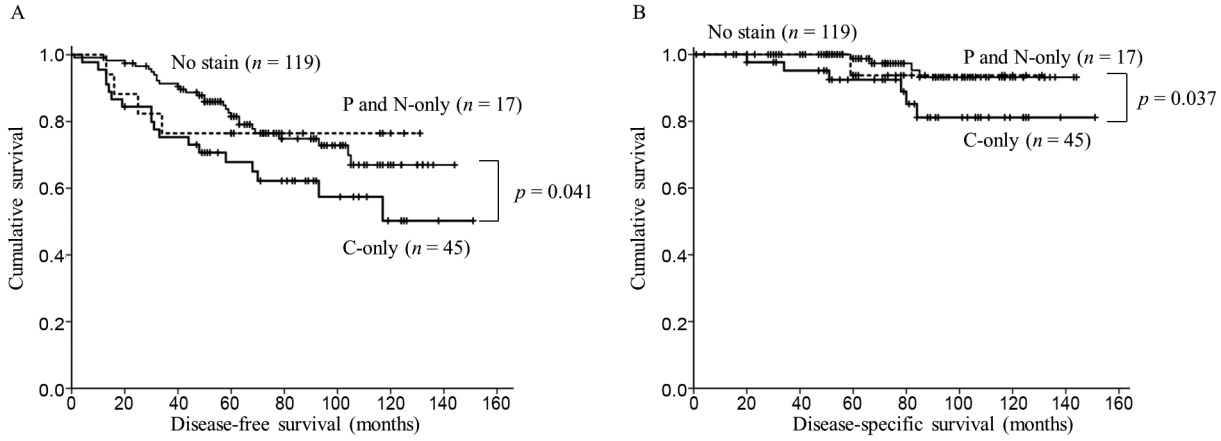

Figure 3. *Kaplan–Meier survival curves for disease-free (A) and disease-specific (B) survival of 181 patients according to subcellular localization of maspin expression. C-only: Cytoplasmic-only, P: pancellular (nuclear and cytoplasmic), N-only: nuclear-only.*

adenocarcinoma excluding AIS and MIA. To our knowledge, there is only a single report focusing on the subcellular localization of maspin expression in lung adenocarcinoma in which a tight association of nuclear expression of maspin with the lepidic growth pattern and a strong association of combined nuclear and cytoplasmic (pancellular) expression of maspin with invasion were observed (18). In the present study, the pancellular expression of maspin was not correlated with any clinicopathological factors, including histological subtype and the size of invasion. The main reason for this discrepancy may be the difference in antibody used as described previously (19). Using an in vivo model system, Goulet *et al*. (20) reported that the nuclear localization of maspin was required for its tumor- and metastasis-suppressor functions and that tumor cells expressing nucleus-excluded, cytoplasmic-only maspin were more metastatic than controls. Goulet *et al*. thus speculated that the cytoplasmic-only (but not pancellular) expression of maspin might correlate with an aggressive phenotype. Our present finding that the cytoplasmic-only (but not pancellular) expression of maspin was a predictor for unfavorable prognosis may support these experimental findings. However, our results failed to demonstrate that the cytoplasmic expression of maspin was an independent prognostic factor for DFS and DSS according to the multivariate analysis. The main reason for this failure may be the relatively small number of cases and the relatively short follow-up period in our study.

Table III. *Univariate analysis of various prognostic factors in 181 patients with pathological stage IA lung adenocarcinoma according to the eighth edition of the TNM classification.*

Prognostic factor	Comparison	Disease-free survival			Disease-specific survival		
		HR	95% CI	*p*-Value	HR	95% CI	*p*-Value
Age	≥70 *vs.* <70 Years	1.945	1.070-3.538	0.029	1.740	0.507-5.969	0.378
Gender	Male *vs.* female	1.278	0.732-2.229	0.388	1.319	0.388-4.486	0.658
Smoking history	Ever *vs.* never	1.708	0.974-2.996	0.062	1.446	0.441-4.743	0.543
Surgery	Segmentectomy *vs.* lobectomy	1.365	0.581-3.206	0.475	2.175	0.520-7.405	0.320
T Classification	pT1c *vs.* pT1a+1b	1.869	1.066-3.277	0.029	2.032	0.620-6.658	0.242
Lymphatic invasion	Present *vs.* absent	2.796	1.577-4.959	<0.001	5.044	1.538-16.540	0.008
Cytoplasmic-only maspin expression	Positive *vs.* negative	1.848	1.037-3.294	0.037	3.323	1.011-10.916	0.048

HR, Hazard ratio; CI, Confidence interval.

Table IV. *Multivariate analysis of various prognostic factors in 181 patients with pathological stage IA lung adenoca)rcinoma according to the eighth edition of the TNM classification.*

Prognostic factor	Comparison	Disease-free survival			Disease-specific survival		
		HR	95% CI	*p*-Value	HR	95% CI	*p*-Value
Age	≥70 *vs.* <70 Years	2.163	1.184-3.950	0.012	2.021	0.575-7.103	0.272
T Classification	pT1c *vs.* pT1a+1b	1.483	0.832-2.643	0.182	1.213	0.341-4.316	0.765
Lymphatic invasion	Present *vs.* absent	3.055	1.716-5.440	<0.001	5.044	1.538-16.540	0.008
Cytoplasmic-only maspin expression	Positive *vs.* negative	1.306	0.705-2.422	0.396	2.584	0.770-8.673	0.124

HR, Hazard ratio; CI, Confidence interval.

In conclusion, we demonstrated, for the first time, that the cytoplasmic-only (but not pancellular or nuclear-only) expression of maspin was a predictor of shorter DFS and DSS in patients with p-stage IA lung adenocarcinoma categorized by the new TNM classification. Due to the recent improvements in imaging techniques, stage I lung adenocarcinoma has been detected more often than in the past; it is, therefore, important to identify markers for stratifying patients with a small tumor but of aggressive phenotype so that appropriate treatment strategies can be selected as early as possible. Although further studies with larger series of patients and longer follow-up periods are needed, our findings suggest that the immunohistochemical analysis of maspin could be useful for the prediction of an aggressive tumor phenotype in patients with p-stage IA lung adenocarcinoma.

Conflicts of Interest

The Authors confirm that there are no conflicts of interest in regard to this study.

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