OncoTargets and Therapy

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ORIGINAL RESEARCH

Clinicopathological and immunohistochemical features of lung invasive mucinous adenocarcinoma based on computed tomography findings

This article was published in the following Dove Press journal:
OncoTargets and Therapy
28 December 2016
Number of times this article has been viewed

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Background: We performed an analysis to clarify differences in clinicopathological and molecular features of lung invasive mucinous adenocarcinoma (IMA) based on computed tomography (CT) findings and their impact on prognosis.

Patients and methods: On the basis of CT findings, we divided lung IMA into three subtypes: solid, bubbling, and pneumonic. We then investigated differences in clinicopathological characteristics, prognosis, and the expressions of well-identified biomarkers, including cyclooxygenase-2 (Cox-2), excision repair cross-complementation group 1 (ERCC1), ribonucleotide reductase M1 (RRM1), class III beta-tubulin, thymidylate synthase (TS), secreted protein acidic and rich in cysteine (SPARC), programmed cell death-1 ligand-1 (PD-L1), and *epidermal growth factor receptor* mutation, among the three subtypes.

Results: A total of 29 patients with resected lung IMA were analyzed. Compared with the solid or bubbling type, the pneumonic type had a higher proportion of symptoms, a larger tumor size, a higher pathological stage, and a significantly worse prognosis. The immunohistochemical findings tended to show high expression of RRM1, class III beta-tubulin, and Cox-2 in the tumor and of SPARC in the stroma, but not of ERCC1, TS, and PD-L1 in the tumor. None of the biomarkers with high expression levels in the tumor were prognostic biomarkers, but the expression of SPARC in the stroma was correlated with a poor outcome.

Conclusion: Clinical and pathological features, in conjunction with molecular data, indicate that IMA should be divided into different subgroups. In our results, the pneumonic type was correlated with a significantly worse outcome. Further studies should be performed to confirm our conclusion and to explore its molecular implications.

Keywords: non-small cell lung cancer, invasive mucinous adenocarcinoma, computed tomography finding, prognostic biomarker, secreted protein acidic and rich in cysteine, SPARC

Introduction

Lung cancer is a major cause of death in many developed countries. Surgical resection is the most important curative treatment option for this disease, especially early-stage non-small cell lung cancer (NSCLC). However, the 5-year survival rate of surgically treated NSCLC patients remains at ~70%.[1,2] Several biomarkers have now been reported as predictors of survival and recurrence in patients with NSCLC.

The most common pathological subtype of NSCLC is adenocarcinoma, the prevalence of which has been increasing. A new classification for lung adenocarcinoma was proposed by an international multidisciplinary expert panel of the International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society (IASLC/ATS/ERS) in 2011.[3] Invasive mucinous adenocarcinoma (IMA) was

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http://dx.doi.org/10.2147/OTT.S121059

recognized as a separate subtype of lung adenocarcinoma, whereas the definition of mucinous bronchioloalveolar carcinoma (BAC) is no longer used. IMA was supposed to contain components of columnar or goblet cells with abundant intracellular or extracellular mucus admixed with invasive adenocarcinoma patterns with stromal invasion. Compared with other lung adenocarcinoma subtypes, IMA has different immunohistochemical and molecular features.[4–6] IMA also has a different progress pattern, compared with other subtypes of adenocarcinoma. In IMA, lymph node and distant metastasis are much less common than in other subtypes of adenocarcinoma. However, pulmonary metastasis frequently occurs in patients with IMA.[7]

Two recent studies have discussed subtypes of IMA according to computed tomography (CT) findings; reportedly, these subtypes are closely related to the clinical outcomes of IMA patients.[8,9] However, sufficient evidence of optimal management based on IMA subtypes does not yet exist. Here, we report a retrospective study examining surgically resected lung IMA with the aim of clarifying differences in clinicopathological and immunohistochemical features among IMA subtypes based on CT findings. In addition, we examined the optimal management of IMA based on a biomarker analysis.

Methods

Patients and specimens

From October 2005 to September 2014, a total of 605 patients with primary lung cancer underwent surgical resection at our hospital. Of these 605 patients, 29 patients (4.8%) presented with primary lung IMA. All the patients included in the present analysis met the following criteria: 1) curative resection and 2) neither radiotherapy nor chemotherapy administered prior to surgery. Written informed consent was obtained from each patient for the study of excised tissue samples from the surgical specimens. This study was conducted with the approval of the institutional Ethics Committee of Kawasaki Medical School (number 2159: approved on August 10, 2015).

Evaluation of CT findings

We classified the IMAs into three types based on the high-resolution CT (HRCT) findings as follows: 1) solid type, in which the shadows represented solitary nodules or masses; 2) bubbling type, in which the shadows represented bubbling shadow; and 3) pneumonic type, in which the shadows represented consolidations with or without air bronchograms (Figure 1).[8]

Immunohistochemical analysis

Immunohistochemical analyses of resected, paraffin-embedded lung cancer tissues were performed. After microtome sectioning (4 μm), the slides were processed for staining using an automated immunostainer (Nexes; Ventana, Tucson, AZ, USA). The streptavidin–biotin–peroxidase detection technique using diaminobenzidine as the chromogen was applied. The primary antibodies were used according to the manufacturer's instructions. The antibodies that were used are summarized in Table S1.[10–15] The slides were examined by two investigators who had no knowledge of the corresponding clinicopathological data. The expression of each marker protein was examined and evaluated according to the original protocol, as reported previously.

Nuclear staining for thyroid transcription factor-1 (TTF-1) and excision repair cross-complementation group 1 (ERCC1) and cell membrane or cytoplasmic staining for cyclooxygenase-2 (Cox-2), cytokeratin 7 (CK7), CK20, ribonucleotide reductase M1 (RRM1), thymidylate synthase (TS), class III beta-tubulin, secreted protein acidic and rich in cysteine (SPARC), and programmed cell death-1 ligand-1 (PD-L1) were assessed (Figure S1).

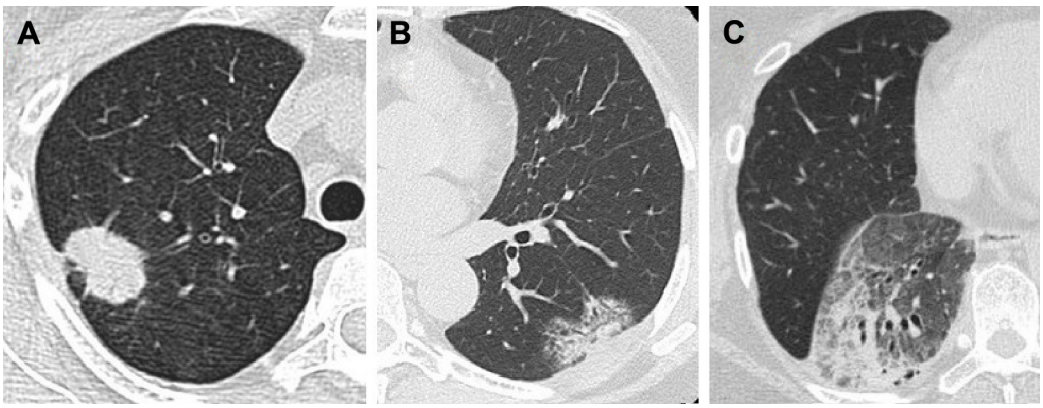


Figure 1 Computed tomography findings of lung invasive mucinous adenocarcinoma.
Notes: (**A**) Solid type, (**B**) bubbling type, and (**C**) pneumonic type.

Immunoreactivity was evaluated semiquantitatively based on the intensity and estimated percentage of tumor cells that were stained. Intensity was quantified as follows: 1+, weak staining (detection required high magnification); 2+, moderate staining (readily detected at medium magnification); and 3+, strong staining (readily detected at low magnification). *H* (histologic)-scores were derived by multiplying the percentage of immunoreactive cells (0–100) by the intensity score (0, 1+, 2+, and 3+), yielding a number between 0 and 300. SPARC staining was also evaluated using the *H*-score and was scored for two separate compartments: peritumoral stromal SPARC in fibroblasts and tumor epithelial SPARC. If the *H*-score exceeded the mean value, the test result was considered to be positive for that marker.

EGFR mutation analysis

An analysis to detect *epidermal growth factor receptor* (*EGFR*) mutations was performed using resected, paraffin-embedded lung cancer tissues and the peptide nucleic acid-locked nucleic acid (PNA-LNA) polymerase chain reaction (PCR) clamp method.[16] The PNA-LNA PCR clamp assay was performed at Mitsubishi Kagaku Bio-clinical Laboratories, Inc, Tokyo, Japan.

Statistical analyses

All the statistical analyses were performed using the SPSS statistical package (version 22.0; SPSS, Chicago, IL, USA). Categorical data were examined using the χ^2 test. The prognostic evaluation was performed by considering the recurrence-free survival (RFS), which was defined as the time until lung cancer recurrence or nonlung cancer-related death. The survival curves were estimated using the Kaplan–Meier method. Two-sided *P*-values of <0.05 were considered statistically significant.

Results

Relationship between the clinical and HRCT findings of IMA

Table 1 shows the clinical characteristics of the patients according to the IMA subtypes based on HRCT findings. Among the clinical findings, the pneumonic type was associated with a higher proportion of symptoms (*P*=0.004) and a large tumor size (*P*=0.001). The bubbling type was associated with a lower maximal standardized uptake value (SUVmax) on fluorodeoxyglucose–positron emission tomography (*P*=0.002).

Relationship between pathological and HRCT findings of IMA

Among the pathological findings, no statistically significant associations were observed between lymph node metastasis, lymphatic invasion, vascular invasion, or pleural involvement and the HRCT findings among the subtypes (Table 2). However, the pneumonic type was associated with a higher pathological stage because of its relatively large tumor size (*P*=0.017). As many previous reports have indicated, the expression of TTF-1 was low and the expressions of CK7 and 20 were high for all subtypes.[11] *EGFR* mutation was positive in only 13.8% (4/29) cases.

Surgical procedure and prognosis

All patients with pneumonic type received standard lobectomy. Five patients with solid or bubbling type received wedge resection, who were considered at increased risk for standard resection because of poor physiologic respiratory reserve. Nine patients received adjuvant chemotherapy, who were pathologically diagnosed with large tumor size (>4 cm) or lymph node metastasis.

Table 1 Clinical characteristics of patients based on computed tomography findings

Characteristics	Solid type	Bubbling type	Pneumonic type	*P*-value
Patients	10	9	10	
Age (years)	70.9±12.4	71.3±7.7	73.6±4.7	0.458
Sex				0.975
Male	6	5	6	
Female	4	4	4	
Smoking history				0.645
Negative	4	5	6	
Positive	6	4	4	
Symptom				0.004
Negative	1	0	6	
Positive	9	9	4	
Tumor size (mm)	23.6±7.2	22.2±10.8	65.0±29.9	0.001
SUVmax	4.7±4.3 (8/10)	1.6±1.2 (7/9)	6.1±2.3 (9/10)	0.002

Note: Data presented as n, mean ± SD, or (n/N).
Abbreviation: SUVmax, maximal standardized uptake value.

Table 2 Pathological characteristics of patients based on computed tomography findings

Characteristics	Solid type	Bubbling type	Pneumonic type	*P*-value
Patients	10	9	10	
Tumor size (mm)	21.8±6.5	23.8±11.4	59.7±17.8	0.001
Lymph node metastasis				0.996
Negative	9	8	9	
Positive	1	1	1	
Lymphatic invasion				0.352
Negative	8	9	8	
Positive	2	0	2	
Vascular invasion				0.617
Negative	9	9	9	
Positive	1	0	1	
Pleural involvement				0.152
Negative	7	9	9	
Positive	3	0	1	
Pathological stage				0.017
IA/IB	5/4	7/1	0/3	
IIA/IIB	0/0	1/0	3/3	
IIIA	1	0	1	
TTF-1 expression				0.283
Negative	9	7	10	
Positive	1	2	0	
CK7 expression				0.316
Negative	0	1	0	
Positive	10	8	10	
CK20 expression				0.861
Negative	3	2	2	
Positive	7	7	8	
***EGFR* mutation**				0.677
Negative	9	7	9	
Positive	1	2	1	

Note: Data presented as n, mean ± SD, or (n/N).
Abbreviations: CK, cytokeratin; EGFR, epidermal growth factor receptor; TTF-1, thyroid transcription factor-1.

Seven (24.1%) patients developed cancer recurrence after resection. A significant association between pneumonic-type IMA and a high rate of recurrence (60.0% vs 10.0% for solid type and 0% for bubbling type, *P*=0.004) was observed. The initial recurrence sites are shown in Table 3. All the initial recurrences were intrathoracic metastases, such as lung metastasis, pleural dissemination, and mediastinal lymph node metastasis. No cases of extrathoracic metastasis of IMA occurred in this series.

The median follow-up time was 980 days. The 5-year RFS rate was 87.5% for solid type, 88.9% for bubbling type, and 25.0% for pneumonic type. Compared with the solid or bubbling types, the pneumonic type had a significantly poorer RFS (*P*=0.018) (Figure 2).

Table 3 Prognosis of patients based on computed tomography findings

Characteristics	Solid type (n)	Bubbling type (n)	Pneumonic type (n)	*P*-value
Patients	10	9	10	
Recurrence				0.004
Intrathoracic	1[a]	0	6	
Extrathoracic	0	0	0	
Intrathoracic recurrence				
Lung	0	0	6	
Dissemination	1[a]	0	1[b]	
Lymph node	0	0	1[c]	
Death				
Lung cancer	1	0	2	
Other cancer	0	1	0	
Noncancer	0	0	0	

Notes: [a]Case of wedge resection due to compromised patient. [b]Lung + pleural dissemination (n=1). [c]Lung + mediastinal lymph node metastasis (n=1).

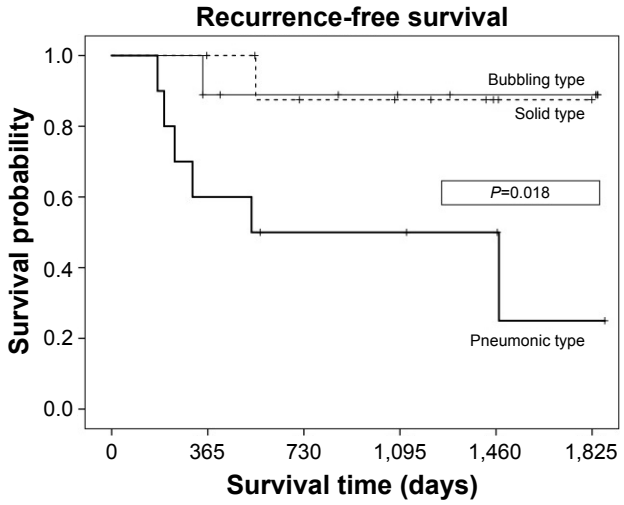


Figure 2 Kaplan–Meier recurrence-free survival curve based on computed tomography findings.

Biomarker analysis

For the immunohistochemical analysis, the *H*-score for each marker expression profile was shown according to the subtypes based on the CT findings (Figure 3). In an overall analysis, the *H*-score of the markers were as follows: ERCC1, 66.2±67.6; RRM1, 178.8±81.5; class III beta-tubulin, 103.1±71.7; TS, 2.4±6.4; SPARC in tumor tissues, 32.4±34.6; SPARC in stroma, 126.0±65.2; PD-L1, 2.0±3.5; and Cox-2, 170.5±68.6. The expressions of RRM1, class III beta-tubulin, Cox-2 in tumor, and SPARC in stroma were relatively high in all the IMA subtypes, whereas those of ERCC1, TS, and PD-L1 were relatively low. Of these, only the *H*-score of SPARC in stroma was significantly higher in the pneumonic type than in the solid (P=0.029) or bubbling type (P=0.048).

As to the RFS, none of the biomarkers were prognostic factors. However, the RFS of patients with positive SPARC expression in stroma tissue was significantly poorer than that of patients with negative SPARC expression in stroma tissue (P=0.028; Figure 4).

Discussion

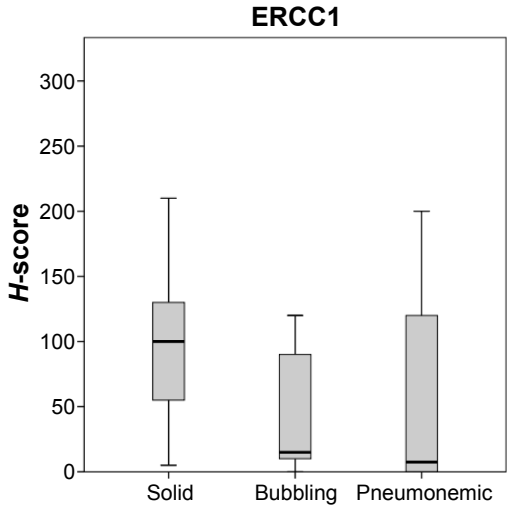
We undertook this study to investigate whether it is reasonable to divide IMA into separate subtypes. To our knowledge, this is the first study to focus on the differences in prognosis and molecular alterations in IMA subgroups with different morphological features. Since the appearance of the IASLC/ATS/ERS classification for lung adenocarcinoma in 2011, many studies have focused on the influence of the pathological subtype on prognosis and molecular changes.[17–19] IMAs, which account for 2%–10% of lung adenocarcinoma cases in East Asia, Europe, and the US, were considered to be more malignant than other common subtypes of lung adenocarcinoma, such as lepidic and acinar subtypes.[19–22] Nonmucinous adenocarcinomas originate from type-II pneumocystis or Clara cells, whereas IMAs most likely originate from bronchiolar lining cells that have undergone mucinous (goblet cell) metaplasia. IMAs show a strong tendency for multicentric, multilobar, and bilateral lung involvement, which may reflect aerogenous spread.[11] IMA is also strongly correlated (76%) with *KRAS* mutation and almost entirely lacks *EGFR* mutations, whereas nonmucinous adenocarcinoma in situ/minimally invasive adenocarcinoma/lepidic predominant adenocarcinoma exhibit the opposite trends with 45% of cases positive for *EGFR* mutation but only 13% positive for *KRAS* mutation.[23]

Generally, mucinous adenocarcinomas in other organs are also associated with lymph node metastasis and a poor prognosis.[24–26] The reason is that the mucus is considered to play a critical role in the development of cancer.[27] In this study, the prognosis of resected IMA of the lung was relatively good, because lung IMA was not associated with lymph node metastasis. However, patients with pneumonic type had a poorer prognosis than those with other types. This subtype was also observed to have a higher rate of intrathoracic metastasis and was associated with a poor prognosis.

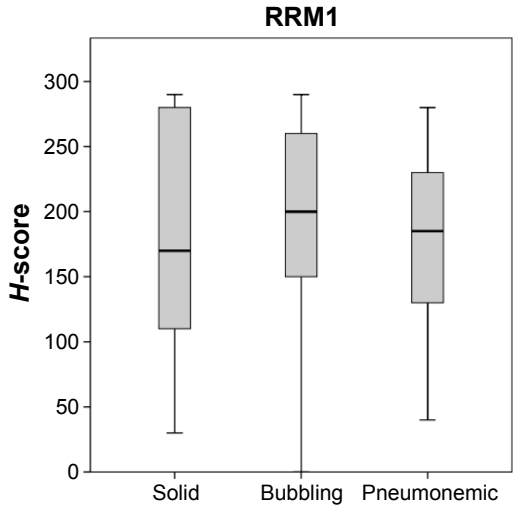
Recently, both experimental and clinical studies have revealed that many molecules contribute to various biological behaviors of malignant tumors, including NSCLC. New strategies based on a better understanding of tumor biology are, thus, needed to maximize the efficacy of current treatments. The associations between these strategies and the response to chemotherapy have been investigated, and the selection of effective chemotherapy regimens based on the evaluation of these biomarkers may improve the clinical outcome of NSCLC patients. Platinum-based chemotherapy remains the scaffold upon which combination chemotherapy regimens are assembled for NSCLC patients. As a predictor of the efficacy of platinum-based chemotherapy, the intratumoral expression of ERCC1, a major component of the nucleotide excision repair pathway, is reported to be associated with a lower responsiveness of patients to cisplatin.[28,29] In addition, the intratumoral expression of class III beta-tubulin is likely to be associated with a lower responsiveness to taxanes, such as paclitaxel and docetaxel.[30,31] Moreover, RRM1 is likely to be associated with a lower responsiveness to gemcitabine.[32] The expression of TS protein was associated with a reduced sensitivity of patients to pemetrexed treatment.[33,34] Our results showed that the expressions of RRM1 and class III beta-tubulin were relatively high, whereas those of ERCC1 and TS were relatively low in IMA. Thus, we suspect that

IMA might be susceptible to cisplatin or pemetrexed, but not to gemcitabine or taxans.

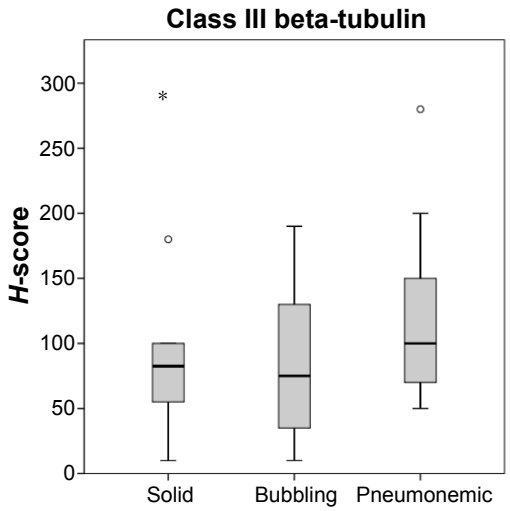
SPARC plays remarkable roles in altering the activity and the microenvironment of cancer cells, modulating cell growth, apoptosis, adhesion, migration, and invasion in human carcinogenesis.[35,36] Thus, many studies have evaluated the effect of SPARC expression in many cancers. SPARC has generated remarkable interest as a potential molecular marker of prognosis for malignant tumors. However, whether a high or low, SPARC expression level is correlated with a poor survival outcome remains controversial. In pancreatic cancer, Han et al reported that high SPARC expression in the stroma, but not in the tumor, was a strong predictor of a shorter survival period.[37] Compared with its expression in tumor cells, the overexpression of SPARC is frequently found in the stroma in pancreatic cancer.[38] In the present



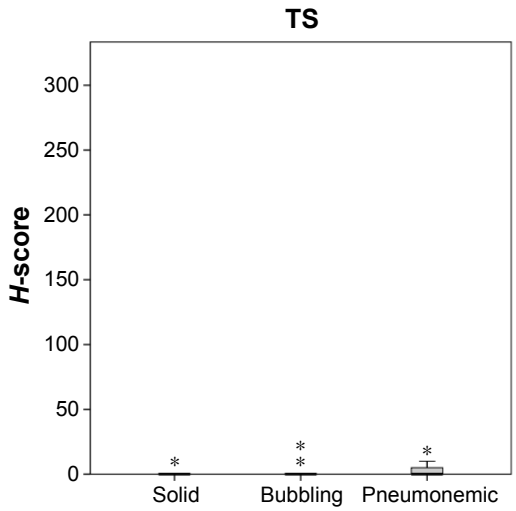
H-score			
Median	93	15	8
Range	5–210	0–120	0–200
Mean	97	48	52
SD	70	49	75



H-score			
Median	167	188	185
Range	30–290	0–290	40–280
Mean	175	186	176
SD	88	88	78

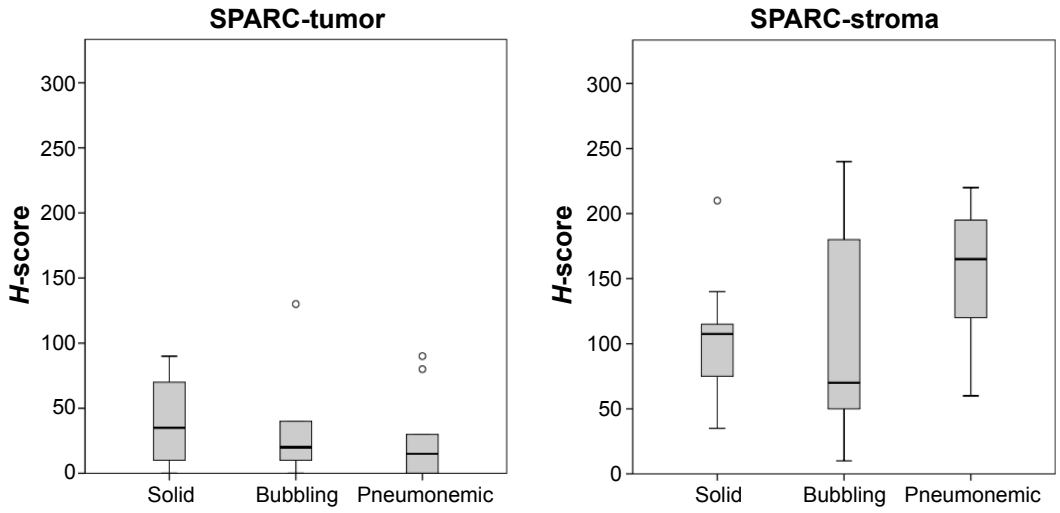


H-score			
Median	85	73	100
Range	10–290	10–190	50–280
Mean	101	85	121
SD	80	65	72



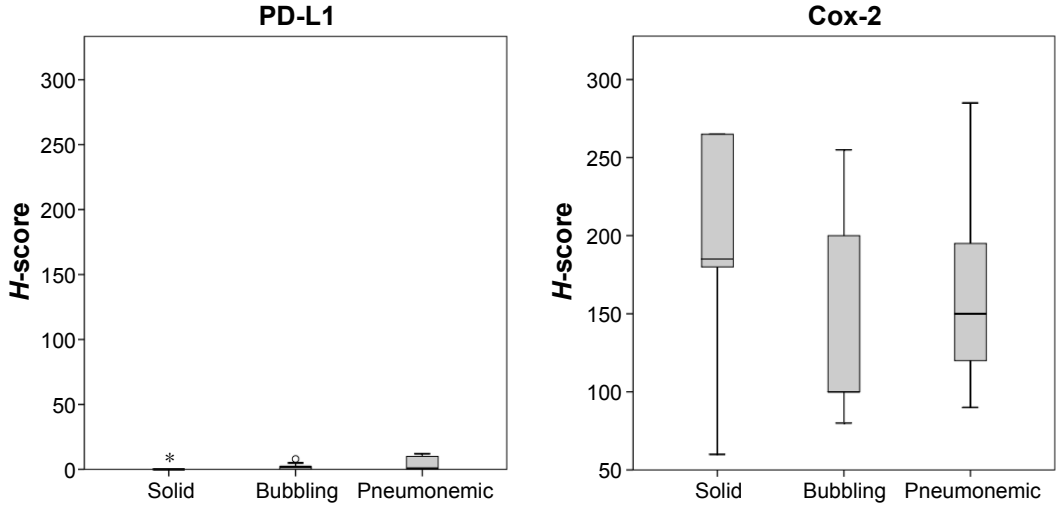
H-score			
Median	0.5	1.3	1.9
Range	0–5	0–15	0–30
Mean	0.5	2.2	4.5
SD	1.6	5.1	9.6

Figure 3 (*Continued*)



H-score			
Median	35	20	15
Range	0–90	0–130	0–90
Mean	40	31	26
SD	33	40	38

H-score			
Median	107	100	163
Range	35–210	10–220	60–220
Mean	106	113	158
SD	47	87	50



H-score			
Median	0.4	1.4	0.8
Range	0–2	0–8	0–12
Mean	0.4	2.1	3.5
SD	0.8	2.8	5.0

H-score			
Median	188	115	150
Range	60–265	80–225	90–250
Mean	199	144	166
SD	66	67	68

Figure 3 The immunohistochemical *H*-score for each marker expression profile based on the computed tomography findings.
Note: °Indicates outliers; *indicates extreme values.
Abbreviations: Cox-2, cyclooxygenase-2; ERCC1, excision repair cross-complementation group 1; *H*-score, histologic score; PD-L1, programmed cell death-1 ligand-1; RRM1, ribonucleotide reductase M1; SD, standard deviation; SPARC, secreted protein acidic and rich in cysteine; TS, thymidylate synthase.

results, the level of SPARC expression in the stroma was relatively high and seemed to be similar to that in pancreatic cancer. SPARC was considered to be a predictive biomarker of response to nab-paclitaxel in three different clinical trials for melanoma, pancreatic, and breast cancer.[39] Our present results showing a high level of SPARC expression in the stroma suggest that IMA, especially pneumonic type, might be susceptible to nab-paclitaxel.

In 2015, nivolumab, a new immunotherapy drug, was approved by the Food and Drug Administration for the treatment of lung cancer after the termination of standard chemotherapy.[15,40] Especially in nonsquamous NSCLC, nivolumab was associated

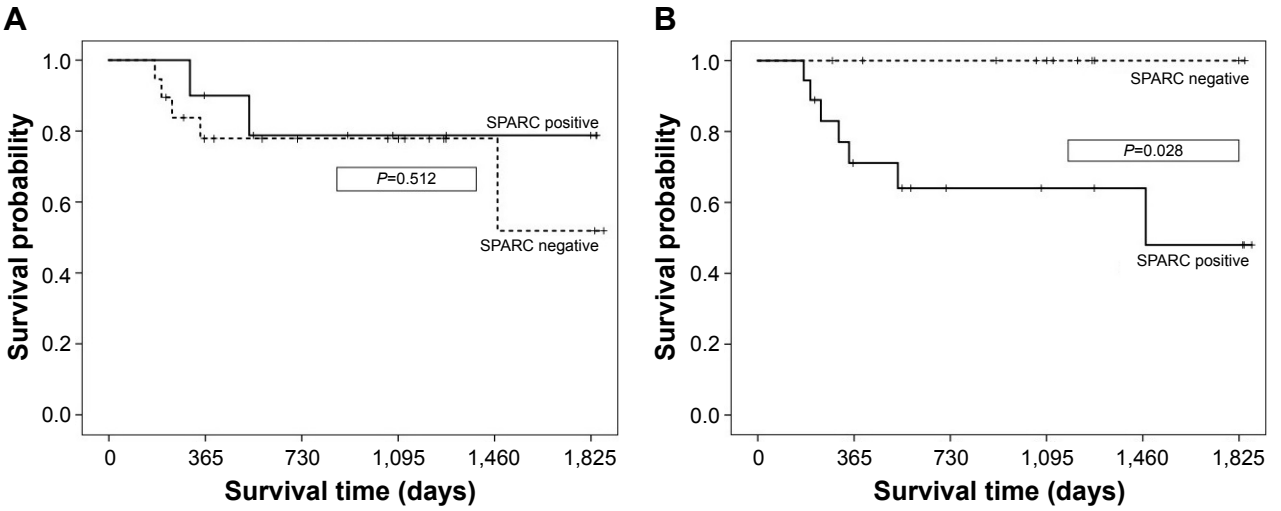


Figure 4 Kaplan–Meier recurrence-free survival curve according to SPARC expression.
Notes: (**A**) Tumor tissue. (**B**) Stroma tissue.
Abbreviation: SPARC, secreted protein acidic and rich in cysteine.

with greater efficacy in subgroups defined according to pre-specified levels of PD-L1. However, our results suggested that IMA might not be susceptible to nivolumab because the IMA specimens had low PD-L1 expression levels.[15]

In this study, the *H*-score for Cox-2 tended to be high in tumor cells. In 2008, Edelman et al reported that Cox-2 expression was a significant prognostic factor in patients with advanced NSCLC (Cancer and Leukemia Group B Trial 30203). Moreover, patients with moderate to high levels of Cox-2 expression had a better tumor response to a Cox-2 inhibitor (celecoxib) in terms of a longer median survival period compared with those not receiving celecoxib.[10]

Limitations

The present study had several limitations. The clinical outcomes were of limited significance because some of the patients had different postoperative treatments. Meanwhile, a sample size of 29 patients is too small to confirm differences among IMA subgroups with different morphological features. The present findings need to be validated by further research with larger samples.

Conclusion

We found three subtypes of lung IMAs based on CT findings. This study represents the first comparison of clinical features and biomarker expressions in IMAs. Clinical and pathological features in conjunction with molecular data indicate that IMA should be divided into different subgroups. Further studies should be performed to confirm our conclusions and to explore the underlying molecular functions.

Acknowledgments

This work was supported, in part, by a Research Project Grant (number 26–64) from Kawasaki Medical School. The authors thank Kiyomi Maitani for providing technical assistance and IMIC for the English language review.

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

Table S1 Antibodies and conditions

Antibody	Clone	Duration	Localization	Source	References
Cox-2	CX-294	1:50	Cytoplasm	DAKO, Carpinteria, CA, USA	Edelman et al[1]
TTF-1	8G7G3/1	1:50	Nuclei	Abcam, Cambridge, MA, USA	Simsir et al[2]
CK7	SP52	1:100	Cytoplasm	Abcam	Simsir et al[2]
CK20	EPR1622Y	1:100	Cytoplasm	Abcam	Simsir et al[2]
ERCC1	8F1	1:100	Nuclei	Abcam	He et al[3]
RRM1	Polyclonal	1:50	Cytoplasm	Abcam	He et al[3]
TS	ATYMSMAB	1:500	Cytoplasm	Immuno-Biological Laboratories Co., Ltd., Fujioka-Shi, Japan	He et al[3]
Class III beta-tubulin	TUJ1	1:400	Cytoplasm	BioLegend, San Diego, CA, USA	Sève et al[4]
SPARC	Polyclonal	1:500	Cytoplasm	Atlas Antibodies AB, Bromma, Sweden	Hidalgo et al[5]
PD-L1	SP142	1:100	Cell membrane	Spring Bioscience, Pleasanton, CA, USA	Borghaei et al[6]

Abbreviations: CK, cytokeratin; Cox-2, cyclooxygenase-2; ERCC1, excision repair cross-complementation group 1; PD-L1, programmed cell death-1 ligand-1; RRM1, ribonucleotide reductase M1; SPARC, secreted protein acidic and rich in cysteine; TS, thymidylate synthase; TTF-1, thyroid transcription factor-1.

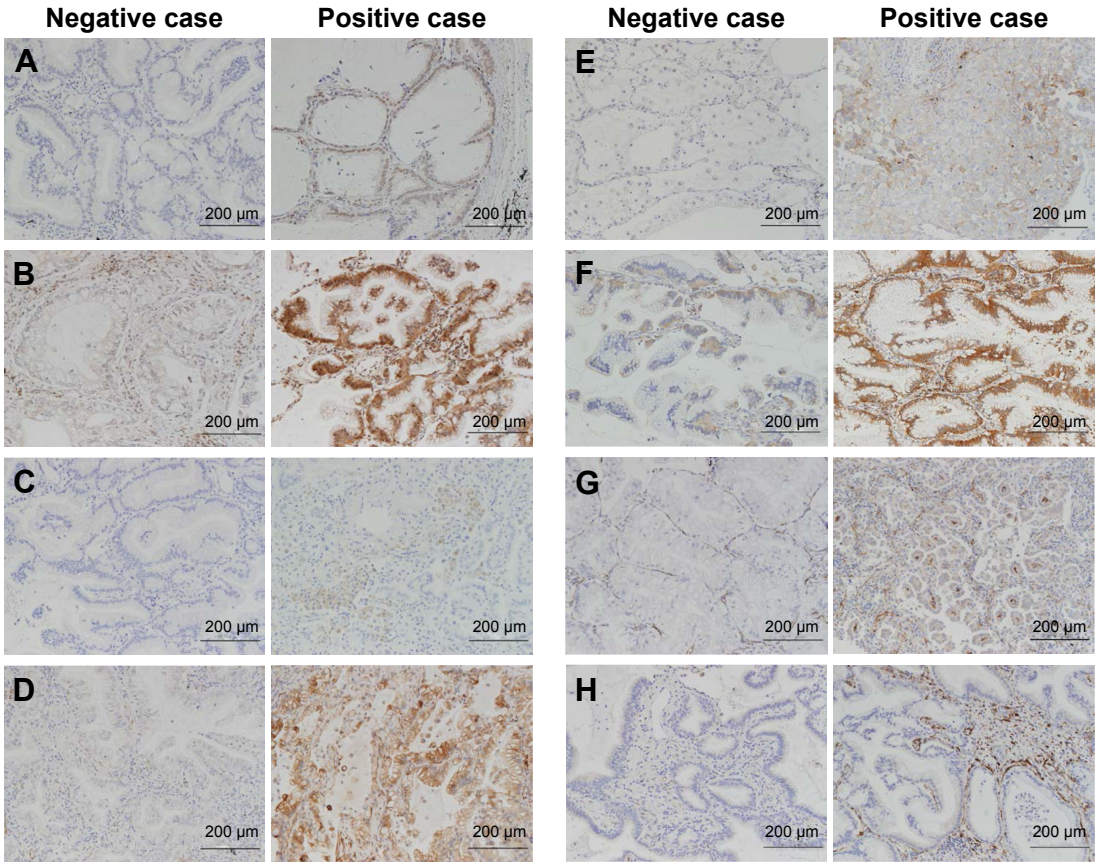

Figure S1 Representative photomicrographs of biomarker expression.
Notes: Magnification ×200. (**A**) ERCC1; nuclear staining, mean *H*-score: 66, negative case score: 0, positive case score: 190. (**B**) RRM1; cytoplasmic staining, mean *H*-score: 178, negative case score: 0, positive case score: 260. (**C**) TS; cytoplasmic staining, mean *H*-score: 2.4, negative case score: 0, positive case score: 30. (**D**) Class III beta-tubulin, cytoplasmic staining, mean *H*-score: 126, negative case score: 10, positive case score: 280. (**E**) PD-L1, membrane staining, mean *H*-score: 4.8, negative case score: 0, positive case score: 50. (**F**) Cox-2; cytoplasmic staining, mean *H*-score: 181, negative case score: 60, positive case score: 270. (**G**) SPARC in tumor tissue; cytoplasmic staining, mean *H*-score: 32, negative case score: 0, positive case score: 90. (**H**) SPARC in stroma tissue; cytoplasmic staining, mean *H*-score:122, negative case score: 10, positive case score: 240.
Abbreviations: Cox-2, cyclooxygenase-2; ERCC1, excision repair cross-complementation group 1; *H*-score, histologic score; PD-L1, programmed cell death-1 ligand-1; RRM1, ribonucleotide reductase M1; SPARC, secreted protein acidic and rich in cysteine; TS, thymidylate synthase.

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