Received: 13 July 2018 | Revised: 7 December 2018 | Accepted: 26 February 2019

DOI: 10.1002/dc.24171

WILEY

ORIGINAL ARTICLE

Cytological differences between invasive and noninvasive or minimally invasive lung adenocarcinomas diagnosed in Japanese patients using needle biopsy specimens of pulmonary lesions ≤3 cm in diameter

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Background: According to the WHO classification for lung cancer, adenocarcinoma in situ (AIS) and minimally invasive adenocarcinoma (MIA) have a better prognosis than invasive adenocarcinoma (IAD). However, detecting the foci of invasion in lung adenocarcinomas radiologically remains difficult. The present study examined whether or not differences in the cytological characteristics between IAD and AIS or MIA (noninvasive or minimally invasive adenocarcinomas [NMIAD]) plays a role in the differential diagnosis.

Methods: Seventy surgical resection specimens of primary lung adenocarcinoma with preoperative cytology, in which several parameters were evaluated and assessed.

Results: The histopathological diagnoses of surgical resection specimens were AIS in 8, MIA in 31, IAD in 31 including lepidic adenocarcinoma in 9, and papillary adenocarcinoma in 22. NMIAD had a 100% 5-year recurrence-free survival (RFS), while IAD had an 82.8% 5-year RFS. The numbers of tumor cells (at ×10 magnification in 10 fields) were 60.3 ± 40.5 in IAD and 39.8 ± 28.7 in NMIAD (P = 0.0017). A univariate analysis of cytological parameters revealed significant differences in large tumor cell clusters, three-dimensional (3D) tumor cell clusters, and irregular nuclear contours between the two groups. The frequency of irregular nuclear contours continued to be significantly different according to a multivariate analysis.

Conclusion: Large or 3D tumor cell clusters and irregular nuclear contours may be important cytological factors for distinguishing IAD from NMIAD, with the latter being potentially more important for distinguishing between the two groups.

1 | INTRODUCTION

The detection of early lung carcinomas has been facilitated in Japan by chest radiography imaging being commonly performed in annual health checkups and chest computed tomography (CT) now being widely used in routine investigations. Furthermore, recent developments in imaging modalities and the widespread use of low-dose helical CT for the screening of lung cancer have led to an increase in the detection rate of pulmonary peripheral lesions.[1] Noguchi et al. previously reported that bronchioloalveolar carcinoma (BAC), which is regarded as adenocarcinoma in situ (AIS), has a favorable prognosis

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with a 5-year survival rate of 100%, while adenocarcinoma with an invasive component has a poor prognosis despite the small tumor size.[2] Furthermore, according to the International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society (IASLC/ATS/ERS) classification of lung adenocarcinomas, patients with AIS and minimally invasive adenocarcinoma (MIA) show a more favorable course than those with invasive adenocarcinoma (IAD).[3] MIA is defined as a small (≤3 cm in diameter), solitary adenocarcinoma with a predominantly lepidic pattern and ≤5 mm invasion in the greatest dimension. MIA is excluded if the tumor invades the lymphatics, blood vessels, air spaces, or pleura; contains tumor necrosis; or spreads through the air spaces.[3] In 2015, the 4th edition of WHO classification of lung adenocarcinomas, which was based on the IASLC/ATS/ERS classification, was published, emphasizing the significance of histological subtypes for assessing the prognosis of patients. This has been validated in independent cohorts.[4,5]

Previous studies have examined the relationship between the IASLC/ATS/ERS classification of histological subtypes and radiological features in lung adenocarcinomas.[6–8] We reported that high-resolution CT (HRCT) findings correlated with the IASLC/ATS/ERS classification and were useful for evaluating the histological features of tumors.[8] This study revealed that the consolidation/tumor ratio and consolidation size in partly solid tumors are important factors for predicting various pathological invasion factors (PIFs), that is pleural invasion, vascular invasion, and lymphatic permeation, as well as for diagnosing IAD. Furthermore, most purely solid tumors are considered to represent a potentially high-grade malignancy, featuring lymph node metastasis and various PIFs. A multicenter registration study showed that the solid tumor size on HRCT and maximum standardized uptake values on positron emission tomography (PET)/CT have greater predictive value for high-grade malignancy and the prognosis in clinical stage IA lung adenocarcinoma than the whole tumor size.[9] However, difficulties remain with accurately identifying the foci of invasion in lung adenocarcinomas clinically due to the challenges associated with distinguishing central scars from the foci of invasion on CT.[8] Furthermore, while active fibroblasts and a papillary growth pattern are histological hallmarks of invasion in lung adenocarcinomas,[2,10] it is difficult to detect these findings in small biopsy or cytology specimens.

We investigated the cytological differences between IAD and noninvasive or minimally invasive adenocarcinoma (NMIAD) diagnosed in Japanese patients based on the new WHO classification using CT-guided needle aspiration cytology specimens of peripheral pulmonary lesions ≤3 cm in diameter to investigate whether or not differences in the cytological characteristics between IAD and NMIAD may contribute to the differential diagnosis. Therefore, the objective of the present study was to identify more readily discernible differences in the cytological characteristics of IAD and NMIAD.

2 | MATERIALS AND METHODS

2.1 | Patient selection

A total of 1752 consecutive patients underwent pulmonary resection for primary lung cancer between January 1997 and December 2008 at Tokyo Medical University Hospital. Among these patients, 70 were pathologically proven to have primary peripheral lung adenocarcinomas ≤3 cm in diameter that showed pure ground-glass opacity (GGO) or partly solid nodules on preoperative CT-guided fine-needle aspiration cytology due to the difficulty of a transbronchial approach. Any patients with pure solid nodules on CT were excluded from this study, as were patients with synchronous multiple adenocarcinomas.

2.2 | Survival analyses

Data collection and analyses were approved and the need to obtain written informed consent from each patient was waived by the Institutional Review Board of Tokyo Medical University. The overall survival (OS) and recurrence-free survival (RFS) were estimated using the Kaplan-Meier method, and differences in the survival rates were determined using a Log-rank analysis (Mantel Cox method). The OS was defined as the time elapsed from the date of pulmonary resection to the date of death from any cause. Among patients in our series, the RFS was defined as the interval from the date of pulmonary resection to the date of recurrence, lung cancer-related death, or the last follow-up. The last follow-up observation was censored if the patient was alive or lost to follow-up.

A univariate analysis was conducted among the different groups. Categorical variables were analyzed using the chi-squared test. Differences between two groups were tested using the Mann-Whitney *U* test. All *P*-values were two-sided, and *P*-values of <0.05 were considered to indicate a significant difference. All statistical calculations were performed using the StatView software program for Windows, version 5.0 (SAS Institute Inc, Cary, North Carolina).

2.3 | Histological and cytological evaluations

All resected specimens were fixed in formalin and embedded in paraffin using routine methods and then stained with hematoxylin and eosin. Elastica-van-Gieson stains were performed where appropriate to evaluate the tumor structure and vascular invasion. These cases were all reclassified by two pathologists (J.M., T.N.) according to the 4th edition of the WHO classification based on the IASLC/ATS/ERS classification for lung adenocarcinomas. All patients had diagnostic preoperative CT-guided fine-needle aspiration cytology specimens available for review. Cytology specimens were fixed in 95% ethanol and stained using the Papanicolaou method. They were then evaluated by one pathologist (J.M.) and one experienced cytotechnologist (S.M.) for the following parameters: (a) the number of tumor cells (×10 magnification in 10 fields), (b) the number of stromal clusters in two slides, (c) the presence of tumor cell clusters composed of ≥20 tumor cells (large tumor cell clusters), (d) three-dimensional (3D) tumor cell clusters, (e) irregular nuclear contours (mainly means multinodular-shaped nucleus with prominent notch), (f) nuclear pleomorphisms (mainly means nuclear-sized variety), (g) prominent nucleoli, and (h) intranuclear cytoplasmic inclusion. In the histologic and cytologic examinations, each researcher evaluated all specimens independently and in random order. Regarding cytological parameters (a) and (b), differences in these values between AIS or MIA (NMIAD) and IAD were examined using a *t* test. We evaluated cytological parameters (c)-

(h) based on three criteria (score 0 [absent], score 1 [a few or slight], and score 2 [prominent]). If the results differed between the two observers, then one other pathologist (T.N.) evaluated these parameters, and three authors (ie, two pathologists and one cytotechnologist) ultimately made a consensus diagnosis after carefully discussing the findings.

The significance of differences between a score of 0 or 1 and a score of 2 was determined using the chi-squared test. All *P* values were two-sided, and *P* values of <0.05 were considered to indicate a significant difference. All statistical analyses were performed using the Statview software program (SAS Institute Inc).

3 | RESULTS

3.1 | Patient characteristics and the survival analysis

Table 1 summarizes the patient characteristics. Of the 70 cases examined, 32 (45.7%) were male, and 38 (54.3%) were female. The median age was 67 years old (range 38-83). The median tumor size was 1.5 cm (range 0.7-3.0 cm). The histopathological diagnoses of the 70 surgical resection specimens were AIS in 8, MIA in 31, IAD in 31 including lepidic adenocarcinoma (means lepidic-predominant adenocarcinoma) in 9, and papillary adenocarcinoma (means papillary-predominant adenocarcinoma) in 22. There were no acinar, solid, micropapillary, or mucinous adenocarcinoma cases in the IAD group in the present study. The median clinical follow-up period was 2873 days (range: 6-5095 days).

The 5-year OS rates were 100%, 93.3%, 88.9%, 90.2%, 94.7%, and 90.0% for AIS, MIA, lepidic adenocarcinoma, papillary adenocarcinoma, AIS + MIA, and lepidic adenocarcinoma + papillary adenocarcinoma, respectively (Table 2, Figure 1A). No significant differences were observed in the OS between patients with AIS and papillary adenocarcinoma tumors, between those with MIA and papillary adenocarcinoma tumors, between those with lepidic adenocarcinoma and papillary adenocarcinoma tumors, or between patients with AIS + MIA and lepidic adenocarcinoma + papillary adenocarcinoma tumors (*P* = 0.192, *P* = 0.431, *P* = 0.596, and *P* = 0.298, respectively; Table 2, Figure 1A). Furthermore, AIS, MIA, lepidic adenocarcinoma, and AIS + MIA had a 100% 5-year RFS, while papillary adenocarcinoma and lepidic adenocarcinoma + papillary adenocarcinoma had a 76.2% and 82.8% 5-year RFS, respectively (Table 3, Figure 1B). Significant differences were observed in the RFS between patients with MIA and papillary adenocarcinoma tumors and between those with AIS + MIA and lepidic adenocarcinoma + papillary adenocarcinoma tumors (*P* = 0.002 and *P* = 0.003, respectively), while no significant differences were found in the RFS between patients with AIS and papillary adenocarcinoma tumors or between those with lepidic and papillary adenocarcinoma tumors (*P* = 0.101 and *P* = 0.106, respectively; Table 3, Figure 1B).

As a significant difference was observed in the RFS between patients with AIS + MIA and lepidic adenocarcinoma + papillary adenocarcinoma tumors, we evaluated both groups cytologically.

TABLE 1 Patient characteristics (n = 70)

Variable	Category	
Sex	Men	n = 32 (45.7%)
	Women	n = 38 (54.3%)
Median age (range)		67 (38-83)
Median whole tumor size (range)		1.5 cm (0.6-3.0 cm)
Histopathological diagnosis	AIS	n = 8 (11.4%)
	MIA	n = 31 (44.3%)
	Lepidic AD	n = 9 (12.9%)
	Papillary AD	n = 22 (31.4%)

Abbreviations: AD, adenocarcinoma; AIS, adenocarcinoma in situ; MIA, minimally invasive adenocarcinoma.

TABLE 2 The overall survival

Group	n	5-y OS (%)	*P*-value
AIS	8	100	
MIA	31	93.3	
Lepidic AD	9	88.9	NS (0.596)
Papillary AD	22	90.2	NS (0.431), NS (0.192)
			Log-rank test

Abbreviations: 5-y OS, 5-year overall survival; AD, adenocarcinoma; AIS, adenocarcinoma in situ; MIA, minimally invasive adenocarcinoma; NS, not significant.

3.2 | The cytological analysis

The numbers of tumor cells were 39.8 ± 28.7 in the NMIAD group and 60.3 ± 40.5 in the IAD group (*P* = 0.0017; Figure 2A). The numbers of stromal clusters were 11.8 ± 11.5 in the NMIAD group and 13.3 ± 11.2 in the IAD group (*P* = 0.59; Figure 2B).

A univariate analysis revealed significant differences between the groups in the rates of large tumor cell clusters (*P* = 0.0106), 3D tumor cell clusters (*P* = 0.0017), and irregular nuclear contours (*P* = 0.0007; Figure 3A,C). However, no significant differences were found in the frequency of nuclear pleomorphisms (*P* = 0.2008), prominent nucleoli (*P* = 0.2484), or intranuclear cytoplasmic inclusion (*P* = 0.8689; Figure 3D,F). A multivariate analysis identified a significant difference between the groups in only one factor: the frequency of irregular nuclear contours.

4 | DISCUSSION

A number of studies have described the cytological features of lung adenocarcinoma.[11–14] Sigel et al. examined 113 fine-needle aspiration cytology specimens of excised pathological stage I lung adenocarcinoma.[11] The surgically resected specimens were histologically graded according to the predominant pattern. In summary, grade 1 tumors are those with a predominantly lepidic pattern, grade 2 tumors are those with predominantly acinar or papillary patterns, and grade 3 tumors are those with predominantly solid or micropapillary

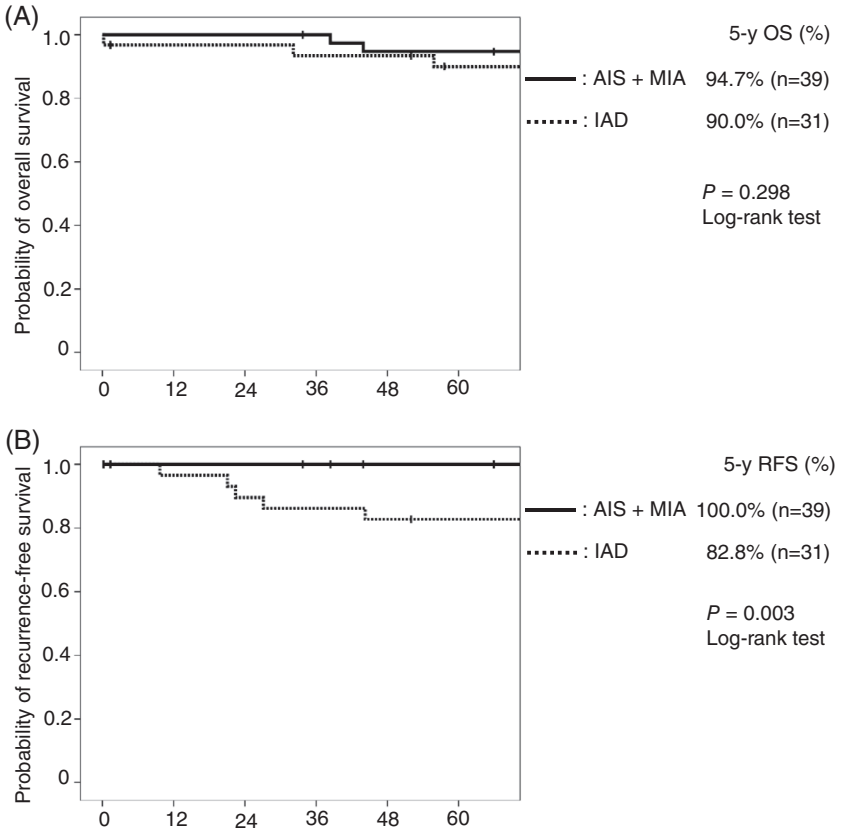

FIGURE 1 A, The overall survival (OS) curve for AIS + MIA (NMIAD) and lepidic and papillary adenocarcinoma (IAD). The 5-year OS rates were 94.7% and 90.0% for NMIAD and IAD, respectively (P = 0.298). B, The RFS curve for AIS + MIA (NMIAD) and lepidic and papillary adenocarcinoma (IAD). The 5-year RFS rates were 100% and 82.8% for NMIAD and IAD, respectively (P = 0.003)

patterns. Three variables (nuclear size, chromatin pattern, and nuclear contours) among several cytological features showed a significant association with the histological grade and disease-free survival. Furthermore, Sigel et al. proposed the establishment of a composite original cytological scoring system based on the above three cytological features. By grouping the cytological scores, they stratified the tumors into three groups (low, intermediate, and high rate of DFS). They also revealed that there was a significant association between the cytological scoring system and histological grading, as defined by the predominant histological architectural patterns of the excised tumors. Rodriguez et al. examined 133 consecutive surgical resection specimens of primary lung adenocarcinoma with preoperative cytology specimens, including samples obtained via CT-guided needle aspiration. Sixty-six patients had diagnostic cytology specimens available for review.[12] Sufficient cellularity was noted on cytologic smears in 58 of 66 cases for subtyping, whereas eight cases could not be subclassified based on their cytology because of scant cellularity or indefinite patterns. Subtyping of adenocarcinoma was concordant between the dominant pattern on resection and cytology specimens in 26 cases (40%) and discordant in 32 (48%) cases. The acinar pattern was the most common in concordant cases, whereas discordant cases had a predominantly solid pattern. The authors emphasized that the identification of solid and micropapillary patterns was prognostically important but might be unreliable and difficult with cytology specimens. Furthermore, several studies have described the cytological features of lung BAC, that is, AIS in the 4th edition of WHO classification.[13,14] In 2004, Ohori and Santa Maria reported the cytological characteristics of 42 lung adenocarcinoma cases, including 6 BACs (three non-mucinous, three mucinous), 17 mixed adenocarcinoma, 6 papillary adenocarcinoma, and 12 IAD.[13] The distribution of types of cytological specimens was as follows: fine-needle aspiration, 30; bronchial washing, 4; bronchial brushing, 4; bronchoalveolar lavage, 2; and sputum 1. On comparison of the 6 BACs with the 12 IADs, they identified 9 statistically significant cytological features

TABLE 3 The recurrence-free survival

Group	n	5-y RFS (%)	*P*-value
AIS	8	100	NS (0.101)
MIA	31	100	0.002
Lepidic AD	9	100	NS (0.106)
Papillary AD	22	76.2	

Abbreviations: 5-y RFS, 5-year recurrence-free survival; AD, adenocarcinoma; AIS, adenocarcinoma in situ; MIA, minimally invasive adenocarcinoma; NS, not significant.

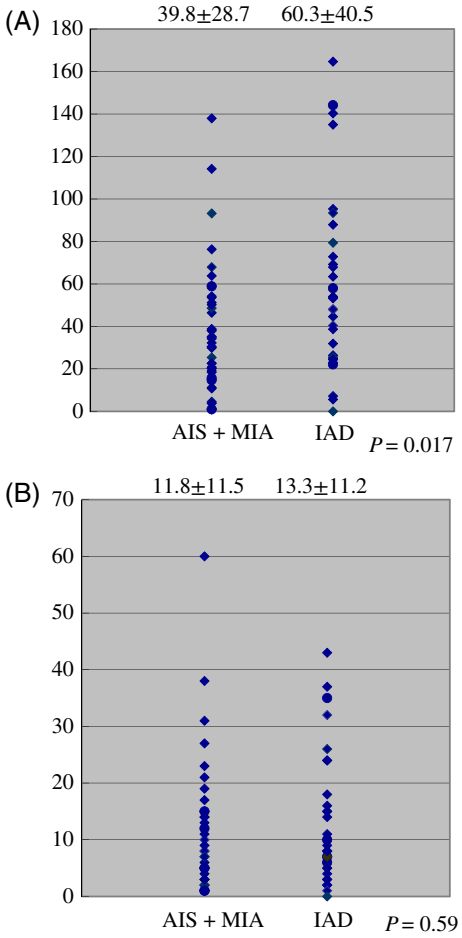

FIGURE 2 A, The numbers of tumor cells (×10 magnification in 10 fields) were 39.8 ± 28.7 in the AIS + MIA (NMIAD) group and 60.3 ± 40.5 in the IAD group (P = 0.017). B, The numbers of stromal clusters in two slides were 11.8 ± 11.5 in the NMIAD group and 13.3 ± 11.2 in the IAD group (P = 0.591)

as the features of BAC cases: a clean background, the absence of 3D clusters, neoplastic cells in flat sheets, the orderly arrangement of cells with round uniform nuclei, a predominance of mucinous cells, the absence of nuclear overlap, the absence of irregular nuclear membranes, fine granular chromatin, and nuclear grooves. MacDonald and Yazdi reported on 49 patients diagnosed by a fine-needle aspiration biopsy (FNAB) and/or surgical biopsy.[14] Twenty-four of the patients diagnosed with BAC by a FNAB had histologic confirmation. Surgical pathology revealed BAC in 15 patients with a cytologic diagnosis of large-cell carcinoma or adenocarcinoma. Nine patients diagnosed with BAC by FNAB were found to have adenocarcinoma histologically. One unsatisfactory FNAB aspirate diagnosed as BAC histologically was due to a sampling error. A review of 15 FNAB specimens with a diagnosis of large cell carcinoma or adenocarcinoma revealed cytologic features typical of BAC. In six aspirates of these cases, additional features, such as pronounced nuclear crowding and overlapping, variation in nuclear size, and an increased number of pleomorphic cells, interfered with the FNAB diagnosis of BAC. Nine FNABs with a diagnosis of BAC were found histologically to have adenocarcinoma with a focal BAC growth pattern.

For small-sized peripheral lung adenocarcinoma, it is especially difficult to obtain a preoperative diagnosis.[15,16] A CT-guided needle biopsy (CTNB) was first reported in 1976[17] and has since become a widely used diagnostic tool for the management of patients with suspected lung cancer. CTNBs are a relatively accurate method of diagnosing peripheral pulmonary lesions.[18–21] In the present study, all cases evaluated were obtained using a CTNB because of their small size and peripheral location. Therefore, all of the cytology specimens were evaluated under the same conditions, morphologically. Furthermore, the IAD and NMIAD groups evaluated in the present study contained 31 and 39 cases, respectively, both of which are greater than in previous cytological studies of small lung adenocarcinomas.

To our knowledge, this is the first report of the cytological differences between IAD and NMIAD, which had pure GGO or partly solid nodules on preoperative CT in small peripheral pulmonary lesions, with a focus on invasion in early lung adenocarcinoma. Patients with pure solid nodules on CT were excluded from this study because in general, tumors in patients with purely solid nodules on preoperative CT histologically have much more prominent nuclear atypia and/or structural atypia than those in patients with pure GGO or partly solid nodules. Although there were some cases with acinar, solid, micropapillary, or mucinous adenocarcinoma components, there were no acinar, solid, micropapillary, or mucinous-predominant adenocarcinoma cases in the IAD group in the present study. The reason why no mucinous adenocarcinoma cases were observed may have been because the patients in our study were all Japanese and therefore included many cases of adenocarcinoma with EGFR mutations. The findings from the present study suggest that several cytological parameters—namely irregular nuclear contours, large tumor cell clusters, and 3D clusters—may be useful for distinguishing IAD from NMIAD. A multivariate analysis showed that the frequency of irregular nuclear contours was significantly higher in the IAD group than in the NMIAD group. Furthermore, the number of tumor cells was higher in the IAD group than in the NMIAD group, whereas no significant differences were observed in the number of stromal clusters between the two groups. These cytological characteristics, which were significantly higher or more prominent in the IAD group than in the NMIAD group, appear to support peripheral pulmonary lesions featuring pure GGO or partly solid nodules being IAD pathologically.

Adenocarcinoma is the most common histological subtype of lung cancer in Japan; it accounts for approximately 70% of all surgically resected lung cancers, and most cases occur as pulmonary peripheral lesions.[22] Patients with GGO-dominant small lung adenocarcinomas were previously reported to have a favorable prognosis.[23] Limited resection of the lung for early-stage lung cancers has recently been advocated from a clinical standpoint for the preservation of the lung function.[24] Therefore, GGO-dominant peripheral lung lesions may be candidates for limited surgical resection.[25] However, there are currently no established criteria for the indication of limited resection.

The new WHO classification clearly emphasizes the significance of the histological subtype for determining the prognosis of patients, and this has been validated in independent cohorts. Under the new classification system, AIS is defined as a preinvasive lesion, while MIA is in a new, separate category; both are distinguished from IAD because AIS and MIA have 100% disease-free survival and RFS rates if the tumor is completely resected.[4,26] We also revealed in the present study that AIS and MIA had a more favorable prognosis

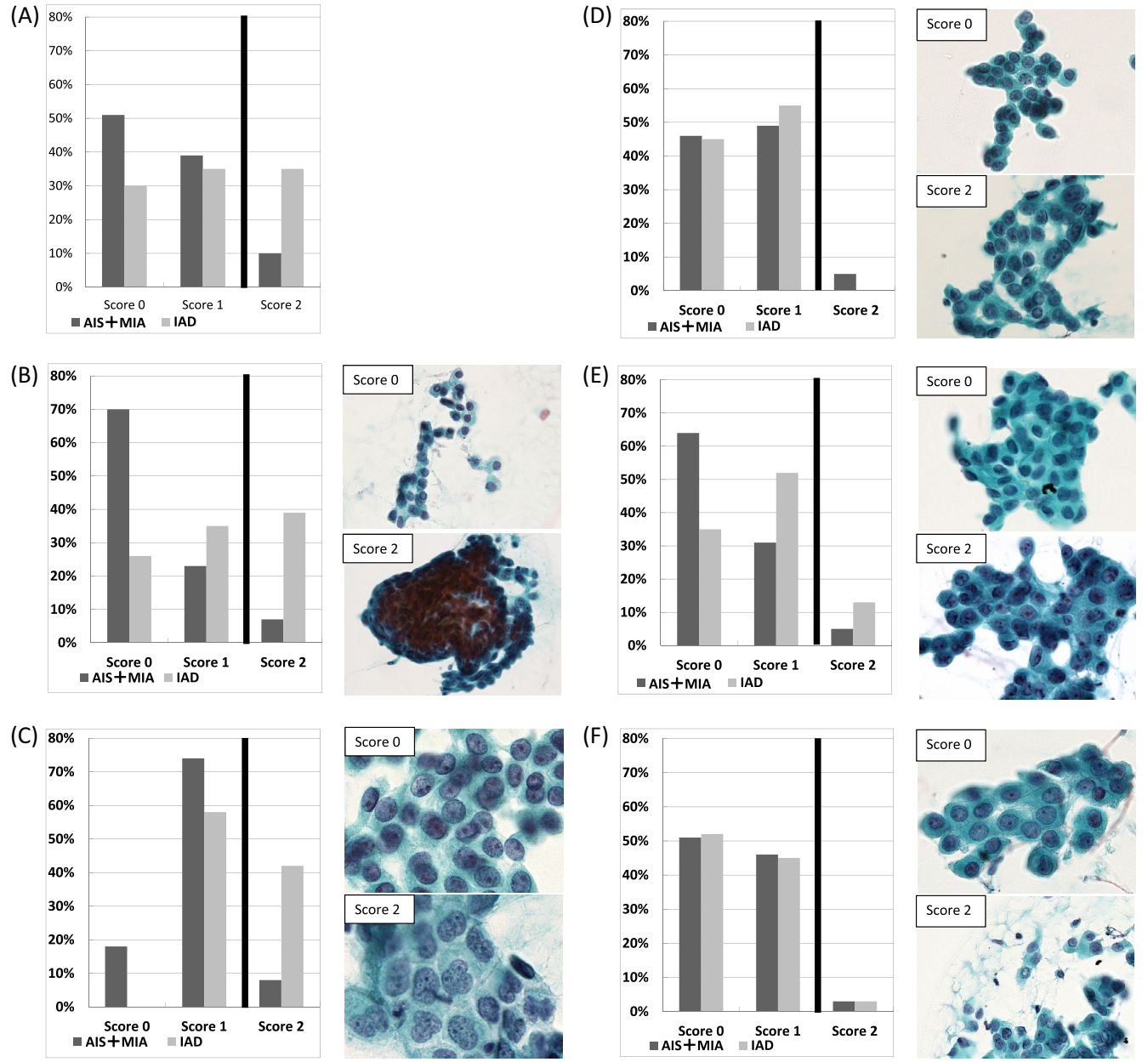

FIGURE 3 (A) Large tumor cell clusters (tumor cell clusters composed of ≥20 tumor cells; $P = 0.0106$), (B) three-dimensional tumor cell clusters ($P = 0.0017$), (C) irregular nuclear contours ($P = 0.0007$), (D) nuclear pleomorphisms ($P = 0.2008$), (E) prominent nucleoli ($P = 0.2484$), and (F) intranuclear cytoplasmic inclusion ($P = 0.8689$)

than IAD; the NMIAD group had a 100% 5-year RFS, while the IAD group had an 82.8% 5-year RFS. There was no difference in the RFS between the patients with NMIAD and those with lepidic adenocarcinoma tumors in our cohort, possibly because our study included only nine cases with lepidic adenocarcinoma. The difference in the RFS was significant between patients with NMIAD and IAD tumors. In IAD tumors with recurrence, there were some cases with tiny foci of a solid (two cases) or micropapillary (one case) adenocarcinoma component, which are poor prognostic factors. Of note, the RFS in our study was defined as the interval from the date of pulmonary resection to the date of recurrence, lung cancer-related death, or the last follow-up. In contrast, no significant differences were observed between NMIAD and IAD in the 5-year OS. The OS in the present study was defined as the time elapsed from the date of pulmonary resection to the date of death from any cause, which is why the OS was lower than the RFS in several groups. Therefore, limited resection may be indicated for adenocarcinomas classified as NMIAD, that is, AIS or MIA.

Chest CT may be useful for detecting the foci of invasion in lung tumors. However, the foci of invasion are indistinguishable from central scars in lung cancer when only chest CT is performed, as NMIAD with a central scar may have partly solid nodules on chest CT. A multicenter registration study recently showed that the solid tumor sizes on HRCT and maximum standardized uptake values on PET/CT have greater predictive value than the whole tumor sizes for high-grade malignancy and the prognosis with clinical stage IA lung adenocarcinoma.[9] Although PET-CT is useful for detecting the foci of invasion in lung adenocarcinomas, detecting particularly small foci can be difficult.

Furthermore, it is not possible to diagnose AIS or MIA using biopsy specimens alone, as central scars cannot be distinguished from the foci of invasion in lung cancer using only preoperative biopsy specimens.[14] In the present study, no significant differences were observed in the number of stroma between the IAD and NMIAD groups. This result indicates that it is difficult to distinguish the foci of invasion in IADs from the scars in NMIAD.

Therefore, in addition to the chest CT and PET findings, the cytological characteristics of irregular nuclear contours, large tumor cell clusters, and 3D tumor cell clusters may play a role in making more accurate preoperative judgment of IAD.

ACKNOWLEDGMENT

English language editing support was provided by Brian Quinn (Japan Medical Communication).

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How to cite this article: Matsubayashi J, Miyake S, Kudo Y, et al. Cytological differences between invasive and noninvasive or minimally invasive lung adenocarcinomas diagnosed in Japanese patients using needle biopsy specimens of pulmonary lesions ≤3 cm in diameter. *Diagn Cytopathol*. 2019;47:688–694. https://doi.org/10.1002/dc.24171