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■ ORIGINAL ARTICLE ■

Cytologic Features of *ALK*–Positive Pulmonary Adenocarcinoma

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Background: The aim of this study was to determine the cytologic features of anaplastic lymphoma kinase (*ALK*) expressing pulmonary adenocarcinoma. **Methods:** We analyzed the cytopathological findings of 15 cases of endobronchial ultrasound guided aspiration and a case of bronchial washing. These cases were selected based on the histomorphology of *ALK*-rearranged lung adenocarcinoma. **Results:** Cytology showed mucinous (81.3%) and hemorrhagic (50%) backgrounds. The cells were arranged in tubulopapillary or tubulocribriform patterns (93.8%), and clusters (56.3%) admixed with signet ring cell features (87.5%). The tumor cells were monotonous and uniform with vesicular nuclei and a small nucleolus. **Conclusions:** The characteristic findings were sheets showing a tubulopapillary or tubulocribriform appearance, with vesicular nuclei and a bland chromatin pattern ($p < 0.001$). Scattered signet ring cells were helpful in suggesting *ALK*-positive adenocarcinoma ($p < 0.001$).

Key Words: Adenocarcinoma; Lung; Anaplastic lymphoma kinase; Carcinoma, non-small-cell lung; Cytology

Lung cancer is the leading cause of cancer death worldwide.[1] Non-small cell lung carcinoma (NSCLC) is the main type (>80%) of lung cancer, and the incidence of adenocarcinoma, the most common type, has increased.[2] Adenocarcinoma is classified according to five major patterns, including acinar, papillary, solid, lepidic (former bronchioloalveolar carcinoma), and micropapillary patterns.[3] In the previous studies, adenocarcinoma showed frequent inactivation of tumor suppressor genes (*TP53* and $p16^{INK4a}$), and mutated oncogenes, including tyrosine kinases (TKs), *HER2*, multiple ephrin receptor genes (notably *EPHA3*), neurotropic TK (*NTRK*) genes, and vascular endothelial growth factor receptor.[4-6] Recently, the molecular alteration of TK receptors, especially epidermal growth factor receptor has been reported to be associated with the prognosis of lung cancer.[7] Echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase (*EML4-ALK*) was identified as a transforming fusion oncogene causing NSCLC (1% to 5% of NSCLC).[2,8,9]

The gold standard in the diagnosis of NSCLC is morphologic findings. Recently targeted therapies have been developed, and immunohistochemical staining and ALK fluorescence *in situ* hybridization (FISH) are required for the diagnosis of *EML4-ALK* fusion. Several researchers have described the characteristic histologic findings of *EML4-ALK*-positive lung adenocarcinomas; an acinar growth pattern, extracellular mucus production, cribriform pattern, and signet ring cells.[10-12] Only one case showing the cytological features of *EML4-ALK*-positive lung adenocarcinoma showing a signet ring cell pattern has been reported.[13]

We evaluated the cytomorphological features of pulmonary adenocarcinoma with positivity for *ALK* rearrangements. The findings of this study may be useful in the diagnosis of adenocarcinoma expressing *ALK*.

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MATERIALS AND METHODS

Patient selection

We selected 107 cases of pulmonary adenocarcinoma diagnosed at Samsung Medical Center and Gachon University Gil Medical Center between March 2010 and July 2012. All tumors, except one, were collected using endobronchial-ultrasound guided biopsy (EBUS). In 15 cases, the patients had undergone EBUS aspiration, and had undergone bronchial washing. The 15 cases were diagnosed by biopsy, and examined by immunohistochemistry using ALK protein (1:40, clone 5A4, Novocastra, Newcastle upon Tyne, UK). *EML4-ALK* rearrangements were confirmed in all ALK-positive immunoreactive cases by FISH analysis using an *ALK* break-apart probe (Vysis LSI ALK Dual Color, break-apart rearrangement probe, Abbott Molecular, Abbott Park, IL, USA). We identified 16 cases of immunohistochemically and FISH proven *ALK*-positive adenocarcinoma. Fine needle aspiration cytologies from the 16 cases of *ALK*-negative pulmonary adenocarcinoma were also evaluated. All of these *ALK*-negative patients underwent lobectomy. The histologic finding was conventional acinar-type adenocarcinoma.

Cytologic and histologic analysis

Hematoxylin and eosin stained slides of histologic sections from 32 cases were retrospectively reviewed. All smeared cytology slides were reviewed by two pathologists. Scoring was as follows: 0, no evidence of cytologic features; 1, $<30\%$ of cytologic features in smeared slides; 2, $\geq 30\%$ of cytologic features in smeared slides.

Each cytologic smear was evaluated for the following features: 1) background pattern, mucin, hemorrhage, and necrosis; 2) cellular arrangement, such as tubulopapillary, tubulocribriform, acini, sheet, cluster, and single; 3) individual cell morphology such as vesicular nuclei, presence of nucleoli, dense chromatin, and signet ring cell features. 'Acinus' refers to a small saclike dilatation, particularly one of glandular appearance. A two dimensional arrangement was 'sheet.' 'Cluster' was used to indicate a three dimensional group of cells.

Histologic sections were assessed for histologic patterns (solid, micropapillary, acinar, lepidic, mucinous, and signet ring cells scored as two parts: $<10\%$, $\geq 10\%$) and nuclear grade (I, II, III).

Statistical analysis

We used the chi-square test to evaluate the differences in cytologic features between *ALK*-positive adenocarcinomas and *ALK*-negative adenocarcinomas. Fisher exact test was used instead of the chi-square test in cases in which 50% of the cells had expected counts of less than 5.

RESULTS

The patients ranged in age from 25 to 67 years (mean age, 52 years). There were nine female and seven male patients with *ALK*-positive adenocarcinoma. Twelve patients with *ALK*-negative adenocarcinoma were females.

Most EBUSs of *ALK*-positive adenocarcinomas showed mucinous (13/16, 81.3%), hemorrhagic (8/16, 50.0%), and necrotic (3/16, 18.8%) backgrounds. An extensive mucinous background was observed in two cases, and 11 cases were focally mucinous admixed with tumor cells. These contained cellular aspirates, even though they were less cellular than fine needle aspiration cytology. The tumor cells were arranged in sheets (15/16, 93.8%), clusters (9/16, 56.3%), and single cells (11/16, 68.8%). The clusters were relatively loose and the sheets were somewhat tubulopapillary or tubulocribriform in appearance ($p<0.001$) (Fig. 1G, H), and contained cells showing intracytoplasmic mucin. The scattered single cells showed vesicular nuclei (14/16, 87.5%; $p<0.001$) and signet ring cell features (14/16, 87.5%; $p<0.001$) (Fig. 1J, N). The nuclei had an open chromatin appearance-similar to the nuclei of papillary carcinoma of the thyroid (ground glass appearance). The nuclear membrane is distinct and thickened. The single cells had the appearance of small histiocyte-like cells and sometimes also contained intracytoplasmic mucin vacuoles. The nucleoli were mostly small in size (10/16, 62.5%). Large prominent nucleoli were observed in two cases (2/16, 12.5%) (Fig. 1K, L). A summary of the cytological features of 16 cases is shown in Table 1. When the cytologic features were scored, a high score (more than 2) was found to be associated with a single cell pattern (four cases), a signet ring cell pattern (six cases), and significant mucinous background (two cases).

The cellularity and background of *ALK*-negative pulmonary adenocarcinomas showed: cells were in loosely cohesive groups, syncytial tissue fragments, acini (13/16, 81.3%), and tubule. Individual cells were variable in size: from small to large. Cytoplasms were pale to dense, foamy, or lacy containing small several intracytoplasmic vacuoles, not single vacuoles. The nuclei displayed relatively uniform, smooth to irregular nuclear membranes with small to large prominent nucleoli, that were larger compared with those of *ALK*-positive adenocarcinomas. These

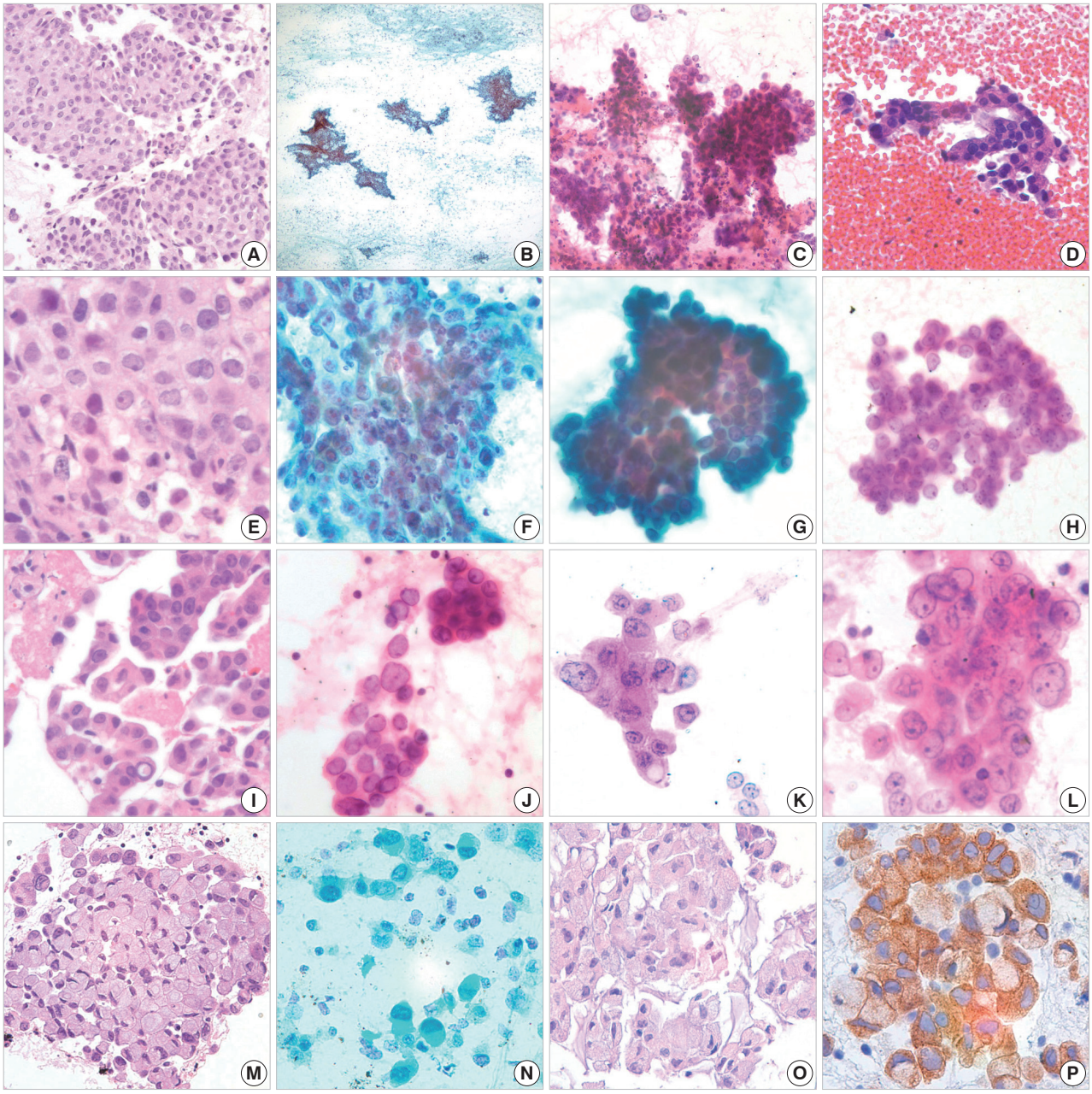

Fig. 1. Pathological findings of anaplastic lymphoma kinase (*ALK*)-positive pulmonary adenocarcinoma: histologic findings (A, E, I, M) and cytologic findings (B-D, F-H, J-L, N-P). (A) The tumor cells show solid-type adenocarcinoma. (B) A low power view of a Papanicolaou stain shows clusters of tumor cells in a mucinous background. (C) Tubulopapillary clusters are present in the background of a small amount of mucin. (D) The tumor cells are arranged in a hemorrhagic background. (E) Individual tumor cells are monotonous with bland nuclei. (F) The tumor cell aggregates show an acinar appearance. (G, H) Tubulopapillary and tubulocribriform patterns are seen. The tumor cells are monotonous. (I) The tumor cells have intranuclear inclusions. (J) The nuclei are vesicular and bland looking with a small nucleolus. (K) The tumor cells are pleomorphic, however the chromatin pattern is bland-looking. (L) The nucleus has a prominent nucleolus. (M) Signet ring cells are seen in histologic sections. (N, O) Cytologic finding of signet ring cells. The cells have eccentric nuclei with plump cytoplasms. (P) Immunohistochemical staining for ALK antibody showing cytoplasmic expression in the tumor cells.

features are summarized in Table 1. The specificity of the tubulopapillary or tubulocribriform patterns and the vesicular chromatin in cytologic smears to predict for *ALK*-positive adenocarcinoma was 81.3%. Those of signet ring cell morphology and single cell distribution were 93.8% and 81.3%, respectively. The sensitivity of tubulopapillary-tubulocribriform pattern and

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Table 1. Comparison of the cytologic features of anaplastic lymphoma kinase (*ALK*)-positive and *ALK*-negative adenocarcinomas

Cytologic feature		ALK-positive adenocarcinoma (%)	ALK-negative adenocarcinoma (%)	p-value[a]
Background	Mucin	13/16 (81.3)	6/16 (37.5)	0.012
	Hemorrhagic	8/16 (50.0)	5/16 (31.3)	0.280
	Necrotic	3/16 (18.8)	8/16 (50.0)	0.063
Architecture	Tubulopapillary	15/16 (93.8)	3/16 (18.8)	<0.001
	Tubulocribriform	15/16 (93.8)	3/16 (18.8)	<0.001
	Acini	6/16 (37.5)	13/16 (81.3)	<0.001
	Sheet	15/16 (93.8)	14/16 (87.5)	0.387[b]
	Cluster	9/16 (56.3)	11/16 (68.8)	0.465
	Single	11/16 (68.8)	3/16 (18.8)	0.004
	Signet ring cell	14/16 (87.5)	1/16 (6.3)	<0.001
Nucleus	Vesicular chromatin	14/16 (87.5)	2/16 (12.5)	<0.001
	Small nucleoli	10/16 (62.5)	8/16 (50.0)	0.476

[a]p-value for chi-square test; [b]p-value for Fisher exact test.

vesicular chromatin was 93.8%. Those of signet ring cell morphology and single cell distribution were 87.5% and 68.8%, respectively.

In histologic findings, backgrounds were hemorrhagic (8/16, 50.0%), mucinous (4/16, 25.0%), and necrotic (0/16, 0%). Eight (50.0%) cases showed a cribriform formation with a rigid or flaccid appearance. A solid pattern was observed in four (25.0%) cases. Significant extracellular mucus was observed in four (25.0%) cases. Fifteen cases showed intraglandular or intracribriform mucinous lumen and intracytoplasmic mucin. One case showed a psammoma body. Individual signet ring cells were identified in 14 (87.5%) cases, accounting for 0% of tumor cellularity (2/16, 12.5%), <10% (7/16, 43.8%), and ≥10% (7/16, 43.8%) (Fig. 1).

DISCUSSION

Molecular targeted therapy has played an increasingly important role in cancer treatment, particularly in genetically defined subsets of patients. Identification of genetic alterations is extremely important for treatments using specific molecular targeted agents. *ALK* gene rearrangements in NSCLC carcinogenesis were first reported in 2007.[14] The *EML4-ALK* fusion gene has been identified in 1-5% of cases of NSCLCs.[2,8-11] ALK inhibitors have been developed and have shown effective activity in *ALK*-rearranged NSCLCs. Confirmation of *EML4-ALK* gene rearrangement is necessary for NCSLC patients.

The histologic findings of *ALK*-rearranged NSCLCs included an acinar growth pattern, extracellular mucus production, and signet-ring cell morphologies in associated with solid growth.[9-12] Some researchers have reported findings of solid or acinar growth patterns, cribriform structure, presence of mucous cells (signet ring cells or goblet cells), abundant extracellular mucus, lack of lepidic growth, and lack of a significant nuclear pleomorphism. In particular, a combination of two findings, such as a solid signet ring cell pattern and a mucinous cribriform pattern, were observed in the majority of *ALK*-positive tumors.[15]

In this study, most cytologic smears of *ALK*-positive adenocarcinomas showed mucinous background (81.3%), even though it was focal, not extensive, except in two cases. Other backgrounds, such as hemorrhage (50.0%) and necrosis (18.8%), might be nonspecific for malignant tumors. The background of *ALK*-negative adenocarcinoma was variable, rather than specific as with malignant tumors. The majority of cells of *ALK*-positive tumors were arranged in sheets with tubulopapillary or tubulocribriform patterns (93.8%). These findings were significant on statistical analysis ($p < 0.001$). However acinus pattern was also found to be significant on statistical analysis, and was relatively frequently observed for glandular forming adenocarcinoma and therefore might be an unspecific feature. Cluster formation showed a relatively loose arrangement (56.3%), however clusters contained cells having mucous vacuoles (goblet-cell-like feature). Scattered signet ring cells, goblet-like cells, or mucous cells, were found in 87.5% of cases ($p < 0.001$). Some of these showed a histiocyte-like appearance with uniform nuclei. Song *et al.*[16] suggested that the histologic findings of adenocarcinomas expressing *ALK* show intra- and extra-cytoplasmic mucin and show a signet ring cell appearance, or a cribriform pattern with extracytoplasmic mucin.[16] These histologic findings are compatible with cytologic features. Most individual cells had vesicular monotonous nuclei without or with a small nucleolus. Prominent nucleoli and dense chromatin were found in only two cases. The chromatin pattern was homogenous, showing a ground-glass appearance. These findings were reminiscent of *ALK*-rearranged anaplastic large cell lymphoma, which tends to show less nuclear pleomorphism than the *ALK*-wild type va-

riety.[17] The specificity and sensitivity of the tubulopapillary or tubulocribriform patterns and vesicular chromatin in cytologic smears to predict *ALK*-positive adenocarcinoma were higher than those of other cytologic findings. However there were only 16 cases of *ALK*-positive adenocarcinoma, it will be necessary to study a larger group.

The morphologic criteria of fine needle aspiration cytology for classic adenocarcinoma include small aggregates with glandular, acinar, and papillary architecture. The cell membrane is poorly defined, and cells show scanty and vacuolated cytoplasm with prominent nucleoli.[18] The differences in features between classic adenocarcinoma (*ALK*-negative adenocarcinoma) and *ALK*-positive adenocarcinoma are cell architecture (tubulopapillary, tubulocribriform, and scattered single cells) and in chromatin pattern (vesicular and bland, and small tiny nucleoli). Based on the specificity and sensitivity findings, the specific cytological findings to predict *ALK*-positive adenocarcinoma might be tubulopapillary or tubulocribriform architectures, signet ring cells, and vesicular chromatin. One of the cytologic features of mucinous adenocarcinoma is the presence of abundant extracellular mucin admixed with a variable number of neoplastic cells, histiocytes, multinucleated giant cells, fibroblasts, and stromal fragments.[19] The tumor cells showed sharply delineated cell borders and unevenly distributed nuclei, forming a characteristic "drunken honeycomb" pattern with tall columnar cells.[20] On the other hand, *ALK*-positive adenocarcinoma frequently showed a relatively small amount of mucin, and did not have tall columnar cells. In addition, no multinucleated giant cells or fibroblasts were observed. The nuclear membrane was smooth and thickened. However, that of mucinous adenocarcinoma was slightly irregular. A lepidic pattern in pulmonary adenocarcinoma tends to exfoliate as single cells, papillary fronds, and sheets. The nuclei are characteristically round to oval and uniform in size, with finely granular or powdery chromatin and inconspicuous nucleoli. *ALK*-positive adenocarcinoma had similar nuclear features of a lepidic pattern.[20] The tumor cells were arranged in a tubulopapillary or tubulocribriform pattern and had small nucleoli. Scattered single cells were more common in the fields of cytologic smears. Making a differential diagnosis may be difficult, however there are several morphological features that can help provide a diagnosis.

In conclusion, a tubulopapillary or tubulocribriform arrangement and scattered single monotonous cells (signet ring cell) with uniform vesicular nuclei are characteristic features of *ALK*-expressing pulmonary adenocarcinoma ($p < 0.001$). Areas of mucinous background may be helpful. These finding might not be definitive; however, they are helpful in suggesting a diagnosis.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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