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Expression of mucins (MUC1, MUC2, MUC5AC and MUC6) in ALK-positive lung cancer: Comparison with EGFR-mutated lung cancer

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ABSTRACT

ALK-positive (ALK +) lung adenocarcinoma usually shows a more advanced-staged disease with frequent nodal metastasis and highly aggressive outcomes compared with EGFR-mutated lung cancers. The aim of this study was to investigate the expression profiles of several mucins in ALK + lung cancers to gain insight into the relationship between the more aggressive biological nature of ALK + lung cancers and the role of mucins.

We examined the immunohistochemical profiles of mucins MUC1, MUC2, MUC5AC, and MUC6 in 19 ALK + lung cancers compared with 42 EGFR-mutated lung cancers.

ALK + cancers were found to occur in younger patients and were characterized by a solid-predominant histologic subtype with frequent signet ring cells and peritumoral muciphages. By contrast, EGFR-mutated cancers lacked ALK-specific histological patterns. Although all MUC1 and MUC5AC were expressed in both subtypes, MUC1 expression in ALK + cancers was visualized exclusively through cytoplasmic staining, whereas those in EGFR-mutated cancers were predominantly membranous staining in apical area (92.9%) and focally in cytoplasmic staining (7.1%). MUC5AC expression in ALK + cancers was exclusively visualized through cytoplasmic staining (100%), whereas EGFR-mutated cancers showed predominantly perinuclear dot-like patterns (90.5%) and focal cytoplasmic staining (9.5%). MUC2 and MUC6 expression was not detected in either type of lung cancer.

Conclusions: The high frequency of both MUC1 and MUC5AC cytoplasmic expression, coupled with a lack of MUC2 and MUC6 expression in ALK + lung cancer may contribute to the biologically aggressive behavior of ALK + cancer. Inhibitors to these types of mucins may thus act as a barrier to cancerous extension reducing their aggressive behavior.

1. Introduction

Aberrant expression of anaplastic lymphoma kinase (ALK), due to rearrangement of the ALK gene, causes ALK-positive (ALK +) lung cancer, a specific type of pulmonary adenocarcinoma that accounts for 4.3%–8% of all pulmonary adenocarcinomas [4,19,22]. Clinically, ALK + lung cancers tend to be aggressive, with locally advanced or metastatic disease detected at the time of diagnosis [21]. ALK + cancers are also associated with a history of never having smoked, young age, and the male gender [4,19,22]. This form of lung cancer

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shows several distinctive features, which imply that its associated molecular alterations are mutually exclusive from those of Epidermal Growth Factor Receptor (EGFR)-mutated lung cancers [21]. Since ALK + lung cancers have unique histologic features, these features may be related to their aggressive behavior. ALK + lung cancer is also associated with multiple mucinous features, such as signet ring cells and intra/extracellular mucin and cribriform patterns [4,19,22]. We therefore focused on evaluating the varying expression of mucins, as these are likely to be involved in the unusual morphology and biologic behavior of ALK + lung cancers.

Mucins are high molecular weight glycoproteins synthesized by a broad range of epithelial tissues. Characterizing the tissue distribution of mucins provides a key to tracing their neoplastic cellular origin and biological features using alveolar (cytoplasmic Mucin 1(MUC1)), intestinal (MUC2), gastric (membrane MUC1, MUC6), and bronchial (MUC5AC) lineage biomarkers [13,23,24]. Furthermore, mucins are highlighted as candidates for additional oncotargets closely related to tyrosine kinases, including ALK and EGFR [12,18]. For example, MUC1-C acts as an oncoprotein through its interaction with various receptor tyrosine kinases, such as EGFR and ErbB2, which subsequently leads to the activation of PI3K-Akt and MEK-ERK signaling pathways [2,20]. Since EGFR and ALK are the most common drivers in lung cancers [21], it is important to characterize whether the mucin profiles of ALK + lung cancers are distinguishable from EGFR-mutated lung cancers, so as to understand the mechanism by which mucin influences the biologic behavior of the respective tumors. This may in turn contribute to the identification of an appropriate treatment regimen. Despite this, the mucin expression profile of ALK + lung cancer has not yet been fully investigated, with only a single case report of a microlesion-harboring ALK rearrangement describing the mucin expression profile of $MUC1^+$/$MUC5AC^-$/$MUC6^-$ [9].

The purpose of the present study was to evaluate mucin expression profiles in a series of ALK + lung cancers compared with EGFR-mutated lung cancers and verify whether the differential expressions of mucins are related to clinical behaviors.

2. Materials and methods

2.1. Patients and histological evaluation

This study was conducted using formalin-fixed paraffin-embedded (FFPE) tissues obtained from 19 patients with ALK + lung adenocarcinoma. Patients underwent biopsy or resection consecutively between 2010 to 2017 at either Hallym University Sacred-Heart Hospital or Kangnam Sacred-Heart Hospital, Korea. EGFR-mutated lung cancer control group samples (n = 42) were randomly selected from lobectomy specimens of primary non-small cell lung cancer without ALK rearrangement during the same period. Inclusion criteria were primary lung origin diagnosed as ALK-rearranged lung cancers confirmed by fluorescence *in situ* hybridization (FISH) or EGFR-mutated lung cancers confirmed by mutation analysis, no prior treatment, complete medical records, and available and well-preserved paraffin-embedded blocks and histopathologic slides of specimens. The medical records of each patient were reviewed for demographic information, radiological data, treatment details, tumor recurrence, and survival status. All slides stained with hematoxylin and eosin (H&E) were re-reviewed by two pathologists (JS and MJK) for histological analysis, and a representative section was selected for subsequent immunohistochemical studies. Diagnosis and histological classification were based on a multidisciplinary classification of lung cancer proposed by the International Association for the Study of Lung Cancer, American Thoracic Society, and European Respiratory Society (IASLC/ATS/ERS) [27]. The predominant histological patterns and histological features of ALK rearrangement (solid signet ring cell, cribriform, and tubulopapillary patterns) were also recorded, as described in previous studies [1,22,28]. TNM stages were based on the tumor registry database, which was classified according to the World Organization Classification of Tumors [26]. This study was approved by the Institutional Review Board at the Hallym University Sacred-Heart Hospital (HALLYM 2018-02-012-001).

2.2. Immunohistochemistry

Immunohistochemical staining was performed on 4 μm thick FFPE sections using a BenchMark XT automated immunostainer (Ventana Medical Systems, Inc., Tucson, AZ, USA), according to the manufacturer's instructions and as previously described [8,10,11]. Briefly, slides were dried at 60 °C for 1 h and deparaffinized using EZ Prep (Ventana Medical Systems) at 75 °C for 4 min. Cell conditioning (heat pretreatment) was performed using CC1 solution containing Tris/Borate/EDTA at 95 °C for 92 min. Anti-ALK primary antibody (mouse monoclonal, clone 5A4, Novocastra, Newcastle, UK) was diluted 1:50, treated, and incubated at 42 °C for 4 h, followed by incubation with secondary antibody (Universal HRP Multimer; Ventana Medical Systems) for 1 h at 37 °C. Additional primary antibodies, including anti-MUC1 (1:50, Novocastra, Newcastle, UK), anti-MUC2 (1:100, Novocastra, Newcastle, UK), anti-MUC5AC (1:100, Novocastra), and anti-MUC6 (1:100, Novocastra) were incubated for 40 min at 37 °C, followed by incubation with secondary antibody (Universal HRP Multimer; Ventana Medical Systems) for 8 min at 37 °C. Thereafter the immunoreaction was developed with the chromogen diaminobenzidine (ultraView Universal DAB Kit; Ventana Medical Systems) for 5 min and then counterstained with hematoxylin for 2 min at room temperature. Positive controls comprised normal pancreatic duct cells for MUC1, mature goblet cells of normal colonic mucosa for MUC2, normal gastric mucosa for MUC5AC, and normal pyloric gland for MUC6.

Semiquantitative assessment was performed by estimating staining intensity and the percentage of tumor cells with positive cytoplasmic staining. Each cell was first scored as 0, 1+, 2+, or 3+, which corresponded to negative, weak, moderate, and strong staining intensities, respectively. ALK immunohistochemical scores were assigned as follows: score 0, no stained cells; score 1+, faint or weak staining intensity with ≤5% tumor cells or any staining intensity with ≤5% tumor cells; score 2+, moderate staining intensity with > 5% tumor cells; score 3+, strong and granular staining intensity with > 5% tumor cells [7]. Scores of 0 and 1+ were classified as negative ALK expression, while cases with scores of 2+ and 3+ were classified as positive ALK expression, as previously described [5,7].

MUC1 expression was regarded as positive if complete or apical membranous and/or cytoplasmic staining was observed in ≥5% of the tumor cells; otherwise, it was classified as negative according to previous reports. For MUC2, MUC5AC, and MUC6, positive expression was defined as cytoplasmic and/or membranous staining in more than 5% of tumor cells.

Two pathologists (JS and MJK) blinded to patients' clinical data interpreted all immunostained slides with good agreement (Kappa value, 0.95) for classification into negative or positive mucin expression. In cases of disagreement, final scoring was determined by consensus.

2.3. Fluorescence in situ hybridization

All cases were performed on FFPE tumor tissues to confirm ALK rearrangement using a break-apart *ALK* probe that hybridizes to the 2p23 band with Spectrum orange (red) and Spectrum green on either side of the *ALK* gene breakpoint (Vysis LSI ALK dual-color, break-apart rearrangement probe; Abbott Molecular, Abbott Park, IL, USA) according to the manufacturer's instructions [7]. Briefly, 3 μm thick FFPE tissue block sections were deparaffinized, dehydrated, immersed in 0.2 N HCl, and washed. The sections were then immersed in 0.01 M citrate buffer (Abbott Molecular), boiled in a microwave for 5 min, treated with pretreatment reagent (Abbott Molecular) at 80 °C for 30 min, and reacted with proteases mixed with protease buffer (Abbott

Molecular). After applying the probe mixture onto the tissue sections, sealed slides were incubated in a humidified atmosphere with Hybrite (Abbott Molecular) at 75 °C for 5 min to denature the probe and target DNA and then sequentially incubated at 37 °C for 16 h to allow hybridization. Tissue sections were thereafter immersed in 0.3% NP-40 (Abbott Molecular)/2× saline sodium citrate for washing. For nuclear counterstaining, 4,6-diamidino-2-phenylindole (DAPI) II with antifade compound, *p*-phenylenediamine, was applied. Signals for each probe were evaluated under a microscope equipped with a triple-pass filter (DAPI/Green/Orange; Abbott Molecular) and an oil immersion for objective x100.

There were two positive *ALK* rearrangement patterns. One was the break-apart pattern with one fusion signal (native *ALK*) and two separated orange and green signals. The distance between two separated signals was estimated using two times the largest signal size. Another was an isolated red signal pattern with one fusion signal (native ALK), and one red signal without corresponding green signal. Positive cases were defined as those with more than 15% break-apart or an isolated red signal in 50 tumor cells, as previously described [7,21].

2.4. EGFR mutation analysis

EGFR mutations were detected using the PNA clamping-assisted fluorescence melting curve analysis assay (PANAMutyper™ EGFR kit), as previously described [6]. The PANAMutyper™ EGFR kit is a newly developed mutation detection kit designed to detect 47 different EGFR variants in exons 18–21 with high sensitivity. All reactions were performed in a total volume of 25 μl containing 10–25 ng DNA template, primer and PNA probe sets, and PCR master mix. All reagents were included with the kit. PCR was performed under the following conditions: 50 °C for 2 min and 95 °C for 15 min as two holding periods; 15 cycles of 95 °C for 30 s, 70 °C for 20 s, 63 °C for 60 s; 35 cycles of 95 °C for 10 s, 53 °C for 20 s, 73 °C for 20 s; and a melting curve step (from 35 °C to 75 °C with gradual increments of 0.5 °C every 3 s). Fluorescence was measured on all four channels (FAM, ROX, Cy5, and HEX) and melting peaks were derived from the melting curve data. Mutations were detected by the melting temperature of each tube for each fluorescent dye as follows: T790 M (HEX) by melting temperature of 58 °C–62 °C; L858R (ROX) by 55.5 °C–59.5 °C for p.L858R (c.2573 T > G), and 44 °C–47 °C for p.L858R (c.2573_2574 TG > GT); E19del (HEX) by 58 °C–67 °C; S768I (HEX) by 58 °C–62 °C; G719X (FAM) by 57 °C–60 °C for p.G719 A, 46 °C–48.5 °C for p.G719S, 50 °C–53 °C for p.G719C; and L861Q (ROX) by 48.5 °C–53.5 °C.

2.5. Statistical analysis

Chi-square test or two-tailed Fisher's exact test was used to analyze the association between ALK + or EGFR-mutated lung cancers and dichotomous factors. Overall survival was defined from first surgery until death. The time of analysis for overall and disease-free survivals was set as January 2018. Disease-free survival was defined as the time from first surgery until documented relapse, including locoregional recurrence and distant metastasis. Survival differences among groups were calculated using the Kaplan-Meier method with a log-rank test. The Cox proportional hazards model was used for multivariate analyses of overall and disease-free survival. SPSS statistical software (version 18, Statistical Package for the Social Sciences) was used for all statistical analyses. *P*-values < 0.05 were considered statistically significant.

3. Results

3.1. Patient demographic characteristics

While ALK protein expression scored 0 in none of the cases (0%), 1+ in 2 (10.5%), 2+ in 5 (26.3%), and 3+ in 12 (63.2%) (Fig. 1A), FISH analysis demonstrated all 19 cases harbored ALK gene rearrangement (Fig. 1B). Clinical demographic characteristics for all patients with ALK + and EGFR-mutated lung cancers are outlined in Table 1. There was no gender predilection in both ALK + and EGFR-mutated lung cancers ($P = 0.376$). Clinically, the mean age of patients with ALK + lung cancers was 58 years, which was approximately 7 years younger than patients with EGFR-mutated lung cancers ($P = 0.048$). Most patients were never smokers in both ALK-rearranged (63.2%) and EGFR-mutated lung cancers (73.8%). Smoking history and tumor site showed no significant difference between ALK + and EGFR-mutated lung cancers ($P = 0.398$ and $P = 0.495$, respectively). ALK + lung cancers were associated with more advanced stage disease than those with EGFR-mutated lung cancers ($P < 0.001$); most patients with ALK + lung cancers had stage IV (42.1%) and stage III (36.8%) disease, whereas most patients with EGFR-mutated lung cancers had stage I disease (76.2%). Two (10.5%) ALK + and three (7.1%) EGFR-mutated tumor patients died of disease, while four (21.0%) ALK + and nine (21.4%) EGFR-mutated tumor patients experienced tumor recurrence.

3.2. Histological features and mucin expression profiles

Comparison of detailed histological features and mucin expression profiles based on ALK and EGFR status are summarized in Table 2. The most common histological subtypes of ALK + lung cancers were acinar- and solid-predominant (both 42.1%) (Fig. 1C), followed by papillary-predominant (15.8%), the frequency of which was statistically different from the lepidic-predominant histological subtype (52.4%) of EGFR-mutated tumors ($P < 0.001$). Solid signet ring cells and peritumoral muciphages were significantly associated with ALK + lung cancers (Fig. 1D–F), as compared to those of EGFR-mutated tumors (both $P < 0.001$). By contrast, apical snouts were more predominant in EGFR-mutated tumors (100%) than in ALK+ lung cancers (31.6%) ($P < 0.001$). ALK+ lung cancers primarily had smooth apical cytoplasmic borders. There was no significant correlation of tubulopapillary or cribriform patterns with ALK+ lung cancers ($P = 0.225$ and $P = 0.558$, respectively) (Fig. 1G–H).

The expression rates of MUC1 were entirely positive (100%) in both ALK + and EGFR-mutated lung cancers. However, the expression patterns were statistically different between the two lung cancer types ($P < 0.001$). MUC1 was primarily expressed in association with the cytoplasm and cell membrane in ALK + lung cancers, as well as within mucus-filled alveolar spaces (Figs. 2A–B). By contrast, 92.9% of EGFR-mutated lung cancers (39/42) were characterized primarily by staining along the apical membranes of tumor cells (Fig. 2C and G), with only three cases (7.1%) showing the cytoplasmic and membranous staining pattern. We did not observe psammomatous calcification in either ALK + or EGFR-mutated lung cancer.

Similar to MUC1, MUC5AC exhibited statistically different expression patterns between ALK + and EGFR-mutated lung cancers ($P < 0.001$). All 19 ALK + lung cancers (100%) showed a cytoplasmic pattern for MUC5AC immunoreactivity (Fig. 2E). On the other hand, the majority of EGFR-mutated lung cancers [38/42 (90.5%)] exhibited weak MUC5AC expression in a primarily perinuclear dot-like pattern (Fig. 2H), with only a small subset (4/42 [9.5%]) showing cytoplasmic staining. Unlike MUC1 and MUC5AC, MUC2, and MUXC6 expression was completely negative in both ALK + and EGFR-mutated lung cancers. Therefore, ALK + lung cancers were characterized by a $MUC1^+$(cytoplasmic)/$MUC5AC^+$(cytoplasmic)/$MUC2^-$/$MUC6^-$ staining pattern, whereas EGFR-mutated lung cancers tended to show a $MUC1^+$(apical membranous)/$MUC5AC^+$(perinuclear dot-like)/$MUC2^-$/$MUC6^-$ staining pattern.

Kaplan-Meier survival curves were constructed to assess the prognostic significance of the MUC1 or MUC5AC expression patterns (Fig. 2I). Subgrouping the MUC1 or MUC5AC expression patterns showed the prognostic relevance to overall survival ($P = 0.019$); the $MUC1^+$ (cytoplasmic)/$MUC5AC^+$ (perinuclear dot) subgroup tended

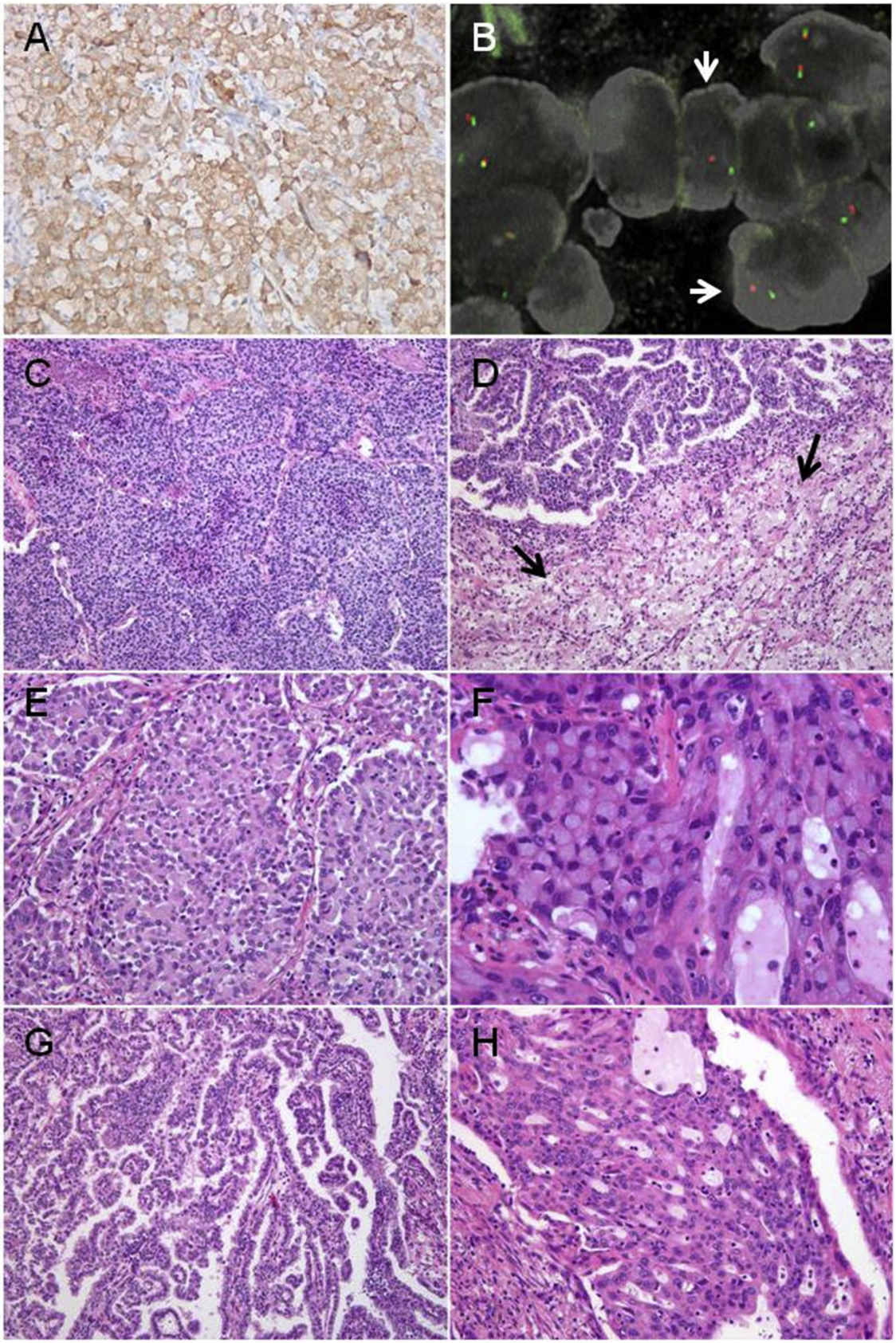

Fig. 1. Representative images of ALK-positive lung adenocarcinomas. (A) Cytoplasmic and membranous ALK immunohistochemical expression. (B) ALK FISH using break-apart probe revealed ALK rearrangement with splitting of green and red signals (white *arrow*) in 25% of tumor cells. (C–H) Histological features of ALK-positive lung cancers. (C) Low-power view shows solid predominant growth pattern. (D) The periphery of tumor mass is surrounded by peritumoural muciphages (black *arrow*). (E) The signet-ring cells form loosely cohesive clusters within solid nests of tumor cells. (F) Tumor cells commonly have signet-ring cell feature with abundant intracellular mucin and a crescentic nucleus displaced toward one end of the cells. Tubulopapillary pattern (G) and cribriform sheets (H) are rarely found. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 1
Clinicopathological parameters of ALK-positive lung cancers and EGFR-mutated lung cancers.

Characteristic	ALK-positive n = 19 (%)	EGFR-mutated n = 42 (%)	*P*
Gender			0.376
Male	10 (52.6)	25 (59.5)	
Female	9 (47.4)	17 (40.5)	
Age (years)			
Mean ± SD	58.68 ± 14.38	65.52 ± 11.20	0.048*
Range	33-79	42-82	
Smoking			0.398
Ever	7 (36.8)	11 (26.2)	
Never	12 (63.2)	31 (73.8)	
Tumor site			0.495
Right	10 (52.6)	26 (61.9)	
Left	9 (47.4)	16 (38.1)	
Stage			< 0.001*
IA	1 (5.3)	24 (57.1)	
IB	3 (15.8)	8 (19.1)	
IIA	0 (0)	0 (0)	
IIB	0 (0)	6 (14.3)	
IIIA	4 (21.0)	3 (7.1)	
IIIB	0 (0)	0 (0)	
IIIC	3 (15.8)	0 (0)	
IVA	8(42.1)	1 (2.4)	
Death	2 (10.5)	3 (7.1)	0.656
Recurrence	4 (21.0)	9 (21.4)	1.000

* Statistically significant, *P* value < 0.05.

to have the worst overall survival rate, which was significantly different from the overall survival rate for MUC1$^+$ (apical membranous)/MUC5AC$^+$ (perinuclear dot) ($P = 0.005$). However, no significant associations were observed between the disease-free survival and MUC1 or MUC5AC expression patterns (all, $P > 0.05$).

4. Discussion

The purpose of this study was to determine mucin expression profiles in ALK + lung cancers, specifically in comparison to EGFR-mutated lung cancers. We report that cytoplasmic MUC1 staining was more frequently expressed in ALK + than EGFR-mutated lung cancers. These results suggest that cytoplasmic MUC1 immunostaining is useful for distinguishing between ALK + - and EGFR-mutated lung cancers.

In the present study, ALK + lung adenocarcinomas were associated with solid signet ring cells and peritumoral muciphages, which are consistent with previous studies [4,19,22]. However, cribriform or tubulopapillary patterns, both previously reported histological patterns associated ALK + lung cancers [4,19,22], were not observed with any statistical significance in ALK + tumors in our study. We further demonstrated that ALK + lung cancers were characterized by a MUC1$^+$(cytoplasmic)/MUC5AC$^+$(cytoplasmic)/MUC2$^-$/MUC6$^-$ staining pattern, whereas EGFR-mutated lung cancers frequently exhibited a MUC1$^+$(apical membranous)/MUC5AC$^+$(perinuclear dot-like)/MUC2$^-$/MUC6$^-$ staining pattern. Although the high expression rates of MUC1 and MUC5AC were frequent in both ALK + and EGFR-mutated lung cancers, the specific expression patterns differed.

MUC1 is generally distributed apically on normal glandular epithelial tissue [23]. In lung cancers, membranous MUC1 is expressed in the terminal bronchiole and part of the respiratory bronchiole [23], whereas aberrantly cytoplasmic MUC1 is expressed in the proximal, juxtabronchial progenitors of alveolar structures and tends to be expressed with less differentiated histology [23,29]. Depolarized MUC1 has been linked to cell-cell detachment [20,25], which may result in the loosely cohesive histology of solid signet ring cells in ALK + lung cancer. We also found MUC1 expression was predominantly positive in the intraluminal mucin of ALK + cancers, which was consistent with other reports of MUC1 being expressed in the intraluminal mucin of

Table 2
Comparison of histological features of ALK-positive lung cancers and EGFR-mutated lung cancers.

Characteristic	ALK-positive n = 19 (%)	EGFR-mutated n = 42 (%)	*P*
Predominant growth pattern			< 0.001*
Lepidic	0 (0)	22 (52.4)	
Acinar	8 (42.1)	14 (33.3)	
Papillary	3 (15.8)	4 (9.5)	
Micropapillary	0 (0)	2 (4.8)	
Solid	8 (42.1)	0 (0)	
Solid-signet rings			
Absent	3 (15.8)	42 (100)	< 0.001*
Present	16 (84.2)	0 (0)	
Tubulopapillary pattern			0.225
Absent	11 (57.9)	32 (76.2)	
Present	8 (42.1)	10 (23.8)	
Cribriform pattern			0.558
Absent	18 (94.7)	41 (97.6)	
Present	1 (5.3)	1 (2.4)	
Peritumoral muciphages (%)			< 0.001*
Absent	8 (42.1)	42 (100)	
Present	11 (57.9)	0 (0)	
Apical snouts (%)			< 0.001*
Absent	13 (68.4)	0 (0)	
Present	6 (31.6)	42 (100)	
MUC1			
Positive	19 (100)	42 (100)	
Cytoplasmic	19 (100)	3 (7.1)	< 0.001*
Apicalmembranous	0 (0)	39 (92.9)	
Negative	0 (0)	0 (0)	
MUC1 in luminal mucin			< 0.001*
Positive	19 (100)	3 (7.1)	
Negative	0 (0)	39 (92.9)	
MUC2			–
Positive	0 (0)	0 (0)	
Negative	19(100)	42 (100)	
MUC5AC			
Positive	19 (100)	42 (100)	< 0.001*
Cytoplasmic	19 (100)	4 (9.5)	
Perinuclear dot	0 (0)	38 (90.5)	
Negative	0 (0)	0 (0)	
MUC6			–
Positive	0 (0)	0 (0)	
Negative	19 (100)	42 (100)	

* Statistically significant, *P* value < 0.05.

78% of ALK + cancers [23]. Since these histologic findings were not identified in EGFR-mutated lung cancers, intraluminal MUC1 expression was suggested to account for the abundant intraluminal mucin production and mucinous features of ALK + cancers. Accordingly, cytoplasmic MUC1 expression in ALK + tumors may be indicative of an alveolar cellular origin and the less differentiated histology in ALK + tumors than EGFR-mutated tumors.

Aberrant MUC1 overexpression has been correlated with worse patient outcomes in lung cancer and is thought to play a role in progression and metastasis [3,12]. In the present study, the MUC1 (cytoplasmic)/MUC5AC (perinuclear dot) subgroup tended to show the worst overall survival rate. Since MUC1 is a transmembrane mucin anchored to the cell surface via its transmembrane domain (with an N-terminal extracellular region), and is involved in various signaling pathways through its short cytoplasmic tail (C-terminal subunit) [17], it is suggested as a good candidate for target therapy using extracellular epitopes. Indeed, the C-terminal subunit cytoplasmic domain of MUC1 (MUC1-C) acts as an oncoprotein through its interaction with various receptor tyrosine kinases such as EGFR and ErbB2, which leads to the activation of PI3K-Akt and MEK-ERK signaling pathways in lung cancers [20]. Interestingly, human H2228 NSCLC cells, containing translocated *EML4-ALK,* express MUC1-C at somewhat higher levels than H1650 cells containing an EGFR mutation (delE746-A750), and were also more sensitive to MUC1-C peptide inhibitors [20]. Treatment with

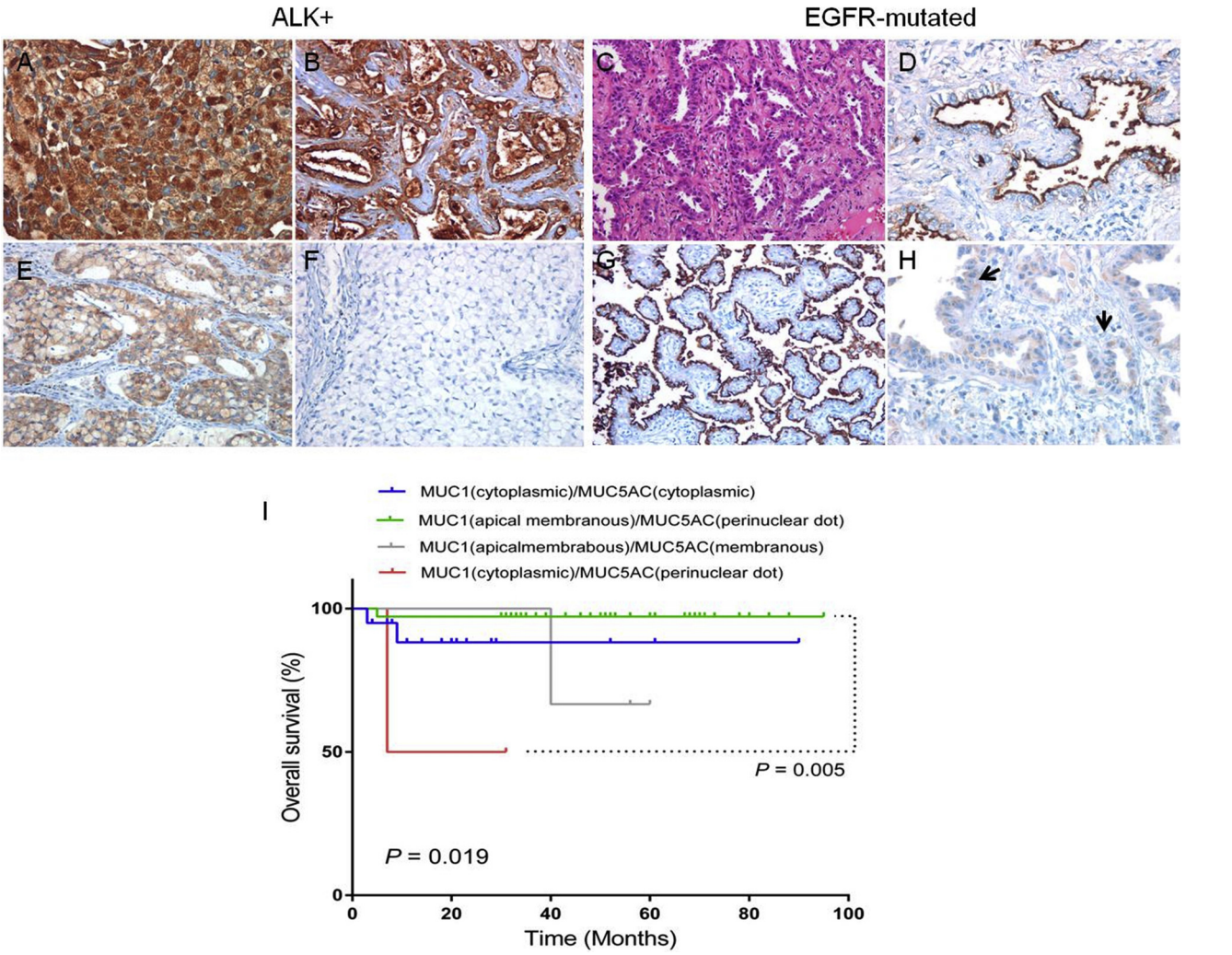

Fig. 2. Comparison of ALK + (A,B,E,F) and EGFR-mutated (C,D,G,H) lung cancers in mucin profiles. (A) ALK + cancers show cytoplasmic MUC1 expression (A) as well as MUC1 positivity in the intraluminal secretion (B). EGFR-mutated cancers having acinar growth pattern (C) show apical membranous MUC1 expression along intraluminal surface (D). Cytoplasmic MUC5AC expression (E) but MUC2 or MUC6 negativity (F) in ALK + cancers. EGFR-mutated lung cancers with papillary growth pattern show apical membranous MUC1 expression along apical surface of tumor cells (G) and perinuclear dot-like MUC5AC expression (black *arrow*) (H). (I) Prognostic impact according to MUC1 and MUC5AC expression pattern in overall survival.

GO-203 (a MUC1 inhibitor) has been seen to be highly effective in H1975 cells containing EGFR mutations (L858R and T790 M), with a complete response being achieved, resulting in tumor regression in xenografts grown in nude mice [20]. This suggests that MUC-1 is a very attractive target for ALK + lung cancer treatment.

MUC5AC, MUC2, and MUC6 are all secreted mucins that lack transmembrane domains [17]. MUC5AC is normally expressed in the trachea and bronchi, but not in bronchioles and smaller alveolar epithelial cells [18,24]. In lung cancers, MUC5AC is diffusely expressed in mucinous bronchiole-alveolar carcinomas (BACs) and adenocarcinoma expressing mucinous cell features, indicating that they originate from airway basal cells of the bronchi or bronchioles [2,17,24]. Sonzogni et al. [23] categorized ALK + lung cancers as a tumor group showing cytoplasmic MUC1 expression with/without MUC5AC. However, that study did not describe in detail the MUC5AC expression pattern in ALK + lung cancers [23]. A single case report of a microlesion harboring ALK rearrangement showed a MUC expression profile of $MUC1^{+}/MUC5AC^{-}/MUC6^{-}$ [9], which contradicts our result of cytoplasmic MUC5AC expression in ALK + lung cancers. A possible explanation for this discrepancy may be that the small size or very early lesion of ALK + lung cancer may not express MUC5AC. It has also been reported that MUC5AC expression in tumors may be related to a favorable prognosis in cancer patients, including patients with breast or colorectal cancers [14,16].

MUC2 is an intestinal type secretory mucin primarily expressed in goblet cells of the intestine, while MUC6 is a gastric pyloric gland type secretory mucin [2,17,24]. MUC2 or MUC6 expression has been reported to be associated with less aggressive, indolent tumor behavior [14]. MUC2 and MUC6 expression were negative in ALK + lung cancers in the present study. This therefore suggests the high expression levels of MUC1 and MUC5AC, coupled with the absence of MUC2 and MUC6 expression, may contribute to tumor behavior in ALK + lung cancers. However, the MUC1 (cytoplasmic) expression pattern in particular appears to be most related to aggressive tumor behavior than any other expression pattern. The distinct mucin expression pattern between ALK + and EGFR-mutated lung cancers was highly related to cell morphology and genetics in the present study.

Patients with ALK + lung cancer tend to be younger and are more likely to be have never been smokers than those without ALK rearrangement [8]. In the present study, we also found that patients with ALK + lung cancers were mostly non-smokers who were younger than patients with EGFR-mutated lung cancer. This suggests that lifestyle modification, such as smoking cessation does not improve therapeutic outcome. Although ALK rearrangement is a target of crizotinib,

resistance to crizotinib eventually occurs after approximately 8 months [15]. Under these circumstances, mucin-targeted therapy may provide for a novel alternative therapeutic modality in non- or light-smoking, and young patients.

In conclusion, we demonstrated that high MUC1 and MUC5AC cytoplasmic expression were the characteristic mucin profile in ALK + lung cancers, as they did not present in EGFR-mutated lung cancers. The high frequency of both MUC1 and MUC5AC cytoplasmic expression, coupled with a lack of MUC2 and MUC6 expression in ALK + lung cancer may contribute to the biologically aggressive behavior of ALK + cancer. Inhibitors to these types of mucins may thus act as a barrier to cancerous extension reducing their aggressive behavior.

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