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Associations between mutations and histologic patterns of mucin in lung adenocarcinoma: invasive mucinous pattern and extracellular mucin are associated with *KRAS* mutation

Kyuichi Kadota, MD, PhD[1,3,6], **Yi-Chen Yeh, MD**[1], **Sandra P. D'Angelo, MD**[2], **Andre L. Moreira, MD, PhD**[3], **Deborah Kuk, ScM**[4], **Camelia S. Sima, MD, MS**[4], **Gregory J. Riely, MD**[2], **Maria E. Arcila**[3], **Mark G. Kris, MD**[2], **Valerie W. Rusch, MD**[1], **Prasad S. Adusumilli, MD, FACS, FCCP**[1,5], and **William D. Travis, MD**[3]

[1]Division of Thoracic Service, Department of Surgery, Memorial Sloan-Kettering Cancer Center, New York, NY, USA

[2]Thoracic Oncology Service, Division of Solid Tumor Oncology, Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY, USA

[3]Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York, NY, USA

[4]Department of Epidemiology and Biostatistics, Memorial Sloan-Kettering Cancer Center, New York, NY, USA

[5]Center for Cell Engineering, Memorial Sloan-Kettering Cancer Center, New York, NY, USA

[6]Department of Diagnostic Pathology, Faculty of Medicine, Kagawa University, Kagawa, Japan

Abstract

Multiple reports indicate that epidermal growth factor receptor (*EGFR*) mutations are associated with lepidic-pattern lung adenocarcinoma, and that *KRAS* mutations are associated with invasive mucinous adenocarcinoma. We sought to investigate the association between *EGFR* and *KRAS* mutations and specific morphologic characteristics, such as predominant histologic subtype and mucinous features. Clinical data for 864 patients with resected lung adenocarcinoma that underwent molecular testing for *EGFR* and *KRAS* mutations were collected. Histologic subtyping was performed according to the IASLC/ATS/ERS lung adenocarcinoma classification, with attention given to signet-ring cell feature and extracellular mucin. *EGFR* mutations were detected using a polymerase chain reaction–based sizing assay, *KRAS* mutations were detected using Sanger sequencing, and ALK expression was detected using immunohistochemistry. Invasive mucinous adenocarcinoma was associated with *KRAS* mutation ($P<0.001$). Among invasive mucinous adenocarcinomas with *KRAS* mutation, a pure mucinous pattern was more common than a mixed mucinous/nonmucinous pattern ($P=0.002$). Invasive mucinous adenocarcinoma was associated with *KRAS* transition mutations (G→A) but not transversion mutations (G→T or G→C) compared to non-mucinous tumors ($P=0.009$). The lepidic-predominant group was

Correspondence: William D. Travis, MD, Department of Pathology, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10065 (travisw@mskcc.org), Phone: 212-639-3325, Fax: 212-717-3576; 646-422-2016.

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associated with *EGFR* mutation compared to nonlepidic-predominant tumors (*P*=0.011). Extracellular mucin was associated with *KRAS* mutation (*P*<0.001), whereas signet-ring cell feature was not associated with *EGFR* or *KRAS* mutation (*P*=0.517). ALK expression was associated with signet-ring cell feature (*P*=0.001) but not with extracellular mucin (*P*=0.089). Our study shows that histologic patterns of mucin in lung adenocarcinoma - including invasive mucinous adenocarcinoma and extracellular mucin - are associated with *KRAS* mutation.

Keywords

lung; adenocarcinoma; subtype; mutation; mucin

INTRODUCTIONS

Activating mutations in the tyrosine kinase domain of *epidermal growth factor receptor* (*EGFR*) can predict sensitivity to *EGFR* tyrosine kinase inhibitors (TKIs) in patients with non-small cell lung cancer (NSCLC).[1–3] Such mutations are most frequently observed in adenocarcinomas, in women, in never smokers, and in Asian patients.[1–5] Previous reports indicate that *EGFR* mutation is associated with lung adenocarcinoma with lepidic-pattern, formerly known as bronchioloalveolar carcinoma (BAC) pattern.[5–8] This has led to the hypothesis that tumors with lepidic (formerly BAC) pattern may be associated with *EGFR* mutation, and that lepidic pattern may predict responses to TKIs.[9–11] *KRAS* is one of the downstream molecules in the *EGFR* signaling pathway,[12, 13] but *EGFR* and *KRAS* mutations are mutually exclusive.[4–6] In contrast to *EGFR* mutations, *KRAS* mutations predict primary resistance to TKI treatment in patients with NSCLC,[14, 15] and they are associated with a history of cigarette smoking and with poor prognosis.[5, 16–18] Interestingly, *KRAS* transversion mutations (G→T or G→C) are more common in ever smokers (because of the bulky carcinogens in cigarette smoke), whereas *KRAS* transition mutations (G→A) are more common in never smokers.[19, 20] *KRAS* mutation has been reported to be associated with invasive mucinous adenocarcinoma, formerly known as mucinous BAC.[21–25] *EGFR* mutations are detected in 20% to 50% and *KRAS* mutations are detected in 10% to 40% of adenocarcinomas.[4, 5, 16, 18, 26] Therefore, approximately half of adenocarcinomas potentially have either *EGFR* or *KRAS* mutations, the presence of which influences clinical decisions regarding TKI treatment. It has recently been reported that *anaplastic lymphoma kinase (ALK)* rearrangement is associated with mucinous features, such as signet-ring cell feature and extracellular mucin, in lung adenocarcinoma.[27–29] However, the relationship between *KRAS* mutations and these mucinous features are not well-documented.

A new lung adenocarcinoma classification that is based on predominant histologic patterns was proposed by the International Association for the Study of Lung Cancer (IASLC), American Thoracic Society (ATS), and European Respiratory Society (ERS) in 2011. This classification clearly emphasizes the prognostic significance of histologic subtypes,[30] which have been validated in independent cohorts.[31–34] However, the associations between the new classification of histologic subtypes and molecular features have not been thoroughly investigated.

We sought to investigate the associations between histologic subtypes (according to the new classification) and *EGFR/KRAS* mutations and to determine whether mucinous features, such as signet-ring cell feature and extracellular mucin, are associated with *EGFR* and *KRAS* mutations in patients with lung adenocarcinoma.

MATERIALS AND METHODS

Patients

Institutional review board approval was obtained for this retrospective study. Clinical data on 864 patients with lung adenocarcinoma who underwent surgical resection and molecular testing for *EGFR* and *KRAS* mutations at Memorial Sloan-Kettering Cancer Center between 2002 and 2009 were collected through the prospectively maintained Thoracic Service database. Patients were staged according to the seventh edition of the *American Joint Committee on Cancer TNM Staging Manual*.[35]

Histologic Evaluation

All available hematoxylin and eosin (H&E) - stained slides from study patients were reviewed jointly by two pathologists (K.K. and W.D.T.) using an Olympus BX51 microscope (Olympus, Tokyo, Japan) with a standard 22-mm diameter eyepiece. Histologic subtyping was performed according to the IASLC/ATS/ERS lung adenocarcinoma classification.[30] Each tumor was reviewed by means of comprehensive histologic subtyping, with the percentage of each histologic component recorded in 5% increments.[31] Tumors were classified as adenocarcinoma in situ (AIS), minimally invasive adenocarcinoma (MIA), and invasive adenocarcinoma, which was further divided into lepidic-predominant, papillary-predominant, acinar-predominant, micropapillary-predominant, and solid-predominant, as well as the variant forms invasive mucinous and colloid-predominant.

AIS and MIA were divided into nonmucinous, mucinous, and mixed mucinous/nonmucinous. Invasive mucinous adenocarcinoma was defined as a tumor with goblet or columnar cells, with abundant intracellular mucin (Fig. 1A) and with lepidic, acinar, papillary, or micropapillary pattern. Invasive mucinous adenocarcinoma was divided into two groups: pure mucinous (>90% invasive mucinous pattern) and mixed mucinous/nonmucinous (≥10% of each component).[30] Colloid-predominant adenocarcinoma was defined as a tumor with mucin pools within airspaces, with destruction of alveolar walls (Fig. 1B).[30]

On the basis of the presence of mucinous pattern (according to the IASLC/ATS/ERS lung adenocarcinoma classification), the histologic subtypes were grouped into two categories: nonmucinous subtype (composed of nonmucinous AIS, nonmucinous MIA, and nonmucinous invasive adenocarcinoma) and mucinous subtype (composed of mucinous AIS, mucinous MIA, invasive mucinous adenocarcinoma, and colloid-predominant adenocarcinoma).

The percentage of cribriform pattern,[36] which our group has recently published as a distinct histologic pattern in acinar predominant subtype with poor prognosis in stage I lung adenocarcinoma,[37] was also recorded in 5% increments, and designations of cribriform-

predominant subtype were made using criteria similar to the IASLC/ATS/ERS classification.

We also evaluated two additional mucinous features: signet-ring cell feature and extracellular mucin. Both of these mucinous features were reported to be positive for histochemical mucin stain.[29] Signet-ring cell feature is characterized by abundant intracellular mucin and a crescentic nucleus displaced toward one end of the cell (Fig. 1C), and represent a cytologic change that can occur in multiple histologic subtypes of invasive adenocarcinoma (acinar, papillary, micropapillary, and solid predominant), the percentage of signet-ring cell feature was recorded regardless of histologic subtype of each tumor, and this feature was recorded as being present when any percentage of signet-ring cell features was found. Extracellular mucin is characterized by abundant mucin pools in tumoral alveolar or intraglandular spaces, which can also occur in multiple histologic subtypes including lepidic (Fig. 2A), acinar (Fig. 2B), papillary (Fig. 2C), and micropapillary pattern (Fig. 2D). Since extracellular mucin can occur in most tumors with mucinous subtypes (invasive mucinous and colloid-predominant), to delineate extracellular mucin as an additional category that is independent from mucinous subtypes, extracellular mucin was recorded to be present only in tumors with nonmucinous subtypes. Extracellular mucin was considered to be positive when observed in ≥10% of the tumor.

Mitotic counts were investigated using high-power fields (HPFs) at ×400 magnification (0.237 mm^2 field of view). Mitoses were counted at 50 HPFs in areas with the highest mitotic activity and were assessed as the average number of mitotic figures per 10 HPFs.[38–40] In addition, we investigated the following histologic factors: visceral pleural invasion (classified as absent [PL0] or present [PL1, PL2, and PL3]),[35] lymphatic invasion, vascular invasion, and tumor necrosis.

Analysis of Mutations

EGFR exon 19 deletion and exon 21 L858R mutation were detected using a polymerase chain reaction - based assay, as previously described.[41] *KRAS* exon 2 mutation was detected by Sanger sequencing.[14]

Tissue Microarray

Formalin-fixed, paraffin-embedded tumor specimens were used for tissue microarray construction. In brief, six representative tumor areas were marked on H&E-stained slides, and cylindrical 0.6-mm tissue cores were arrayed from the corresponding paraffin blocks into a recipient block using an automated tissue arrayer (ATA-27; Beecher Instruments, Sun Prairie, WI), resulting in 15 tissue microarray blocks. From each tissue microarray, 4-μm-thick paraffin sections were prepared for immunohistochemical analysis. In total, 677 cases with adequate cores were available for immunohistochemical analysis. On average, 5.6 tumor cores per patient were available for analysis.

Immunohistochemical Analysis and Scoring of ALK

Since it was not possible to study all patients in this cohort for *ALK* rearrangements by fluorescence *in situ* hybridization (FISH), we used immunohistochemistry as a surrogate as

it has been shown in multiple studies to correlate highly with FISH results.[42–44] In brief, 4-μm sections from the tissue microarray blocks were deparaffinized in xylene and dehydrated in graded alcohols. The standard avidin-biotin complex peroxidase technique was used for immunohistochemical staining of anti-ALK antibody (clone 5A4; Adcam; diluted at 1:30). Sections were stained using a Ventana Discovery XT automated immunohistochemical stainer (Ventana, Tucson, AZ), in accordance with the manufacturer's guidelines. Diaminobenzidine was used as the chromogen, and hematoxylin was used as the nuclear counterstain. Positive control tissues were stained in parallel with the study cases.

ALK expression was recorded as the intensity of tumor cells with cytoplasmic-positive immunostaining in each tumor core. The intensity of staining was classified as no staining, weakly positive (faint cytoplasmic staining), moderately positive (moderate granular cytoplasmic staining), and strongly positive (strong granular cytoplasmic staining).[42–44] ALK expression was divided into two groups: negative (no staining – weakly positive) and positive (moderately – strongly positive).[43, 44]

Statistical Analysis

Associations between clinicopathologic variables and histologic findings were analyzed using Fisher's exact test (for categorical variables) and the Wilcoxon test (for continuous variables). Two-sided $P < 0.05$ was considered to indicate statistical significance. All analyses were performed using SAS statistical software (version 9.2; SAS Institute, Cary, NC).

RESULTS

Patient Demographic Characteristics

The clinical demographic characteristics for all 864 patients are outlined in Table 1. The median age was 69 years (range, 23 to 96 years); 63% of patients were women. Most patients were white (91%), followed by Asian (5%) and African-American (4%). Most patients were former smokers (67%); 14% were current smokers. Most patients had pathologic stage I disease (77%), followed by stage II (13%) and stage III (11%).

Histologic Subtypes and Mucinous Features

In total, 42 tumors (5%) had mucinous subtypes: 1 mucinous MIA (0.1%), 36 invasive mucinous adenocarcinomas (4%), and 5 colloid-predominant tumors (0.6%). Of the invasive mucinous adenocarcinomas, 20 were pure mucinous, and 16 were mixed mucinous/nonmucinous. No mucinous or mixed mucinous/nonmucinous AIS were identified. There were 822 tumors with nonmucinous subtypes (95%): 2 nonmucinous AIS (0.2%), 31 nonmucinous MIA (4%), 97 lepidic-predominant (11%), 300 acinar-predominant (35%), 151 papillary-predominant (17%), 78 micropapillary-predominant (9%), and 163 solid-predominant tumors (19%) (Table 2). Among acinar-predominant tumors, 34 showed cribriform-predominant pattern.

Signet-ring cell features were identified in 69 tumors (8%): 2 lepidic-predominant, 31 acinar-predominant, 9 papillary-predominant, 4 micropapillary-predominant, 16 solid-

predominant, 5 invasive mucinous, and 2 colloid-predominant (Fig. 3A). Extracellular mucin was identified in 116 tumors (13%): 2 nonmucinous MIA, 11 lepidic-predominant, 56 acinar-predominant (including 6 cribriform predomiant), 26 papillary-predominant, 6 micropapillary-predominant and 15 solid-predominant tumors (Fig. 3B).

Associations between Clinicopathologic Characteristics and Mucinous Patterns

Invasive mucinous adenocarcinoma was associated with non-Asian race (P=0.045) - in particular, with African-American race (P=0.044). In addition, invasive mucinous adenocarcinoma was associated with a lower rate of nodal metastases (P=0.031), less lymphatic invasion (P<0.001), less vascular invasion (P=0.011), absence of necrosis (P=0.030), and lower mitotic count (P<0.001). Signet-ring cell features were associated with higher stage (P=0.037) and presence of lymphatic invasion (P=0.032). Extracellular mucin was associated with history of smoking (P=0.014), higher rate of nodal metastases (P=0.012), and higher stage (P=0.019) and had a tendency to be associated with non-Asian race (P=0.055).

Associations between *EGFR*/*KRAS* Mutations and Histologic Subtypes or Mucinous Features

In total, 127 tumors (15%) had *EGFR* mutations, and 228 (26%) had *KRAS* mutations. The associations between mucinous and nonmucinous subtypes and *EGFR* and *KRAS* mutations are summarized in Table 2. No mucinous subtype tumors had *EGFR* mutations. Mucinous subtype was significantly associated with *KRAS* mutation, compared with *EGFR* mutation (P<0.001) and *EGFR/KRAS* wild-type (P<0.001). One mucinous MIA had *KRAS* mutation. Of the 36 invasive mucinous adenocarcinomas, 22 (61%) had *KRAS* mutations, and none had *EGFR* mutations. Invasive mucinous adenocarcinomas were significantly associated with *KRAS* mutation, compared with *EGFR* mutation (P<0.001) and *EGFR/KRAS* wild-type (P<0.001). Among invasive mucinous adenocarcinomas, pure mucinous tumors were significantly more likely to have *KRAS* mutations, compared with mixed mucinous/nonmucinous tumors (85% vs. 31%; P=0.002) (Fig. 4). Of the 5 colloid-predominant tumors, 1 had *KRAS* mutation, and none had *EGFR* mutation.

Of the 2 tumors with nonmucinous AIS, 1 had *KRAS* mutation. Of the 31 tumors with nonmucinous MIA, 4 (13%) had *EGFR* mutations, and 5 (16%) had *KRAS* mutations. Of the 97 lepidic-predominant invasive adenocarcinomas, 27 (28%) had *EGFR* mutations, and 21 (22%) had *KRAS* mutations. Nonmucinous AIS/MIA was not associated with *EGFR* and *KRAS* mutations (P=0.49). Because of the similarities in their morphologic appearances, nonmucinous AIS, nonmucinous MIA, and lepidic-predominant invasive adenocarcinoma were combined into the lepidic-predominant group for the following analysis. The associations between *EGFR/KRAS* mutations and predominant subtypes/mucinous features among nonmucinous subtype tumors are summarized in Table 3. Tumors in the lepidic-predominant group (n=130) were more likely to have *EGFR* mutations, compared with tumors with the other predominant subtypes (P=0.011 [*EGFR* mutation vs. *EGFR*/*KRAS* wild-type] and P=0.011 [*EGFR* mutation vs. *KRAS* mutation]). Papillary-predominant tumors were less likely to have *EGFR*/*KRAS* wild-type (P=0.032). Solid-predominant tumors were more likely to have *KRAS* mutations (P<0.001) or *EGFR*/*KRAS* wild-type

(*P*<0.001) compared to *EGFR* mutation. However, acinar-predominant, micropapillary-predominant, and cribriform-predominant tumors were not associated with *EGFR* and *KRAS* mutations (*P*=0.18, *P*=0.23 and *P*=0.11, respectively).

Of the 116 tumors with extracellular mucin, 7 (6%) had *EGFR* mutations, and 51 (44%) had *KRAS* mutations (Table 3). Tumors with extracellular mucin were more likely to have *KRAS* mutations, compared with tumors without extracellular mucin (*P*<0.001 [*KRAS* mutation vs. *EGFR*/*KRAS* wild-type] and *P*<0.001 [*KRAS* mutation vs. *EGFR* mutation]). Of note, *KRAS* mutations were most frequent in tumors with ≥50% extracellular mucin, followed by tumors with 10% to 49% extracellular mucin and those with <10% extracellular mucin (62% vs. 40% vs. 22%; *P*<0.001) (Fig. 5). However, the presence of signet-ring cell features was not associated with *EGFR* or *KRAS* mutations (*P*=0.52).

Associations between *EGFR* and *KRAS* Mutation Types and Histologic Subtypes and Mucinous Features

Of the 127 tumors with *EGFR* mutations, 64 (50%) had exon 19 deletions, and 63 (50%) had exon 21 L858R mutations. However, no associations were found between any of the nonmucinous subtypes and *EGFR* mutation types.

Of the 228 tumors with *KRAS* mutations, 170 (75%) had transversion mutations (G→T or G→C), and 58 (25%) had transition mutations (G→A). *KRAS* transversion mutations were more common in ever smokers (76%; 166/218), whereas *KRAS* transition mutations were more common in never smokers (60%; 6/10, *P*=0.019). *KRAS* transition mutations were more frequently observed in invasive mucinous adenocarcinoma (50%; 11/22) compared to non-mucinous subtype tumors (23%; 46/204, *P*=0.009). Extracellular mucin was not associated with *KRAS* mutation types (*P*=0.36).

Associations between ALK Expression and Histologic Subtypes, Mucinous Features, or Clinical Characteristics

Positive ALK expression was identified in 29 of the 677 tumors (4%) that underwent ALK immunohistochemical analysis. Among ALK positive tumors, diffuse positivity (>50% of tumor area) was identified in half of the tumor cores in tissue microarrays. Of the *EGFR*/*KRAS* wild-type tumors in this cohort (*n*=400), positive ALK expression was identified in 27 (7%). In contrast, of the tumors with *EGFR* mutations (n=97), positive ALK expression was identified in only 1 (1%). In addition, of the tumors with *KRAS* mutations (*n*=180), positive ALK expression was identified in only 1 (0.6%).

The associations between histologic subtypes and mucinous features and ALK expression are summarized in Table 4. Of the tumors with signet-ring cell features (*n*=54), 8 (15%) had positive ALK expression. Signet-ring cell features were significantly associated with positive ALK expression (*P*=0.001). In addition, positive ALK expression was identified in 12% (3/26) of cribriform-predominant tumors (*P*=0.095) and in 7% (9/126) of tumors with extracellular mucin (*P*=0.089). Mucinous subtype was not associated with ALK expression, compared with nonmucinous subtype (*P*=0.18).

ALK expression was not associated with patient sex (*P*=0.85) or age (*P*=0.85). In this cohort, ALK expression was more frequently observed in never smokers (7%; 8/120) than in ever smokers (4%; 21/557) but the difference was not statistically significant (*P*=0.21).

DISCUSSION

In this study, we have demonstrated that specific histologic subtypes and mucinous features are associated with *EGFR* and *KRAS* mutations. (1) *KRAS* mutations are associated with invasive mucinous adenocarcinoma and extracellular mucin. (2) Among invasive mucinous adenocarcinomas, pure mucinous tumors are more likely to have *KRAS* mutations, compared with mixed mucinous/nonmucinous tumors. (3) Among tumors with *KRAS* mutations, invasive mucinous adenocarcinoma is associated with transition mutations, rather than transversion mutations. (4) *EGFR* mutations are associated with the lepidic-predominant group (nonmucinous AIS, nonmucinous MIA, and lepidic-predominant invasive adenocarcinoma).

KRAS mutation is associated with the subset of mucinous adenocarcinomas formerly classified as mucinous BAC[21–25] (now called invasive mucinous adenocarcinoma, or mucinous AIS or mucinous MIA).[30] This is supported by a recent study that demonstrated invasive mucinous adenocarcinoma classified according to the IASLC/ATS/ERS classification was associated with *KRAS* mutation.[45] We hypothesized that other mucinous features might also be associated with *KRAS* mutation, and identified that invasive mucinous adenocarcinoma was significantly associated with *KRAS* mutation and a complete absence of *EGFR* mutation. More interestingly, *KRAS* mutations were significantly more frequently detected in pure invasive mucinous adenocarcinomas (85%) than in mixed mucinous/nonmucinous tumors (31%). This finding supports the practical value of subclassifying invasive mucinous adenocarcinoma as either pure mucinous or mixed mucinous/nonmucinous. *KRAS* transition mutations have been reported to occur frequently in never smokers with lung adenocarcinoma.[19, 20] This association was confirmed in our study, and *KRAS* transition mutations were more common in invasive mucinous adenocarcinomas than in nonmucinous subtypes. With regard to the other mucinous subtypes, 1 mucinous MIA tumor had *KRAS* mutation. Of the 5 colloid-predominant adenocarcinomas, 1 had *KRAS* mutation, and none had *EGFR* mutation. However, this finding was based on a small number of cases; thus, further investigation, with more cases, is warranted.

The clinical and pathologic significance of extracellular mucin in lung adenocarcinoma has not yet been investigated. In our study, extracellular mucin was identified in 13% of tumors. *KRAS* mutations were detected in 44% of tumors with extracellular mucin. Of note, of the tumors with ≥50% extracellular mucin, *KRAS* mutations were detected in 62%, indicating a significant association between extracellular mucin and *KRAS* mutation. Whether extracellular mucin in lung adenocarcinomas is associated with resistance to TKI treatment may require additional investigation.

Recent studies have reported that *ALK* rearrangement is associated with mucinous features, such as signet-ring cell feature and extracellular mucin, and cribriform pattern in lung adenocarcinoma[27–29]; however, the associations between *EGFR* and *KRAS* mutations and

these mucinous features have not been thoroughly investigated. In our study, signet-ring cell features were not associated with *EGFR* or *KRAS* mutations. Although our study did not specifically assess for *ALK* rearrangement, ALK expression was used as a surrogate method. Based on ALK status by immunohistochemistry (using monoclonal antibody clone 5A4), signet-ring cell features were associated with positive ALK expression. Tumors with extracellular mucin more frequently had positive ALK expression, although the association was not statistically significant. In studies of Japanese cohorts, cribriform pattern was associated with *ALK* rearrangement in lung adenocarcinoma.[29, 46] In contrast, a study from the United States did not identify an association between cribriform pattern and *ALK* rearrangement.[47] Therefore, it is not clear whether cribriform pattern is universally associated with *ALK* rearrangement. In the current study, cribriform-predominant tumors had more frequent ALK expression although the association was not statistically significant. One limitation of these findings is that, in our study, *ALK* rearrangement was not confirmed by FISH. However, a strong association between ALK immunohistochemical staining and *ALK* FISH- which has led to proposals to use immunohistochemical analysis as a screening method for *ALK* rearrangement - has been demonstrated in recent studies. Of note, anti-ALK monoclonal antibody 5A4, which was also used in our study, has demonstrated 95% to 100% sensitivity and specificity for the identification of tumors with *ALK* rearrangement confirmed by FISH in NSCLC.[42–44] *EGFR* and *KRAS* mutations and *ALK* rearrangement have been reported to be mutually exclusive.[43, 48] However, several studies have demonstrated that a very small number of tumors (<1%) concomitantly harbor *EGFR* or *KRAS* mutations and *ALK* rearrangement in NSCLC.[42, 49, 50] In our study, positive ALK expression was identified in 1% of tumors with *EGFR* mutations and in 0.6% of tumors with *KRAS* mutations.

EGFR and *KRAS* mutations are detected even in preinvasive lesions, such as atypical adenomatous hyperplasia and AIS.[51–53] In our study, *EGFR* mutations were identified in nonmucinous MIA, and *KRAS* mutations were identified in nonmucinous AIS and mucinous and nonmucinous MIA. *EGFR* mutations were associated with nonmucinous lepidic-predominant growth. This finding is compatible with previous studies that reported an association between *EGFR* mutations and presence of lepidic (formerly BAC) features.[5–8] In the current study, furthermore, solid-predominant tumors were associated with *KRAS* mutations compared *EGFR* mutations, however; acinar-, papillary- and micropapillary-predominant tumors were not associated with *EGFR* and *KRAS* mutations. In the previous study from our group using a smaller cohort (n=100) of lung adenocarcinoma, papillary and micropapillary-predominant tumors were associated with EGFR mutation.[54] In the recent study from our group using lung adenocarcinoma samples (n=180), in which mutation analyses were performed by Sequenom Mass ARRAY system (Sequenom), KRAS mutation was associated with solid-predominant tumors, and EGFR mutation was associated with lepidic, papillary and acinar-predominant tumors.[55] There would be 2 possible explanations of the discrepancy regarding the association between predominant histologic subtype and mutation status: 1) inter-observer variability and 2) differences in mutation detection method. The reproducibility of histologic subtyping for lung adenocarcinoma was assessed by experienced pulmonary pathologists from multiple countries, with respect to predominant pattern, which reported good reproducibility (kappa score 0.77±0.07) in identifying

predominant subtypes with typical cases.[56] However, the limitations on this study were that the cases were reviewed using a micro-photographic image-based method evaluating selected images of tumors but actual tumor slides were not reviewed. Therefore, to confirm reproducibility of the new classification, further investigation is needed using actual tumor slides. Differences in mutation detection methods (direct sequencing vs. Sequenom anlaysis) could also have some impact on the study comparing morphologic finding to mutation status. Sequenom Mass ARRAY system (Sequenom) is suitable for more sensitive and broader mutation screening than direct sequencing.[57]

In summary, our study has demonstrated that mucinous histologic patterns - particularly invasive mucinous adenocarcinoma and extracellular mucin - are associated with *KRAS* mutations and specific clinicopathologic features. From a clinical standpoint, our findings suggest that there are some tendencies for associations between molecular characteristics and lung adenocarcinoma subtypes. However, the histologic-molecular associations are not 100% specific, so it is difficult to reliably predict the molecular status of *EGFR* and *KRAS* mutations and *ALK* rearrangement on the basis of histologic features alone. Therefore, molecular testing is still necessary, even in tumors for which a strong association between histologic type and *EGFR* or *KRAS* mutation or *ALK* rearrangement status has been demonstrated.

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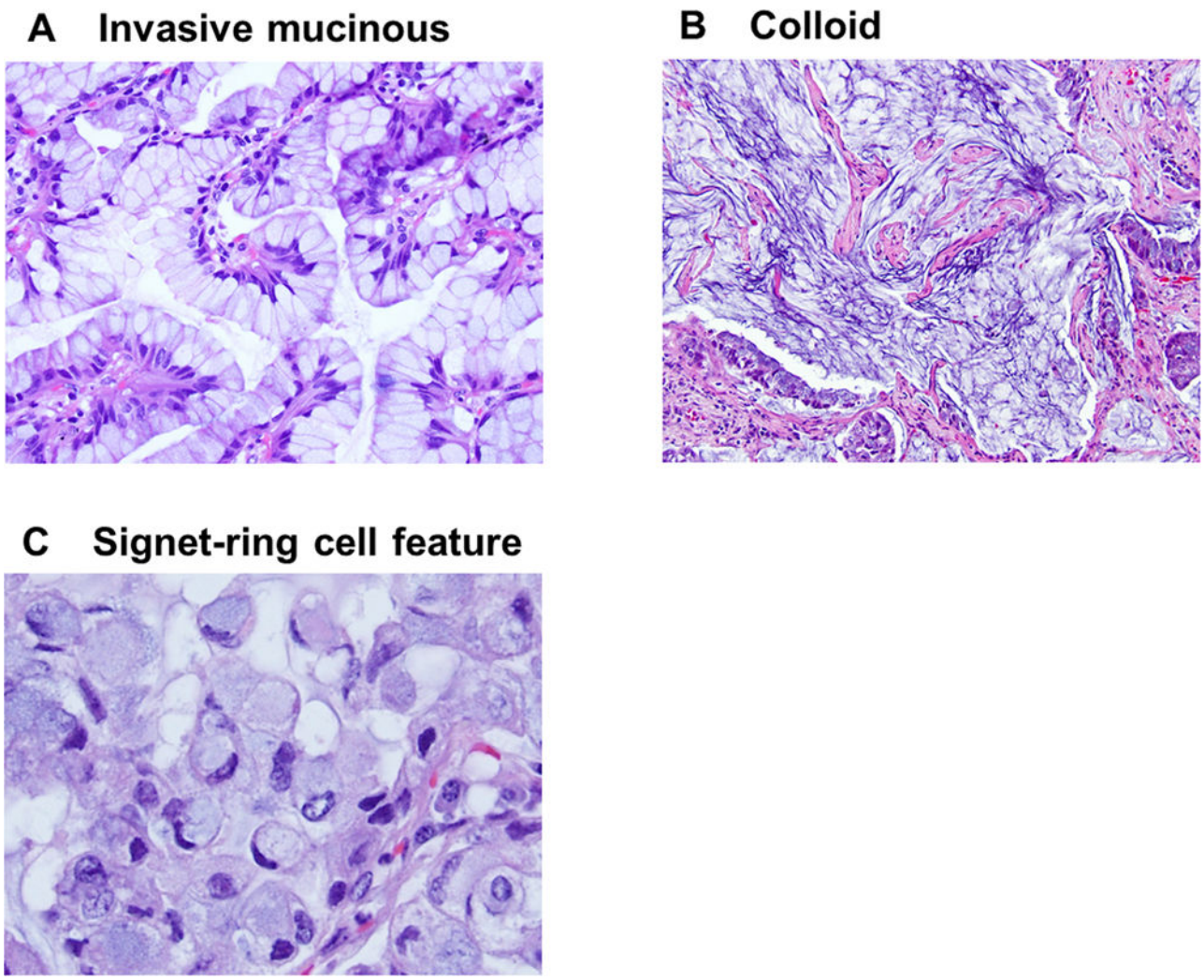

FIGURE 1.

Mucinous subtypes and features. (A) Invasive mucinous adenocarcinoma. Goblet or columnar tumor cells with abundant intracellular mucin. (B) Colloid-predominant adenocarcinoma. A tumor has a mucin pool within tumoral stroma, with destruction of the alveolar wall. (C) Signet-ring cell feature. Tumor cells have abundant intracellular mucin and a crescentic nucleus displaced toward one end of the cells.

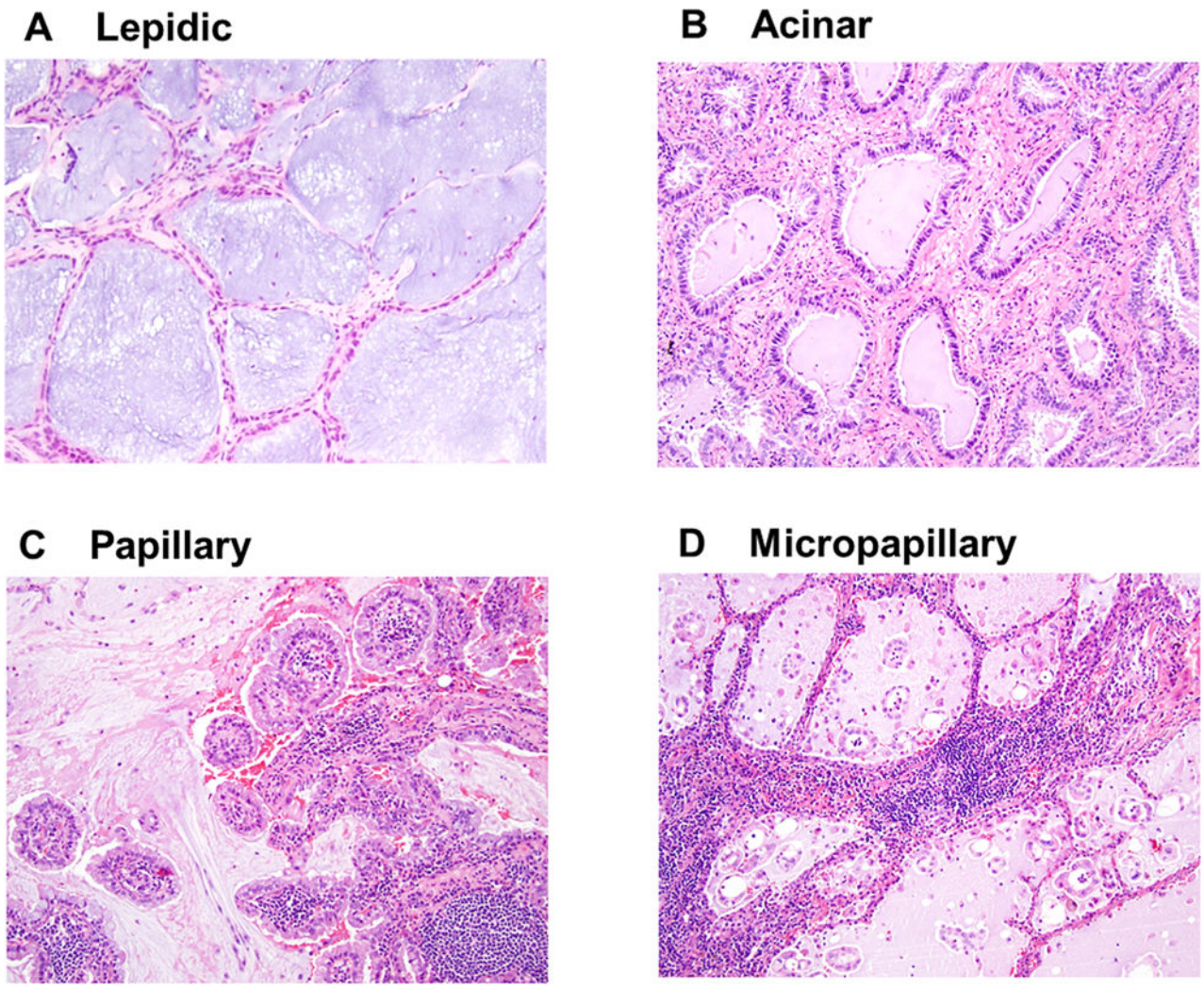

FIGURE 2.

Extracellular mucin in each histologic pattern. Extracellular mucin in (A) lepidic, (B) acinar, (C) papillary, and (D) micropapillary pattern.

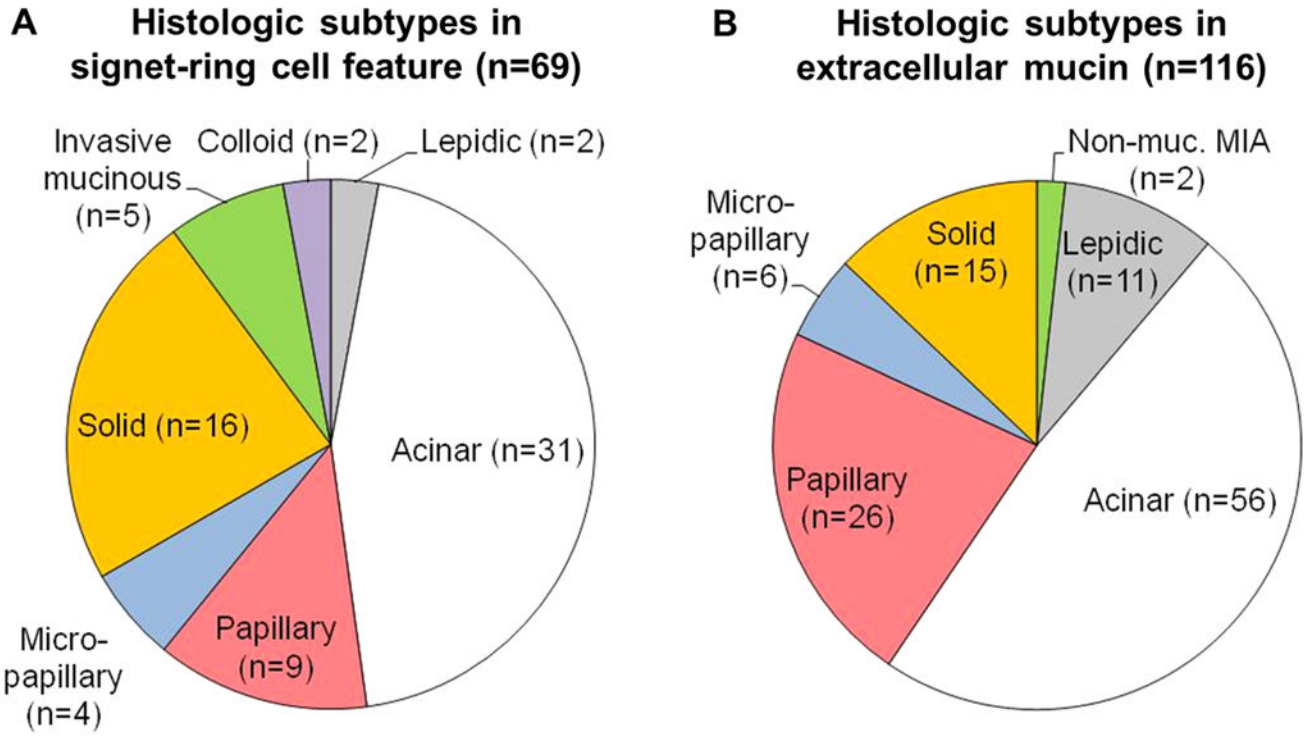

FIGURE 3.

Mucinous features and their predominant subtypes. (A) Signet-ring cell features were identified in 69 tumors (5%): 2 lepidic-predominant, 31 acinar-predominant, 9 papillary-predominant, 4 micropapillary-predominant, 16 solid-predominant, 5 invasive mucinous, and 2 colloid-predominant. (B) Extracellular mucin was identified in 116 tumors (13%): 2 nonmucinous MIA, 11 lepidic-predominant, 56 acinar-predominant, 26 papillary-predominant, 6 micropapillary-predominant, and 15 solid-predominant.

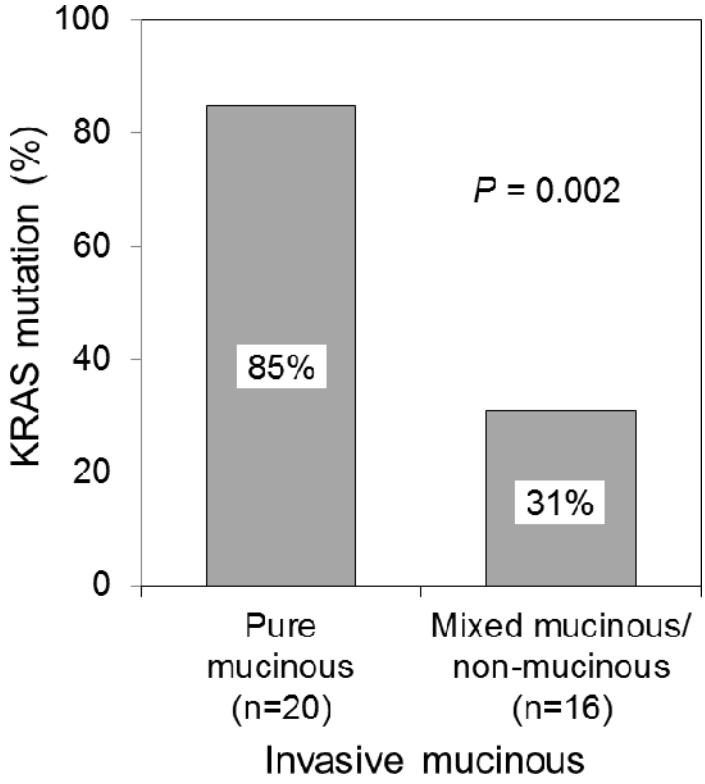

FIGURE 4.

Associations between *KRAS* mutation and invasive mucinous adenocarcinoma (pure mucinous vs. mixed mucinous/nonmucinous). Among invasive mucinous adenocarcinomas (*n*=36), pure mucinous tumors were significantly more likely to have *KRAS* mutations, compared with mixed mucinous/nonmucinous tumors (85% vs. 31%).

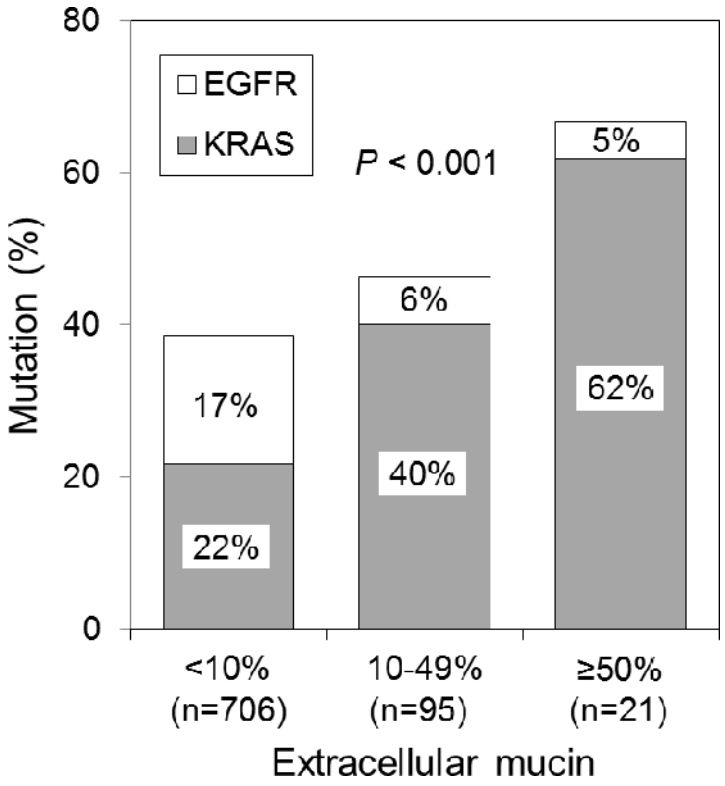

FIGURE 5.

Associations between *KRAS* mutation and percentage of extracellular mucin. *KRAS* mutations were most frequent in tumors with ≥50% extracellular mucin (*n*=21), followed by tumors with 10% to 49% extracellular mucin and those with <10% extracellular mucin (62% vs. 40% vs. 22%).

TABLE 1

Patient demographics

Variable	n (%)
Age, median (range)	69 (23–96)
Sex	
Female	541 (63)
Male	323 (37)
Race	
Asian	41 (5)
African-American	33 (4)
White	790 (91)
Smoking	
Never	160 (19)
Ever	704 (81)
Pathologic TNM stage	
I	663 (77)
II	109 (13)
III	92 (11)

TABLE 2

Associations between *EGFR*/*KRAS* mutations and histologic subtypes

		Mutation, n (%)		
Histologic subtype	**Total**	**Wild-type**	***EGFR***	***KRAS***
Mucinous subtypes	42	18 (43)	0 (0)	24 (57)
Mucinous AIS	0	0 (0)	0 (0)	0 (0)
Mucinous MIA	1	0 (0)	0 (0)	1 (100)
Invasive mucinous	36	14 (39)	0 (0)	22 (61)
Colloid	5	4 (80)	0 (0)	1 (20)
Non-mucinous subtypes	822	491 (60)	127 (15)	204 (25)
Nonmucinous AIS	2	1 (50)	0 (0)	1 (50)
Nonmucinous MIA	31	22 (71)	4 (13)	5 (16)
Lepidic	97	49 (50)	27 (28)	21 (22)
Acinar	300	180 (60)	54 (18)	66 (22)
Papillary	151	76 (50)	29 (19)	46 (30)
Micropapillary	78	49 (63)	7 (9)	22 (28)
Solid	163	114 (70)	6 (4)	43 (26)
Total	864	509 (59)	127 (15)	228 (26)

EGFR, epidermal growth factor receptor; AIS, adenocarcinoma in situ;

MIA, minimally invasive adenocarcinoma

TABLE 3

Associations between *EGFR/KRAS* mutations and histologic subtypes/mucinous features

	Mutation, n (%)			*P*-value (overall)	*P*-value (pairwise comparison)		
Variable	**Wild-type**	***EGFR***	***KRAS***		***EGFR* vs. wild-type**	***KRAS* vs. wild-type**	***EGFR* vs. *KRAS***
Histologic subtype							
Lepidic predominant group	72 (55)	31 (24)	27 (21)	**0.019**	**0.011**	0.72	**0.011**
Nonlepidic	419 (60)	96 (14)	177 (26)				
Acinar	180 (60)	54 (18)	66 (22)	0.18			
Nonacinar	311 (60)	73 (14)	138 (26)				
Papillary	76 (50)	29 (19)	46 (30)	**0.032**	**0.063**	**0.029**	1.00
Nonpapillary	415 (62)	98 (15)	158 (24)				
Micropapillary	49 (63)	7 (9)	22 (28)	0.23			
Nonmicropapillary	442 (59)	120 (16)	182 (24)				
Solid	114 (70)	6 (4)	43 (26)	**<0.001**	**<0.001**	0.62	**<0.001**
Nonsolid	377 (57)	121 (18)	161 (24)				
Cribriform	26 (77)	4 (12)	4 (12)	0.11			
Noncribriform	465 (59)	123 (16)	200 (25)				
Mucinous feature							
Signet-ring cell feature	35 (56)	8 (13)	19 (31)	0.52			
Non–signet-ring cell feature	456 (60)	119 (16)	185 (24)				
Extracellular mucin	58 (50)	7 (6)	51 (44)	**<0.001**	0.050	**<0.001**	**<0.001**
Nonextracellular mucin	433 (61)	120 (17)	153 (22)				

Lepidic-predominant group includes nonmucinous adenocarcinoma in situ, nonmucinous minimally invasive adenocarcinoma, and lepidic-predominant invasive adenocarcinoma.

Significant *P*-values are shown in bold.

EGFR, epidermal growth factor receptor

TABLE 4

Associations between ALK expression and histologic subtypes/mucinous features

		ALK expression, no. (%)		
Variable	**Total, no.**	**Positive**	**Negative**	***P*-value**
Histologic subtype				
Mucinous subtype	35	3 (9)	32 (91)	0.18
Nonmucinous subtype	642	26 (4)	616 (96)	
Cribriform pattern				
Cribriform-predominant	26	3 (12)	23 (88)	0.095
Noncribriform-predominant	651	26 (4)	625 (96)	
Signet-ring cell features				
Positive	54	8 (15)	46 (85)	**0.001**
Negative	623	21 (3)	602 (97)	
Extracellular mucin				
Positive	126	9 (7)	117 (93)	0.089
Negative	551	20 (4)	531 (96)	

Significant *P*-values are shown in bold.

ALK, anaplastic lymphoma kinase