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# *KRAS* mutations are associated with solid growth pattern and tumor-infiltrating leukocytes in lung adenocarcinoma

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***KRAS* mutations define a clinically distinct subgroup of lung adenocarcinoma patients, characterized by smoking history, resistance to EGFR-targeted therapies, and adverse prognosis. Whether *KRAS*-mutated lung adenocarcinomas also have distinct histopathological features is not well established. We tested 180 resected lung adenocarcinomas for *KRAS* and *EGFR* mutations by high-sensitivity mass spectrometry-based genotyping (Sequenom) and PCR-based sizing assays. All tumors were assessed for the proportion of standard histological patterns (lepidic, acinar, papillary, micropapillary, solid, and mucinous), several other histological and clinical parameters, and TTF-1 expression by immunohistochemistry. Among 180 carcinomas, 63 (35%) had *KRAS* mutations (*KRAS*+), 35 (19%) had *EGFR* mutations (*EGFR*+), and 82 (46%) had neither mutation (*KRAS*−/*EGFR*−). Solid growth pattern was significantly over-represented in *KRAS*+ carcinomas: the mean ± s.d. for the amount of solid pattern in *KRAS*+ carcinomas was 27 ± 34% compared with 3 ± 10% in *EGFR*+ ($P<0.001$) and 15 ± 27% in *KRAS*−/*EGFR*− ($P=0.033$) tumors. Furthermore, at least focal (≥20%) solid component was more common in *KRAS*+ (28/63; 44%) compared with *EGFR*+ (2/35; 6%; $P<0.001$) and *KRAS*−/*EGFR*− (21/82; 26%; $P=0.022$) carcinomas. *KRAS* mutations were also over-represented in mucinous carcinomas and were significantly associated with the presence of tumor-infiltrating leukocytes and heavier smoking history. *EGFR* mutations were associated with non-mucinous non-solid patterns, particularly lepidic and papillary, lack of necrosis, lack of cytological atypia, hobnail cytology, TTF-1 expression, and never/light smoking history. In conclusion, extended molecular and clinicopathological analysis of lung adenocarcinomas reveals a novel association of *KRAS* mutations with solid histology and tumor-infiltrating inflammatory cells and expands on several previously recognized morphological and clinical associations of *KRAS* and *EGFR* mutations. Solid growth pattern was recently shown to be a strong predictor of aggressive behavior in lung adenocarcinomas, which may underlie the unfavorable prognosis associated with *KRAS* mutations in these tumors.**
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Kristen rat sarcoma viral oncogene (*KRAS*) mutations are one of the most common oncogenic events in human carcinomas of endodermal origin, occurring at high frequency in adenocarcinomas of lung, pancreatic, and colorectal origin.[1,2] *KRAS* is an 'old oncogene' in lung cancer, having been first described in these tumors in 1984,[3] but recent years have witnessed a revamped interest in the role of *KRAS* in lung adenocarcinoma because of the rapid advances in molecularly targeted therapies. Although the efforts to therapeutically target mutant *KRAS* have thus far proven unsuccessful, *KRAS* has emerged as a useful negative predictive marker because it occurs in a mutually exclusive fashion with several recently identified targetable mutations, including epidermal growth factor receptor (*EGFR*)—the molecular target of EGFR tyrosine kinase inhibitors erlotinib and gefitinib. Thus, routine predictive molecular testing of lung adenocarcinomas now commonly combines screening for *KRAS* together with *EGFR* mutations.[4,5]

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Clinically, *KRAS* and *EGFR* mutations define two distinct and contrasting subgroups of lung adenocarcinoma patients. Although *KRAS* mutations are more common in western than East Asian patients (25–35% *vs* 5–10%, respectively), *EGFR* mutations have an inverse prevalence in these ethnic groups (10–20% *vs* >50%, respectively).[6] In addition, *KRAS* mutations are more common in smokers, whereas *EGFR* mutations in never or light smokers.[6] Although the data on prognostic significance of *KRAS* and *EGFR* mutations has been conflicting across studies, the adverse prognostic impact of *KRAS* mutations and the favorable impact of *EGFR* mutations have been demonstrated in several studies over the years[7–10] and in recent studies from our institution.[11,12] In addition, several studies also suggested that *KRAS* mutations may be markers of resistance not only to EGFR tyrosine kinase inhibitors[4,5] but also to conventional cisplatin-based chemotherapy.[13–15]

Histologically, it is well established that *EGFR* mutations occur preferentially in non-mucinous adenocarcinomas with lepidic/bronchioloalveolar and papillary patterns (reviewed in Travis *et al*[16]). By contrast, *KRAS* mutations are over-represented in mucinous adenocarcinomas.[17–20] However, mucinous carcinomas account for only a minority of lung adenocarcinomas with *KRAS* mutations in western populations,[18,20,21] and therefore this association is unlikely to explain the distinct clinical characteristics imparted by *KRAS* mutations. Several previous studies also suggested that *KRAS* mutations are associated with poor differentiation,[22–24] but this finding has been inconsistent across publications. Furthermore, because grading of lung carcinomas is not well-established, it is not known which morphological features (growth pattern, cytological features, necrosis, etc) may have imparted this association.

The goal of this study was therefore to re-examine potential histopathological correlates of *KRAS* mutations, particularly in non-mucinous adenocarcinomas. In addition to recent clarification regarding adverse prognostic significance of *KRAS* mutations, this re-examination was also prompted by advances in mutation testing methodology, with emergence of methods like mass spectrometry-based genotyping (Sequenom platform), which detect a wide spectrum of *KRAS* mutations with higher analytical sensitivity than standard Sanger sequencing. The use of a higher-sensitivity method to detect *KRAS* mutations can be anticipated to yield a more robust molecular baseline for the study of histological and other clinicopathological correlates of mutations.

With these considerations in mind, we performed a detailed histological and clinicopathological analysis of 180 lung adenocarcinomas annotated for *KRAS* and *EGFR* mutations by mass spectrometry-based genotyping and sensitive PCR-based assays with the main goal to re-examine potential histopathological characteristics associated with *KRAS* mutations.

## Materials and methods

### Study Design

One hundred and eighty surgical resections of primary lung adenocarcinomas, which had undergone routine genotyping for *EGFR* and *KRAS* mutations as part of prospective reflex protocol in 2009–2010, were randomly selected from the archives of Memorial Sloan-Kettering Cancer Center, New York, NY, USA. Only conventional invasive adenocarcinomas were included, whereas adenocarcinomas *in situ* (formerly pure bronchioloalveolar carcinoma) and minimally invasive adenocarcinomas[16] were excluded. All tumors were reviewed by two thoracic pathologists (NR and AM). The study was performed with the approval of Institutional Review Board of Memorial Sloan-Kettering Cancer Center.

### Histological and Immunohistochemical Analysis

Using a modification of histological scoring system proposed by IASLC/ATS/ERS,[16] each tumor was scored semi-quantitatively for the percentage (0–100%) of seven composite histological patterns, including five standard non-mucinous patterns (lepidic/bronchioloalveolar, acinar, papillary, micropapially, and solid) plus 'complex glandular' and mucinous patterns. 'Complex glandular' pattern was defined as either (1) cribriform morphology (resembling mammary ductal carcinoma *in situ*) or (2) complex arborizing intra-glandular proliferations and/or formation of slit-like multilumina (resembling mammary usual duct hyperplasia). Detailed description of morphology and clinicopathological characteristics of complex glandular patterns will be reported separately (Moreira *et al*, in preparation). Solid pattern was defined as pavement-like sheets of cells with no glandular lumina (ie, cells with circumferential attachment to other cells) with or without focal cytoplasmic mucin. In cases where solid pattern had abundant pink cytoplasm with sharp cell borders, imparting a 'squamoid' appearance, the distinction from a true squamous component was made on the basis of positive TTF-1 and/or negative p40 (ΔNp63) immunostains (data not shown). For the purposes of this study, all mucinous carcinomas were analyzed as a single group, which included tumors with non-solid histology with prominent cytoplasmic mucin, including mucinous carcinomas with lepidic growth pattern (former 'mucinous bronchioloalveolar carcinoma'[25]/'invasive mucinous adenocarcinoma'[16]), colloid carcinomas, and carcinomas with mucinous features, not otherwise specified. Carcinomas were classified as 'mucinous' if mucinous component represented ≥20% of the tumor volume. Similarly, non-mucinous patterns were analyzed using a ≥20% threshold (based on previous data that patterns in the amount of <20%

may not be clinically significant as they do not impact the metastatic potential associated with different patterns[26]). All patterns were recorded in 5% increments.

In addition, all tumors were also scored for the following histological parameters:

- Necrosis: scored as 2+/extensive = involving ≥20% of the tumor, 1+/focal = involving <20% of the tumor, and 0/absent.
- Cytological atypia: defined as anisonucleosis, nucleomegaly, irregular nuclear membranes, and/or macronucleoli; scored as 2+ = marked and diffuse; 1+ = moderate or focal; and 0 = minimal.
- Hobnail cytology: defined as cell outlines individually projecting into luminal spaces (as opposed to forming a smooth luminal border), thus resembling type II pneumocytes or Clara cells, analogous to what has been described as a defining feature of terminal respiratory unit-type histology by Yatabe *et al*;[27] scored as 2+ = diffuse; 1+ = focal; and 0 = none.
- Tumor-infiltrating leukocytes: defined as lymphocytes and/or other inflammatory cells involving intra- and peri-tumoral stroma and/or infiltrating in-between tumor cells; scored as 2+/marked = prominent at low power (×4 objective), 1+/moderate = easily noticeable at low power, and 0/none or minimal = inconspicuous at low power.

A representative whole tissue section from each tumor was analyzed for TTF-1 expression by immunohistochemistry, as previously described.[28] Presence of any TTF-1 reactivity was scored as positive. In addition, percentage of immunoreactive cells (0–100%) and intensity of staining (1+, 2+, or 3+) were recorded, and *H* scores were calculated by multiplying the percentage by intensity score (0–300).

### Clinicopathological Analysis

The following clinicopathological parameters were recorded: age, gender, smoking status (never *vs* current/former smoker), pack-year smoking history (defined as packs of cigarettes per day multiplied by years of smoking), tumor size, and tumor stage (American Joint Committee on Cancer seventh edition). Smoking history was collected based on a prospectively administered questionnaire. Never smokers were defined as patients who smoked <100 cigarettes in a lifetime.

### Molecular Analysis

*KRAS* and *EGFR* point mutations were tested by Sequenom Mass ARRAY system (Sequenom)—a mass spectrometry-based multiplex genotyping platform—which detects 22 non-synonymous *KRAS* mutations in codons 12, 13, and 61 and 20 *EGFR* mutations, as previously described.[29] Based on previous studies, Sequenom has analytical sensitivity for a mutated allele of ~5% (ie, required minimal tumor cell content is ~10%).[30] *EGFR* exon 19 deletions were identified by length analysis of fluorescently labeled PCR products, as previously described.[29]

### Statistical Analysis

Comparison of categorical variables was performed by Fisher's exact or Chi-square test, and comparison of continuous variable was performed by a Mann–Whitney test. *P*-values of ≤0.05 were considered statistically significant.

## Results

### Clinicopathological, Molecular and Histological Characteristics

The clinical characteristics of 180 patients with lung adenocarcinoma were as follows: age median (range) 67 (34–87) years, female gender $n=107$ (59%), never smoker $n=31$ (17%), and smoking pack-years median (range) 30 (0–200). Tumor stage was as follows: stage I $n=120$ (67%), stage II $n=35$ (19%), and stage III/IV $n=20$ (11%). Surgical procedures included wedge resection $n=60$, segmentectomy $n=2$, bronchial tumor resection $n=1$, lobectomy $n=116$, and pneumonectomy $n=1$.

As shown in Table 1, mutation analysis revealed that among 180 adenocarcinomas, 63 (35%) had *KRAS* mutations (*KRAS*+), 35 (19%) had *EGFR* mutations (*EGFR*+), and 82 (46%) had neither mutation (*KRAS*−/*EGFR*−). *KRAS* and *EGFR* mutations were mutually exclusive, with no tumor containing both mutations. *KRAS* mutations were distributed in codons 12 ($n=59$), 13 ($n=2$), and 61 ($n=2$).

The distribution of seven histological patterns (lepidic, acinar, papillary, micropapillary, complex

**Table 1** Summary of mutations in 180 lung adenocarcinomas

| *Mutation* | *N (%)* |
|---|---|
| *KRAS* | 63 (35%) |
| G12A | 7 |
| G12C | 24 |
| G12D | 15 |
| G12F | 2 |
| G12R | 1 |
| G12V | 10 |
| G13C | 1 |
| G13D | 1 |
| Q61H | 2 |
| *EGFR* | 35 (19%) |
| L858R | 21 |
| Exon 19 Δ | 13 |
| S768V | 1 |
| No *EGFR* or *KRAS* mutations | 82 (46%) |

glandular, solid, and mucinous) in 180 adenocarcinomas is shown in Table 2. The majority (162/180; 90%) of adenocarcinomas were highly heterogeneous, consisting of a mixture of 2–6 patterns. The number of mixed patterns per tumor was 2 in 45 cases, 3–4 in 99 cases, and 5–6 in 19 cases (mean number of patterns per tumor = 3). At least focal (≥20%) acinar, papillary, solid, complex glandular, micropapillary, and lepidic patterns were present in 59, 48, 28, 27, 22, and 19% of cases, respectively. Mucinous patterns were rare in our unselected patient population, occurring in only 17 (9%) of cases, which included mucinous bronchioloalveolar carcinoma/'invasive mucinous adenocarcinoma' ($n=6$), colloid carcinoma ($n=1$), and carcinoma with mucinous features, not otherwise specified ($n=10$). We also attempted to classify carcinomas based on a single predominant pattern, as recently recommended;[16] however, 29% (53/180) of cases had ≥2 patterns in a similar co-dominant amount, precluding objective assignment of a single predominant pattern.

### Association of Mutations and Histological Patterns

Association of mutations and histological patterns is shown in Table 3, where patterns were analyzed as categorical variables (ie, pattern absent *vs* present), and Figure 1, where patterns were analyzed as continuous variables (ie, by comparing the mean amount of a pattern according to mutation). Overall, no pattern was invariably present or absent in any molecular group, except for the exclusion of mucinous histology in *EGFR*+ carcinomas (Table 3). The only pattern that was significantly over-represented in *KRAS*+ carcinomas compared with the *EGFR*+ and *KRAS*−/*EGFR*− groups was solid: the mean ± s.d. for the amount of solid pattern in *KRAS*+ carcinomas was 27 ± 34% compared with 3 ± 10% in *EGFR*+ ($P<0.001$) and 15 ± 27% in *KRAS*−/*EGFR*− ($P=0.033$) tumors (Figure 1). Conversely, the presence of at least focal (≥20%) solid component was significantly more frequent in *KRAS*+ carcinomas (28/63; 44%) compared with the *EGFR*+ (2/35; 6%, $P<0.001$) and *KRAS*−/*EGFR*− (21/82; 26%; $P=0.022$) groups (Table 3). The rate of *KRAS* mutations in carcinomas with a solid component was 55% (28/63 cases).

Table 3 also shows that of the 63 *KRAS*+ adenocarcinomas, only 7 (11%) cases were mucinous, whereas the rest (89%) of *KRAS* mutations occurred in non-mucinous carcinomas. The rate of *KRAS* mutations in mucinous carcinomas overall was 41% (7/17 cases) and the rate of KRAS mutations specifically in mucinous bronchioloalveolar/'invasive

**Table 2** Distribution of histologic patterns in 180 lung adenocarcinomas

| | *N (%)*[a] *of cases with indicated amount of pattern:* | | | *Pattern amount: mean ± s.d. (range)* |
|---|---|---|---|---|
| | *≥20%* | *≥50%* | *100%* | |
| Lepidic/bronchioloalveolar | 35 (19) | 7 (4) | 0[b] | 7 ± 14 (0–70) |
| Acinar | 106 (59) | 49 (27) | 0 | 28 ± 25 (0–90) |
| Papillary | 86 (48) | 19 (11) | 4 (2) | 19 ± 24 (0–100) |
| Micropapillary | 40 (22) | 5 (3) | 0 | 8 ± 14 (0–80) |
| Complex glandular | 48 (27) | 11 (6) | 1 (1) | 12 ± 18 (0–90) |
| Solid | 51 (28) | 31 (17) | 1 (1) | 17 ± 29 (0–100) |
| Mucinous | 17 (9) | 16 (9) | 12 (7) | 9 ± 27 (0–100) |

[a]The denominator for shown percentages is the total number of cases ($n=180$).
[b]Entirely lepidic carcinomas (pure bronchioloalveolar carcinomas/'adenocarcinomas *in situ*') were excluded from this study.

**Table 3** Distribution of histological patterns according to mutation

| | *Total* N = *180* | *Mutation* | | | P-*value* | | |
|---|---|---|---|---|---|---|---|
| | | *EGFR*+ n = *35* | *KRAS*+ n = *63* | *neg/neg* n = *82* | *KRAS*+ *vs* *EGFR*+ | *KRAS*+ *vs* *neg/neg* | *EGFR*+ *vs* *neg/neg* |
| Lepidic/bronchioloalveolar | 35 (19) | 15 (43) | 7 (11) | 13 (16) | **<0.001** | 0.47 | **0.004** |
| Acinar | 106 (59) | 28 (80) | 34 (54) | 44 (54) | **0.016** | 1.00 | **0.008** |
| Papillary | 86 (48) | 26 (74) | 22 (35) | 38 (46) | **<0.001** | 0.18 | **0.008** |
| Micropapillary | 40 (22) | 9 (26) | 12 (19) | 19 (23) | 0.45 | 0.69 | 0.82 |
| Complex glandular | 48 (27) | 9 (26) | 21 (33) | 18 (22) | 0.49 | 0.14 | 0.64 |
| Solid | 51 (28) | 2 (6) | 28 (44) | 21 (26) | **<0.001** | **0.022** | **0.012** |
| Mucinous | 17 (9) | 0 | 7 (11) | 10 (12) | **0.048** | 1.00 | **0.032** |

The denominator for shown percentages is the total number of cases with each mutation. Patterns were analyzed using a ≥20% threshold (see Materials and methods). neg/neg = cases negative for *KRAS* and *EGFR* mutations. Bold *P*-values are statistically significant.

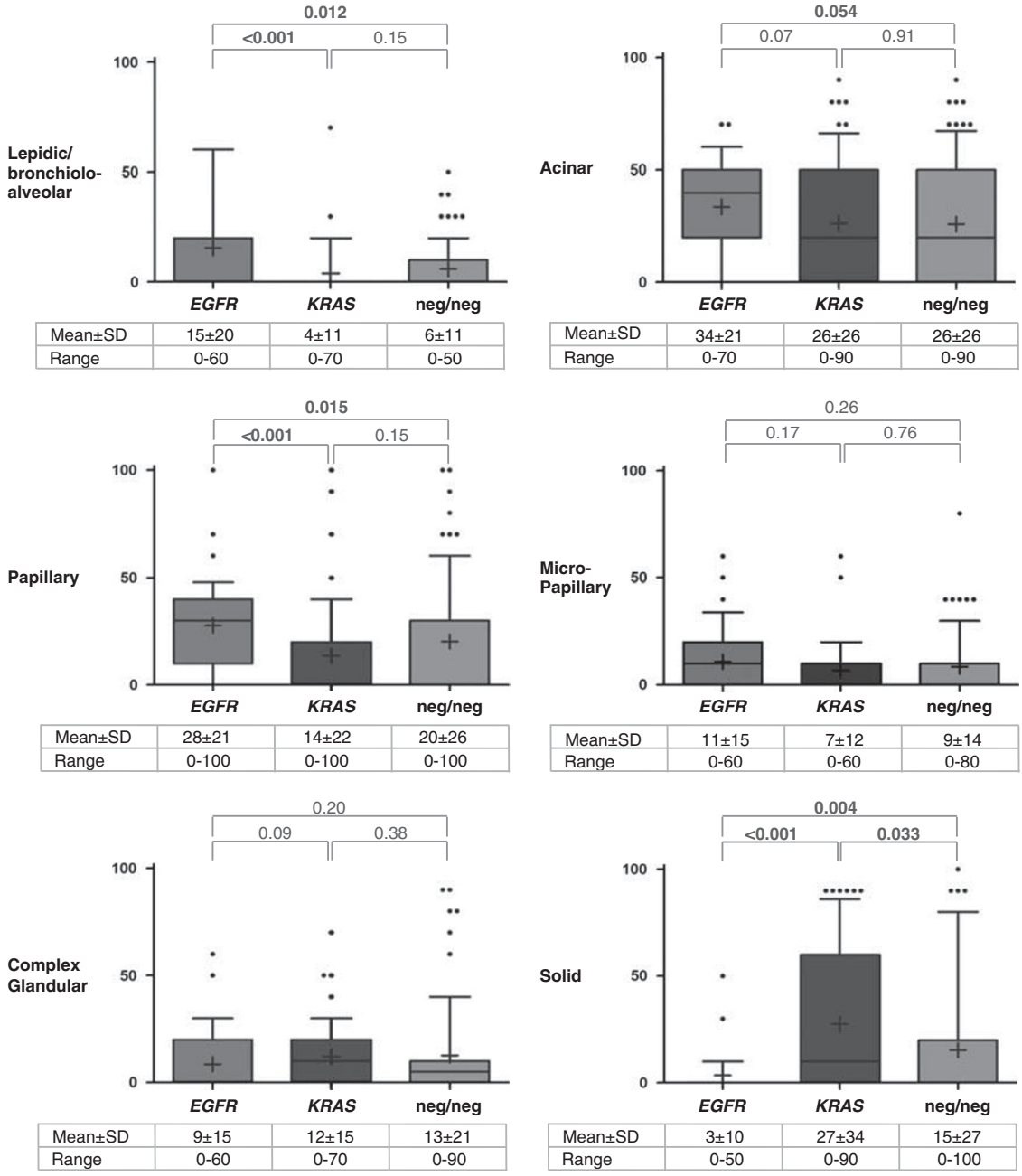


**Figure 1** Distribution of patterns according to mutation: the box plots. Y axis indicates the amount of pattern per tumor (0–100%). Upper and lower box borders = 25th and 75th percentiles, whisker = 10th and 90th percentiles, horizontal line = median, plus sign = mean, and dots = outliers. If box borders or a median are not visible, their value is 0. Bold-faced *P*-values (Mann–Whitney test) are statistically significant. Mucinous pattern was excluded from this analysis due to the overall low number of cases with this pattern.

mucinous' carcinoma subset was 67% (4/6 cases); statistical analysis of these associations was limited by the overall rarity of mucinous carcinomas in our unselected patient population. The association of *KRAS* mutations and solid pattern was a property of non-mucinous carcinomas, as solid component was rare in mucinous tumors (present in only 1/17 cases).

The patterns significantly associated with *EGFR* mutations compared with the *KRAS*+ and *KRAS*−/*EGFR*− groups were non-solid and non-mucinous patterns overall, and specifically lepidic, papillary, and, to a lesser degree, acinar (Table 3; Figure 1).

Notably, the amount of solid growth pattern had a graduated effect on the frequency of *KRAS* and *EGFR* mutations (Figure 2a). The incremental increase in the amount of solid pattern from 0→ ≥20→≥50% lead to the enrichment of *KRAS* mutations from 26→55→ 61% (2.4×), respectively, while the rate of *EGFR* mutations had a pronounced 9.3× decrease. By contrast, the amount of lepidic pattern exerted the opposite graduated effect on the

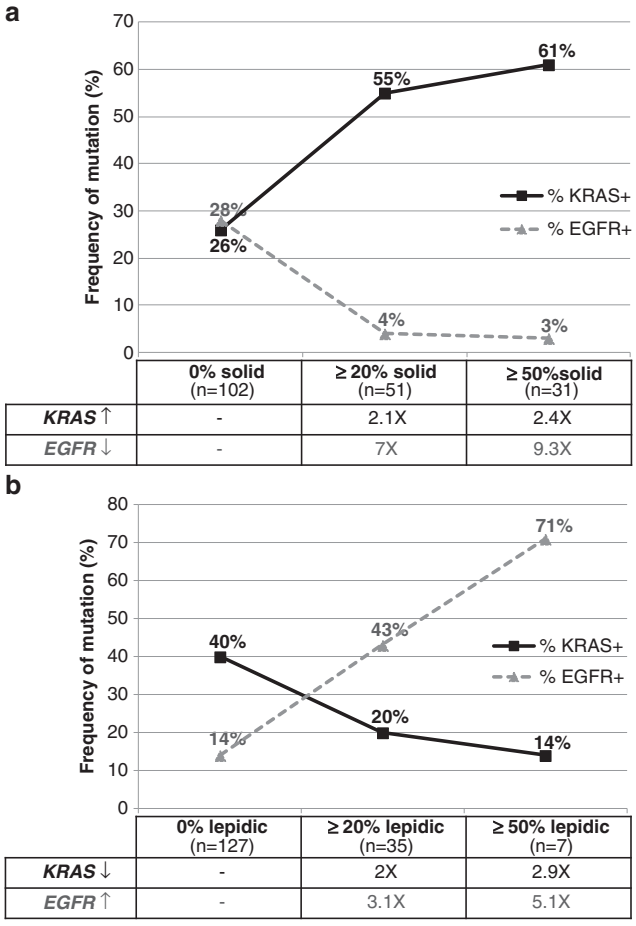


**Figure 2** Enrichment for *KRAS* versus *EGFR* mutations in tumors with increasing amount of solid (**a**) versus lepidic (**b**) patterns, respectively. The denominator for shown percentages is the number of cases with indicated amount of pattern.

likelihood of *KRAS* and *EGFR* mutations (Figure 2b). None of the other patterns showed a similar graduated effect on either *KRAS* or *EGFR* mutations.

### Association of Mutations with Other Histological Characteristics and TTF-1 Expression

In addition to the distribution of patterns, we also analyzed the association of mutations with several other histological features (Table 4). This revealed that *KRAS* mutations were significantly associated with the presence of tumor-infiltrating leukocytes: 86% of *KRAS*+ carcinomas featured moderate-marked tumor-associated inflammation compared with 66% of *EGFR*+ ($P=0.038$) and 67% of *KRAS*−/*EGFR*− ($P=0.012$) tumors. Furthermore, marked (2+) inflammation was uncommon in *EGFR*+ carcinomas relative to other groups but without reaching statistical significance. Similar to solid pattern, association of inflammation with *KRAS* mutations was only seen in non-mucinous but not in mucinous carcinomas (data not shown). *KRAS*+ carcinomas also had more necrosis and cytological atypia relative to the *EGFR*+ and *KRAS*−/*EGFR*− groups, although the differences with the *KRAS*−/*EGFR*− group were not statistically significant. Adenocarcinomas with *EGFR* mutations had less necrosis and cytological atypia than the other two molecular groups, but the strongest histological association for *EGFR* mutations relative to *KRAS*+ and *KRAS*−/*EGFR*− mutations was with hobnail cytology.

Although it is well established that adenocarcinomas with *EGFR* mutations are almost invariably TTF-1-positive, TTF-1 status in *KRAS*+ carcinomas is not well established. We therefore analyzed TTF-1 expression by immunohistochemistry in each molecular group (Table 4). Overall, 162 of 180 (90%) adenocarcinomas were positive for TTF-1. Among the molecular subgroups, 100% of *EGFR*+ carcinomas were TTF-1-positive compared with 89% (56/63) of *KRAS*+ ($P=0.048$) and 87% (71/82) of *KRAS*−/*EGFR*− ($P=0.032$) carcinomas. Notably, the lack of TTF-1 expression was rare in *KRAS*+ non-mucinous carcinomas (3/56; 5%) but was seen in the majority of *KRAS*+ mucinous carcinomas (4/7; 57%); $P=0.002$. Among non-mucinous carcinomas there was no statistical difference in the number of TTF-1-positive tumors between *KRAS*+ (53/56; 95%) and *EGFR*+ (35/35; 100%) tumors; $P=0.52$. Furthermore, the extent of TTF-1 reactivity in non-mucinous carcinomas was similar in the *KRAS*+ *vs* *EGFR*+ groups, which showed mean (range) for TTF-1 *H* scores of 258 (0–300) *vs* 281 (60–300), respectively ($P=0.29$).

Examples of histological findings in *KRAS*+ adenocarcinomas are illustrated in Figure 3.

### Association of Mutations and Patient Characteristics

Distribution of patient characteristics according to mutation is shown in Table 5. Consistent with previous studies, *EGFR* mutations were strongly associated with never-smoker status and lower pack-year smoking history. By contrast, *KRAS* mutations were associated with a greater mean pack-year smoking history than the *EGFR*+ (42 *vs* 13, $P<0.001$) and *KRAS*−/*EGFR*− (42 *vs* 35; $P=0.041$) groups. Finally, women were underrepresented in the *KRAS*−/*EGFR*− group. There were no differences in the distribution of age, tumor size, and stage. The length of the clinical follow-up was too short for survival analysis.

### Comparison between Different Types of *KRAS* and *EGFR* Mutations

We next examined whether histological associations were linked to specific types of *KRAS* and *EGFR* mutations. We thus compared tumors with *KRAS* mutations known to be smoking associated (ie, transversion mutations involving purine↔pyrimidine

**Table 4** Distribution of other histologic features and TTF-1 expression according to mutation

| | *Total* N = *180* | *Mutation* | | | P *value*[a] | | |
|---|---|---|---|---|---|---|---|
| | | *EGFR+* n = *35* | *KRAS+* n = *63* | *neg/neg* n = *82* | *KRAS vs EGFR* | *KRAS vs neg/neg* | *EGFR vs neg/neg* |
| *Necrosis: N (%)* | | | | | | | |
| 2+ | 24 (13) | 1 (3) | 11 (18) | 12 (14) | **0.004** | 0.31 | **0.042** |
| 1+ | 36 (20) | 4 (11) | 16 (25) | 16 (20) | | | |
| 0 | 120 (67) | 30 (86) | 36 (57) | 54 (66) | | | |
| *Cytological atypia: N (%)* | | | | | | | |
| 2+ | 14 (8) | 1 (3) | 7 (11) | 6 (7) | **0.009** | 0.20 | 0.14 |
| 1+ | 34 (19) | 3 (9) | 16 (25) | 15 (18) | | | |
| 0 | 132 (73) | 31 (89) | 40 (63) | 61 (74) | | | |
| *Hobnail cytology: N (%)* | | | | | | | |
| 2+ | 48 (27) | 21 (60) | 12 (19) | 15 (18) | **<0.001** | 0.29 | **<0.001** |
| 1+ | 24 (13) | 5 (14) | 11 (17) | 8 (10) | | | |
| 0 | 108 (60) | 9 (26) | 40 (64) | 59 (72) | | | |
| *Tumor-infiltrating leukocytes: N (%)* | | | | | | | |
| 2+ | 24 (13) | 2 (6) | 11 (18) | 11 (13) | **0.038** | **0.012** | 1.00 |
| 1+ | 108 (60) | 21 (60) | 43 (68) | 44 (54) | | | |
| 0 | 48 (27) | 12 (34) | 9 (14) | 27 (33) | | | |
| *TTF-1: N (%)* | | | | | | | |
| Positive | 162 (90) | 35 (100) | 56 (89) | 71 (87) | **0.048** | 0.80 | **0.032** |
| Negative | 18 (10) | 0 | 7 (11) | 11 (13) | | | |
| *TTF-1 in non-mucinous carcinomas: N (%)* | N = 163 | n = 35 | n = 56 | n = 74 | | | |
| Positive | 152 (94) | 35 (100) | 53 (95) | 64 (89) | 0.52 | 0.29 | 0.09 |
| Negative | 9 (6) | 0 | 3 (5) | 8 (11) | | | |

[a]*P*-values were analyzed for two groups—feature present (1–2+) *vs* absent (0). Bold *P*-values are statistically significant.

substitutions, $n = 47$) *vs* mutations unrelated to smoking (ie, transition mutations involving purine↔purine or pyrimidine↔pyrimidine substitutions, $n = 16$), and tumors with *EGFR* exon 19 deletions ($n = 13$) *vs* L858R mutations ($n = 21$) for the distribution of seven histological patterns, necrosis, cytological atypia, hobnail cytology, tumor-infiltrating leukocytes, and TTF-1 expression. No significant differences between molecular subgroups were identified (data not shown), but this analysis was limited by relatively small number of cases in each subgroup.

## Discussion

In the present study, by combining detailed histopathological analysis with high-sensitivity mutation detection method, we identified a novel association of *KRAS* mutations with solid growth pattern and tumor-infiltrating leukocytes in non-mucinous lung adenocarcinomas. In addition, we expanded on several previously described histological and clinical associations of *KRAS* and *EGFR* mutations.

Although this is the first study to identify a propensity of *KRAS+* lung adenocarcinomas for solid growth pattern, several previous studies did hint at this association. First, several studies showed an association of *KRAS* mutations with poor differentiation.[22–24] Although there is currently no standardized grading system for lung adenocarcinomas, solid growth pattern is the central parameter in grading of adenocarcinomas system-wide, and it is likely that the presence of solid growth pattern, at least in part, explains the association of *KRAS* mutations and poor differentiation in those studies. Furthermore, an association of *KRAS* mutations with a gene expression profile correlating with solid histology was noted in a study by Motoi *et al*.[21] Lastly, an association of *KRAS* mutations and 'tumor islands', which, in turn, were associated with solid growth pattern, was recently reported by Onozato *et al*.[31]

Three potential factors could have contributed to the differences in the reported histological associations of *KRAS* mutations in lung adenocarcinomas across studies:

(1) One potential factor is under-detection of *KRAS* mutations by assays with suboptimal sensitivity, such as Sanger sequencing. The relevance of method sensitivity is particularly supported by our finding that lung carcinomas harboring *KRAS* mutations are enriched with inflammatory cells. Standard macrodissection of such tumors may fail to enrich for tumor cells due to their intimate association with inflammation, and consequently extracted DNA may be diluted by DNA contributed by inflammatory cells.

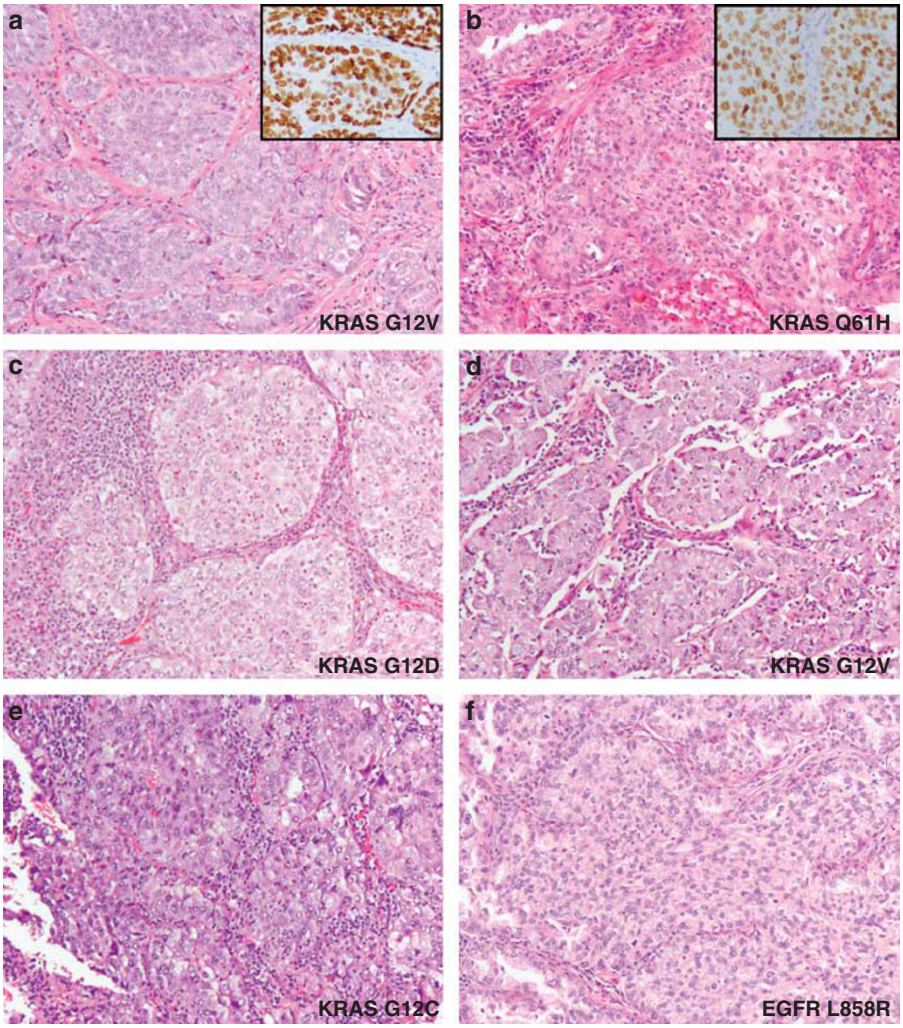


**Figure 3** Examples of solid component in adenocarcinomas with *KRAS* mutations. At least focal (≥20%) solid component was present in 55% of *KRAS*+ carcinomas (**a–e**) compared with 4% of *EGFR*+ carcinomas (**f**). These solid areas, some of which have 'squamoid' appearance, were distinguished from a true squamous component by immunohistochemistry for TTF-1 (inset in **a** and **b**) and/or negative p40/ΔNp63 (not shown). Images also illustrate a spectrum of cytological atypia from minimal (**a**, **b**) to moderate (**c**, **d**) to marked (**e**), and spectrum of tumor-associated leukocytes from minimal (**a**, **b**) to moderate (**d**, **e**) to marked (**c**) in *KRAS*+ carcinomas.

Thus, *KRAS*-mutated carcinomas may be particularly prone to false-negative results by standard Sanger sequencing, which has a notoriously low analytical sensitivity, requiring high tumor cell content (40–50%).[30] By contrast, Sequenom platform, used in this study, requires ~10% tumor cell content.[30] The possibility that *KRAS* mutations may be under-detected by Sanger sequencing is indirectly supported by the data in colorectal carcinomas, where in a matched comparison, Sanger sequencing was found to under-estimate the frequency of exon 2 *KRAS* mutations by 9% compared with more sensitive methods.[30]

(2) The second factor potentially contributing to the variability in molecular/histological correlation results in individual studies could be the variation in the designation of histological patterns. This is illustrated in a recent inter-observer reproducibility study, which showed significant variability in designation of histological patterns in lung adenocarcinomas among pathologists.[32] Although solid pattern showed one of the highest concordances, a potential source of variability comes from the lack of agreement on the designation of complex glandular patterns (such as cribriform), which are currently variably classified as acinar or solid.[32] These patterns were annotated as a distinct category in this study, and, while over-represented in *KRAS*+ carcinomas, they did not reach a statistical association with any molecular group. Another potential confounder is recently recommended classification based solely on a single histological pattern, judged to be predominant relative to other patterns,[16] which we found to be difficult to assign objectively in a fair number (29%) of cases due to ≥2 patterns being present

**Table 5** Distribution of clinicopathological features according to mutation

| | *Total* N = *180* | *Mutation* | | | P-*value* | | |
|---|---|---|---|---|---|---|---|
| | | *EGFR+* N = *35* | *KRAS+* N = *63* | *neg/neg* N = *82* | *KRAS+ vs EGFR+* | *KRAS+ vs neg/neg* | *EGFR+ vs neg/neg* |
| Age: mean (range), years | 66 (38–87) | 65 (51–80) | 66 (38–85) | 67 (34–87) | 0.68 | 0.38 | 0.29 |
| *Gender: N (%)* | | | | | | | |
| Female | 107 (59) | 27 (77) | 41 (65) | 39 (48) | 0.26 | **0.044** | **0.004** |
| Male | 73 (41) | 8 (23) | 22 (35) | 43 (52) | | | |
| *Smoking status: N (%)* | | | | | | | |
| Never | 31 (17) | 17 (49) | 4 (6) | 10 (12) | <**0.001** | 0.27 | <**0.001** |
| Current/former | 149 (83) | 18 (51) | 59 (64) | 72 (88) | | | |
| Smoking pack years: mean (range) | 33 (0–200) | 13 (0–60) | 42 (0–120) | 35 (0–200) | <**0.001** | **0.041** | <**0.001** |
| Tumor size, cm: mean (range) | 2.3 (0–9.4) | 2 (0.7–5) | 2.3 (0.5–7.8) | 2.5 (0.6–9.4) | 0.29 | 0.32 | 0.08 |
| *Stage: N (%)* | | | | | | | |
| I | 121 (67) | 26 (74) | 44 (70) | 51 (62) | 0.82 | 0.38 | 0.29 |
| II–IV | 59 (33) | 9 (26) | 19 (30) | 31 (38) | | | |

Bold *P*-values are statistically significant.

in a similar co-dominant amount. In addition, this annotation may exclude tumors in which a pattern is present in a minor amount but is still biologically significant, as illustrated by our finding that both solid and lepidic patterns have a significant effect on the frequency of *KRAS* and *EGFR* mutations even when present as a minor component of a tumor.

(3) Lastly, genotype/phenotype associations could be influenced by ethnic factors. In particular, a potential confounding factor is still a largely unexplained significant difference in the rate of *KRAS* (and *EGFR*) mutations in lung adenocarcinomas between western and East Asian populations. Specifically, the baseline rate of *KRAS* mutations is low (5-10%) in East Asian populations, with a substantial (40–60%) proportion of mutations concentrated in mucinous carcinomas.[19,33] By contrast, *KRAS* mutations occur in 25–35% of lung adenocarcinomas in western patients, with the majority (89% in this series) of mutations occurring in non-mucinous carcinomas. Thus, both the frequency and histologic correlates of *KRAS* mutations in non-mucinous adenocarcinomas may have geographic differences.

Overall, *KRAS* mutations appear to have a dual histological association in lung adenocarcinomas—one with non-mucinous carcinomas with a solid component, which we found to have *KRAS* mutations in 55% of cases, and the other with mucinous carcinomas formerly designated mucinous bronchioloalveolar carcinoma[25] ('invasive mucinous adenocarcinoma'[16]), which are reported to harbor *KRAS* mutations in 30% to >80% of cases.[17–20] In this study, *KRAS* mutations were also over-represented in the latter tumors, occurring in 67% (4/6) of cases; although statistical analysis of this association was limited by overall rarity of this tumor type in our unselected patient population. In addition to the dual role of *KRAS* in invasive adenocarcinomas, *KRAS* mutations have also been reported to be paradoxically over-represented in pre-invasive glandular lesions - pure bronchioloalveolar carcinomas/adenocarcinomas *in situ*;[34] these lesions were excluded from the present study to focus the analysis on conventional invasive adenocarcinomas. From the perspective of lung cancer pathogenesis, these pleotropic histological associations may hint at the complex role of *KRAS* mutations in stem cells. One hypothesis is that *KRAS* mutations may arise in distinct stem cells, giving rise to neoplasms with divergent histology. Alternatively, *KRAS* mutations may arise in a common pleuripotent stem cell with a broad differentiation potential. These possibilities are in line with pre-clinical data that *KRAS*-mediated tumorigenesis is significantly influenced by the cellular context.[35]

Our finding that *KRAS* mutations are associated with solid histology and tendency for greater necrosis and cytological atypia may represent the underlying link between *KRAS+* genotype and aggressive clinical behavior in lung adenocarcinomas. Several recent studies have demonstrated that solid growth pattern is a strong predictor of adverse clinical outcome, whereas lepidic pattern—associated with *EGFR* mutations—is a predictor of indolent behavior in lung adenocarcinomas.[26,36] Thus, the distinct association of *KRAS* and *EGFR* mutations with aggressive *vs* indolent histologies, respectively, parallels the differences in prognosis. Because the follow-up available for patients in this series was too short for survival analysis, future studies with survival data and multivariate analysis will be needed to determine whether indeed *KRAS*

and *EGFR* mutations exert their prognostic effects via a link to distinct histological subsets or whether these effects are histology-independent.

Of interest, the association of *KRAS* mutations and solid histology in lung adenocarcinomas ties in with our recent description of a high frequency (40%) of *KRAS* mutations in large cell (undifferentiated) carcinomas showing glandular immunophenotype.[37] We proposed that these clinically aggressive tumors represent a spectrum of adenocarcinomas with an extreme amount of solid growth pattern. The high frequency of *KRAS* mutations in conventional adenocarcinomas with partial solid histology reported in this study is in line with that proposal, as is the low-frequency of *EGFR* mutations seen in both adenocarcinomas with solid component and large cell carcinomas with glandular immunoprofile. The propensity for solid growth/poor differentiation of *KRAS*-mutant tumors is also consistent with the finding of a high rate (38%) of *KRAS* mutations in sarcomatoid/pleomorphic lung carcinomas.[38]

The finding that *KRAS*-mutated carcinomas are associated with tumor-infiltrating leukocytes, in addition to representing a potential confounder in molecular testing, may itself have biological and clinical significance. Presence of inflammatory cells has been implicated as both favorable and unfavorable prognostic indicator in several malignances, consistent with the capacity of immunity to exert both anti-tumor and pro-tumor effects depending on both tumor and host factors.[39,40] Non-small cell lung carcinomas are frequently associated with prominent tumor-infiltrating lymphocytes and other inflammatory cells, but their significance remains controversial. Both adverse[41,42] and favorable[43] prognostic effects having been reported, which may be related to different subsets of inflammatory cells, scoring criteria, and patient populations.[44] In this study, only the overall extent of inflammatory infiltrate was analyzed, and further study will be needed to evaluate specific leukocyte subsets. Although association of inflammation and *KRAS* mutations is a novel observation, Dacic *et al*[20] noted that high level of tumor-infiltrating lymphocytes is uncommon in adenocarcinomas with *EGFR* mutations; this trend for pan-inflammatory infiltrate was also seen in the present study. We cannot exclude that the degree of inflammation in adenocarcinomas with *KRAS vs EGFR* mutations reflects tissue response to tumors with more *vs* less aggressive histology, respectively. Nevertheless, these data raise the possibility that patients with *KRAS*-mutated lung adenocarcinomas may be an especially attractive subset for clinical trials of immunomodulatory agents aimed at enhancing the anti-tumor activity of tumor-infiltrating lymphocytes, such as therapeutic antibodies to PD-1 and PD-L1.[45,46]

In addition to describing novel histological associations of *KRAS* mutations, this study also expanded on the previously recognized histological and clinical association of *EGFR* mutations. Previous studies consistently reported that *EGFR* mutations are associated with non-mucinous non-solid histology, as also seen in this study. The association of *EGFR* mutations with individual histological patterns has significant variability in the literature, and includes lepidic, papillary, micropapillary, and in some studies acinar.[16] In this study, individual patterns associated with *EGFR* mutations were lepidic, papillary, and, to a lesser degree, acinar. However, the strongest association of *EGFR* mutations was with hobnail cytological features, which were typically seen in carcinomas with lepidic component and/or tumors with characteristic serrated intra-glandular infoldings, which could be variably described as displaying papillary/micropapillary/acinar patterns (data not shown). Hobnail cytology is proposed as a defining feature of terminal respiratory unit-type adenocarcinomas,[27] and it is possible that several architectural patterns emerging as associated with *EGFR* mutations in individual studies represent variable annotation of architectural manifestations of this type of adenocarcinoma.

It has been suggested that TTF-1 expression represents a feature of terminal respiratory unit-type adenocarcinomas and that while *EGFR*+ adenocarcinomas are uniformly TTF-1-positive, carcinomas with *KRAS* mutations tend to be TTF-1-negative.[47] Here we clarify that the lack of TTF-1 expression in *KRAS*+ carcinomas applies primarily to mucinous carcinomas, of which 57% in this study were TTF-1-negative, whereas the lack of TTF-1 expression in *KRAS*+ non-mucinous carcinomas is rare (5% in this series). Similarly, we clarify that despite propensity for solid growth, a subset of *KRAS*+ non-mucinous carcinomas displays hobnail cytological features in better differentiated areas, suggesting that these tumors do not always belong to a non-terminal respiratory unit lineage, consistent with previous observations.[48]

A notable observation in this study is that *KRAS*+ adenocarcinomas have a greater propensity for solid growth pattern compared not only with *EGFR*+ but also with *KRAS*−/*EGFR*− carcinomas. It is worth noting, however, that *KRAS*−/*EGFR*− is not a molecularly homogenous group but rather a mixture of carcinomas with various low-frequency molecular alterations, including *ALK*, *BRAF*, *HER2*, *ROS1*, and *RET* (frequency of each ranging from <1% to 5%), as well as tumors with yet unidentified molecular events.[49,50] Despite its heterogeneous nature, the pooled clinical outcome for this group was found to be favorable compared with *KRAS*+ tumors and inferior compared with *EGFR*+ tumors in clinical studies,[11,12] which parallels the different propensities of these groups for solid growth pattern identified in this study, although at least some molecular subsets within *KRAS*−/*EGFR*− group—namely *ALK*-rearranged carcinomas—are also known to also show a propensity for solid histology (in addition to

the classic association with signet ring cells)[51] and aggressive behavior.[52] Greater pack-year smoking history in patient with *KRAS*+ carcinomas compared with both the *EGFR*+ as well as with *KRAS*−/*EGFR*− group in this series is in line with a link to never-smokers of the *EGFR*+ group and several known molecular subsets within the *KRAS*−/*EGFR*− group, including *ALK*, *ROS1*, and *RET*.

From a practical perspective on predictive molecular testing, our data support previous conclusion that while both *EGFR* and *KRAS* mutations are associated with propensities for distinct histological and clinicopathological characteristics, none of these associations have sufficient predictive value to allow triage of cases for molecular studies, and therefore all lung adenocarcinomas should undergo molecular testing irrespective of histological and clinical features (with possible exception being the exclusion of mucinous carcinomas from testing for *EGFR* mutations).[53] On the other hand, estimation of pre-test probability of mutations may have value in some clinical settings. For such situations, nomograms, based on clinical +/− histological features, have been recently developed to predict the likelihood of *EGFR* mutations.[54,55] The findings in this study, particularly the predictive effect of solid histology on the likelihood of *KRAS* and *EGFR* mutations, may be of value for refinement of such nomograms. Although *KRAS* mutations have thus far evaded therapeutic targeting, and the current value of testing for these mutations in lung carcinomas is to serve as negative predictors for other targetable mutations, it is hoped that effective targeted therapies for mutant KRAS will emerge in the near future.[56]

In summary, we have described here a novel association of *KRAS* mutations with propensity for solid histology in non-mucinous lung adenocarcinomas, which may explain the adverse clinical outcome portended by *KRAS* mutations. We also describe an association of *KRAS* mutations with tumor-infiltrating leukocytes, which raises the possibility that patients with *KRAS*-mutated adenocarcinomas may benefit from novel immunomodulatory agents.

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## Disclosure/conflict of interest

The authors declare no conflict of interest.

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