Genetic features of pulmonary adenocarcinoma presenting with ground-glass nodules: the differences between nodules with and without growth

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Background: Pulmonary ground-glass nodules (GGNs) include both malignant and benign lesions. Some GGNs become larger, whereas others remain unchanged for years. We have previously reported that smoking history and large diameters are predictors for growth. However, the genetic differences among GGNs remain unclear.

Patients and methods: GGNs with ground-glass component of ≥50% on a thin-section computed tomography scan that were resected between 2012 and 2014 were evaluated for clinicopathological features and the presence of EGFR/KRAS/ALK/HER2 mutations. ‘Incidence of 2-mm growth’ and ‘Time to 2-mm growth’ were analyzed according to the mutational status.

Results: Among 104 GGNs in 96 patients, this study included 3 atypical adenomatous hyperplasia (AAH), 19 adenocarcinoma in situ (AIS), 27 minimally invasive adenocarcinoma (MIA), and 55 invasive adenocarcinoma (IA). Among the 71 lesions evaluable for growth, 30 GGNs exhibited growth and 5 lesions remained unchanged for ≥2 years before surgery was carried out. We identified mutations or rearrangements in 75% of GGNs (78/104). EGFR mutations were noted in 64% of samples, KRAS in 4%, ALK in 3%, and HER2 in 4%. The remaining 26 quadruple-negative tumors were significantly associated with AAH/AIS (P < 0.01) and no-growth (P < 0.01) compared with driver mutation-positive tumors, whereas EGFR mutation-positive tumors were correlated with MIA/AI (P < 0.01) and growth (P < 0.01) compared with EGFR-negative tumors.

Conclusions: Three fourths of resected GGNs were positive for EGFR, KRAS, ALK, or HER2 mutations. Quadruple-negative tumors were associated with a lack of GGN growth, whereas EGFR mutation-positive tumors displayed a correlation with growth.

Key words: ALK, EGFR, ground-glass opacity, HER2, KRAS, lung cancer

introduction

Pulmonary ground-glass nodules (GGNs), hazy lesions on computed tomography (CT) scans that do not obscure underlying bronchial structures or pulmonary vessels [1], are increasingly detected in clinical practice. These lesions include both malignant and benign lesions: lung adenocarcinoma and their preinvasive lesions, focal interstitial fibrosis, inflammation, or hemorrhage [2].

A ground-glass opacity (GGO) proportion of ≥50% has been suggested as a cutoff value for pathological noninvasiveness; the rate of lymph node metastasis ranges from 21% to 26% in ≤3-cm lesions with GGO component of <50% [3–5]. Moreover, the specificities for the diagnosis of pathological noninvasiveness are 96.4% and 98.7% for lesions ≤3 cm with >50% GGO component and lesions ≤2 cm with >75% GGO component,

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respectively [6]. Based on a sample of pulmonary nodules ≤3 cm with ≥50% GGO component, we previously reported that some GGNs exhibit gradual growth, whereas others remain unchanged for years, and that 3-year follow-up is a reasonable benchmark to distinguish these lesions [7]. Approximately 20% of pure GGNs and 40% of mixed GGNs gradually grow or increase their solid component in our recent review article summarizing four reports [8]. Additionally, we demonstrated that smoking and larger diameter were predictors of GGN growth [9]. However, the genetic differences among GGNs remain unclear.

This study aimed to determine the frequency of oncogenic driver mutations in resected GGNs and clarify the association between GGN growth and driver mutations.

**methods**

**patient cohort**

GGNs with ground-glass component of ≥50% that were resected between January 2012 and March 2014 were retrospectively included in this study. Candidates were extracted from a database of surgery patients at Aichi Cancer Center. Radiologic and clinicopathologic features, including the presence of pathological invasion and genetic features, were reviewed. Appropriate approval was obtained from the institutional review committee in addition to written informed consent from the patients.

**radiological evaluation of the lesions**

First, we evaluated CT images carried out within 4 weeks before the surgery. At least one CT imaging study was carried out on each patient before surgery to confirm the presence of a lesion, which excludes patently benign lesions. The maximum diameters of whole GGO lesions and solid component on lung windows were measured, and the ratio was calculated using these linear diameters.

Next, all CT images with 1- or 2-mm thicknesses, if they were available before the surgery, were retrospectively evaluated to analyze changes in size. Intervals between CT examinations, ranging from 1 to 12 months, were determined at the physician’s discretion. Based on the data that an increase in the diameter of >1.72 mm would be necessary to describe growth considering measurement errors [10], we defined growth as ≥2 mm increase in diameter or a solid component in accordance with previous reports on GGNs [9, 11, 12]. Images were acquired using a multidetector CT with the previously reported window settings [9].

**pathological diagnosis and genetic analysis**

The pathological diagnosis was classified according to the International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society International Multidisciplinary Classification of Lung Adenocarcinoma [13].

Invasive portions of individual tumors were examined for molecular analyses, though lack of heterogeneity of driver mutations has been reported by us [14]. Mutation analyses were carried out for epidermal growth factor receptor (EGFR) mutations, Kirsten rat sarcoma (KRAS) mutations, anaplastic lymphoma kinase (ALK) rearrangements, and human epidermal growth factor receptor type 2 (HER2) mutations. Reverse transcriptase-polymerase chain reaction (RT-PCR) direct sequencing of EGFR, KRAS, ALK, and HER2 was used to assess the mutational status if frozen tissues were available. In the case that paraffin-embedded tissues were only available, the mutational status was determined using cycleave methods for codon 719, 858, and 861 of EGFR and codon 12 of KRAS and fragment analyses for exon 19 and 20 of EGFR and exon 20 of HER2 [15]. ALK rearrangement was screened with immunohistochemistry (IHC) in addition to RT-PCR if the RNA could be accessed. When the IHC results were negative and RNA was not available, break-apart fluorescence in situ hybridization (FISH) was used for confirmation as previously reported [16].

Mutation analyses for EGFR and KRAS were carried out in all lesions. Based on the evidence that these four major mutations are mutually exclusive [17–19], ALK and HER2 analyses were carried out in selected lesions so that all 104 lesions were categorized into EGFR, KRAS, ALK, HER2, or quadruple-negative.

**statistical analysis**

Clinical characteristics of mutation-positive tumors were compared with quadruple-negative tumors. Differences were analyzed using the χ² test or Fisher’s exact test as appropriate. To investigate the influence of the most predominant specific mutation on the differences in clinical characteristics, we subsequently compared the characteristics of the most predominant specific mutation-positive tumor with the characteristics of the remaining tumors.

In the analyses of the association between GGN growth and driver mutations, lesions for which 1- or 2-mm thicknesses CT imaging study was carried out more than once were included. First, ‘Incidence of 2-mm growth’ was compared according to the mutational status using the definition of ‘no-growth’ for GGNs that remained unchanged for ≥2 years. Next, ‘Time to 2-mm growth’ curves were estimated using the Kaplan–Meier method, and the differences were compared using the log-rank test. In this analysis, all GGNs that remained unchanged were censored based on the follow-up period.

All statistical analyses were carried out with JMP version 11.1.1 (SAS Institute, Inc., Cary, NC, USA). Differences were considered statistically significant at a two-sided P-value of <0.05.

**results**

**baseline characteristics of patients and pulmonary lesions**

We identified 104 GGNs in 96 patients that fulfilled the inclusion criteria. These lesions were initially detected from a variety...
of clinical situations: routine screening in 56, examination for unrelated extrapulmonary diseases in 45, and preoperative CT examination of an unrelated pulmonary lesion in 3. The median age was 63 years old (range, 32–84 years), and approximately two thirds of the patients were women and never smokers. Among the 96 patients, 91 exhibited solitary lesions, 3 had 2 lesions, and the remaining 2 patients had 3 and 4 lesions, respectively. At the time of surgery, the median lesion size on CT was 1.8 cm (range, 0.6–4.4 cm), and ~80% of the lesions were mixed GGN.

pathologic diagnoses and driver oncogene mutations in 104 lesions

Ninety-one patients with a single lesion underwent the following surgeries: lobectomies in 49, segmentectomies in 26, and wedge resections in 16. Two of three patients with two lesions underwent wedge resections, and the remaining patient underwent lobectomy. A patient with three lesions underwent wedge resection and segmentectomy as well as subsequent second wedge resection. A patient with four lesions underwent wedge resection twice. Lymph node dissection was carried out in 63 patients. This study included 3 atypical adenomatous hyperplasia (AAH), 19 adenocarcinoma in situ (AIS), 27 minimally invasive adenocarcinoma (MIA), and 55 invasive adenocarcinoma (IA). All patients were diagnosed with pathological stage 0 or I tumors.

In the mutation analyses, the majority of tumors exhibited EGFR mutations (Figure 1). All of the four KRAS-positive tumors were G12C-positive. ALK was positive in 3 of 80 lesions. Two lesions were positive by both IHC and RT-PCR, and the remaining lesion was positive by both IHC and FISH. HER2 was positive in 4 of 63 lesions; 3 of the lesions harbored 12-bp insertion in exon 20, and the remaining lesion harbored 3-bp insertion in exon 20. Of note, gene analyses were successfully carried out even in small lesions and pure GGNs; 7 of 10 GGNs ≤1 cm and 14 of 22 pure GGNs were positive for at least 1 of the 4 mutations (Table 1).

Quadruple-negative tumors were significantly associated with AAH/AIS compared with driver mutation-positive tumors, whereas EGFR mutation-positive tumors were correlated with MIA/IA compared with EGFR-negative tumors (Table 1).

| Table 1. The association between clinical characteristics and driver mutations in 104 lesions |
|---------------------------------------------|----------------|----------------|----------------|----------------|----------------|
| Characteristics | EGFR (N = 67) | KRAS (N = 4) | ALK (N = 3) | HER2 (N = 4) | Mut− (N = 26) |
| Age (years)       |                |                |                |                |                |
| ≤60              | 23             | 0              | 3              | 4              | 12             |
| >60              | 44             | 4              | 0              | 0              | 14             |
| Gender           |                |                |                |                |                |
| Female           | 39             | 2              | 2              | 3              | 16             |
| Male             | 28             | 2              | 1              | 1              | 10             |
| Smoking history  |                |                |                |                |                |
| Yes              | 27             | 3              | 0              | 1              | 15             |
| No               | 40             | 1              | 3              | 3              | 11             |
| Lesion diameter (cm) |            |                |                |                |                |
| ≤1               | 7              | 0              | 0              | 0              | 3              |
| 1.1–2            | 32             | 3              | 3              | 4              | 15             |
| ≥2              | 28             | 1              | 0              | 0              | 8              |
| Solid component (%) |              |                |                |                |                |
| 0                | 13             | 0              | 0              | 1              | 8              |
| 1–25             | 8              | 1              | 1              | 1              | 6              |
| 26<50            | 46             | 3              | 2              | 2              | 12             |
| Histologic type  |                |                |                |                |                |
| AAH              | 1              | 0              | 0              | 0              | 2              |
| AIS              | 9              | 0              | 0              | 0              | 10             |
| MIA              | 18             | 2              | 0              | 4              | 3              |
| IA               | 39             | 2              | 3              | 0              | 11             |
| Growth change    |                |                |                |                |                |
| Increased        | 27             | 0              | 1              | 1              | 1              |
| No changes for ≥2 years | 1          | 0              | 0              | 0              | 4              |
| Not evaluable    | 39             | 4              | 2              | 3              | 21             |

*Statistical analyses were carried out for AAH/AIS versus MIA/IA.
*Statistical analyses were carried out except for lesions that were not evaluable.

A AH, atypical adenomatous hyperplasia; AIS, adenocarcinoma in situ; ALK, anaplastic lymphoma kinase; EGFR, epidermal growth factor receptor; HER2, human epidermal growth factor receptor type 2; KRAS, Kirsten rat sarcoma; IA, invasive adenocarcinoma; MIA, minimally invasive adenocarcinoma; Mut+, mutation positive; Mut−, mutation negative.
the association between GGN growth and driver mutations

Before the surgery, thin-section CT was carried out more than once on 71 lesions, and 30 lesions exhibited growth. Among the remaining 41 GGNs, 5 lesions were followed for ≥2 years, and these lesions were included in the no-growth group (Figure 2). As a result, 35 GGNs were analyzed for the ‘Incidence of 2-mm growth’. Quadruple-negative tumors were significantly associated with no growth (growth +/−; 1/4) compared with driver mutation-positive tumors (growth +/−; 29/1) (P<0.01). Additionally, EGFR mutation-positive tumors were correlated with growth (growth +/−; 27/1) compared with EGFR mutation-negative tumors (growth +/−; 3/4) (P<0.01).

‘Time to 2-mm growth’ curves were shown in supplementary Figure S1, available at Annals of Oncology online, and the results concurred with those of the above analyses for 2-mm growth incidence.

discussion

In this study, we found that quadruple-negative tumors were associated with no GGN growth, whereas EGFR mutation-positive tumors demonstrated a correlation with GGN growth. To the best of our knowledge, this is the first study to comprehensively investigate the genetic features of GGNs in terms of targetable or potentially targetable driver mutations including EGFR, KRAS, ALK, and HER2.

The following factors have been reported as predictors for the growth of GGNs: lesion diameter [11, 12], past history of lung cancer [11, 12], and smoking history [9]. Our study demonstrated a novel relationship between the quadruple-negative status and no GGN growth. This finding was also supported by the result that quadruple-negative status was associated with pathological noninvasiveness because lung adenocarcinomas are generally thought to follow a linear multistep progression. Furthermore, these data were obtained by the high-quality gene analyses; approximately half of the tumors were diagnosed as harboring mutations even in AAH and AIS. Lesions in the ‘no-growth’ group might grow during a sufficient follow-up period because 3-year follow-up period is a reasonable benchmark to accurately evaluate growth [7]. However, in clinical practice, it is not acceptable to follow all GGNs for 3 years before surgery due to the potential of malignancy, especially in large GGNs with a solid component. According to the guidelines of the American College of Chest Physicians, the consideration of surgical resection is recommended for GGNs that meet any of the following conditions: any GGN with growth or the development of solid components, pure GGNs >10 mm with confirmed persistence, mixed (ground-glass >50%) GGNs ≥8 mm with confirmed persistence, or mixed GGNs ≥15 mm without follow-up [20]. The Fleischner Society recommends the consideration of surgical resection in GGNs with a solid component ≥5 mm with confirmed persistence [21]. We basically carried out surgical resection for any GGNs with growth or the development of solid components, and GGNs ≥15 mm or solid component ≥5 mm. Regarding these clinical situations, we excluded GGNs without growth that had been observed for <2 years from the growth analyses.

In the subsequent analysis used to investigate the influence of a specific mutation on the differences, EGFR mutations were significantly associated with growth. This result concurs with Yatabe’s hypothesis for the progression of lung adenocarcinoma that EGFR-mutated AAH develops a linear progression schema, whereby AAH progresses to AIS and is followed by IA [22]. Interestingly, he also suggested that KRAS-mutated AAH rarely progresses to more advanced tumors based on the frequencies of these mutations in adenocarcinomas. EGFR-mutated tumors were evenly distributed along each progression step, whereas the incidences of KRAS mutations were 33%, 12%, 8%, and 0% in AAH, AIS, MIA, and well-differentiated adenocarcinomas, respectively [23]. The overall frequency of KRAS mutations in lung adenocarcinoma was limited to 13% [24]. These findings cannot be explained without assuming that some tumors with KRAS mutations might undergo regression. One possible mechanism may be associated with dual role of KRAS. Oncogenic Ras has been shown to cause senescence through the activation of the p53–p21 WAF and p16INK4A–retinoblastoma tumor-suppressor pathways [25, 26]. Therefore, linear progression with unlimited proliferation might represent the subset. In the present study, GGNs with KRAS mutations certainly exhibited no growth (Figure 2).

The demands for appropriate management of GGNs must increase with the prevalence of CT screening. The National Lung Screening Trial (NLST) revealed 20% decrease in lung cancer mortality as a result of low-dose CT screening for patients at high risk of developing lung cancer [27]. Moreover, the Dutch-Belgian randomized lung cancer screening trial (NELSON) is ongoing [28]. These situations are anticipated to assess the usefulness of CT screening for not only smokers but also nonsmokers, thereby likely resulting in increased identification of GGNs. However, >18% of all lung cancers detected by low-dose CT in the NLST appear to be indolent and over diagnosed [29]. Additionally, long-term follow-up results in cumulative radiation exposure. Based on the low-dose CT protocol by NLST, the cumulative dose of a 20-year follow-up was estimated to
exceed those of nuclear workers and atomic bomb survivors [30]. Currently, no evidence is available to suggest when to cease GGN follow-up. Our results potentially suggest that an early genetic diagnosis of GGN could predict growth potential and assist in clinical decision making. It is reasonable to prolong the intervals between CT examinations for mutation-negative GGNs, which would contribute to reducing cumulative radiation exposure. In contrast, mutation-positive GGNs should be carefully followed or surgically resected given the growth potential. To access mutation status of the tumors, CT guided biopsy or transbronchial biopsy is commonly used, and the possible genetic heterogeneity of the tumors and adverse events from invasive biopsies may be concerned. However, heterogeneous distribution of driver mutation is revealed to be extremely rare, thus molecular testing guideline stated that testing of multiple different areas within a single tumor is not necessary [31]. Further development of noninvasive and highly sensitive methods, such as genetic analysis from plasma DNA, is warranted for clinical application.

There are several limitations in our study. First, further gene analyses for the quadruple-negative group would detect minor mutations. Analyses of 10 genes detected mutations other than the major four genes in 4% of tumors, including BRAF in 2%, PIK3CA in 0.8%, MET amplification in 0.7%, NRAS in 0.7%, MEK1 in 0.1%, and NRAS in 0% [17]. Similarly, analyses of seven genes revealed 3.3% of adenocarcinoma harbored other genes, including RET in 2%, ROS1 in 1%, and BRAF in 0.3% [32]. According to these data, approximately 3–4% of GGNs in our quadruple-negative group would be positive for these minor mutations, although these minorities minimally influence the results of statistical analyses. Second, our study was retrospectively conducted at a single institution in an Asian country. The high frequency of EGFR mutations and the low frequency of KRAS mutations in the present study appear to be influenced by ethnic features, gender, and smoking status (supplementary Figure S2, available at Annals of Oncology online). Third, the no-growth group should be defined more strictly with a sufficient follow-up period in a larger cohort. Fourth, measurement error should be considered although objects for growth analyses were limited to thin-section CT images to minimize measurement error.

In conclusion, three fourths of resected GGNs were positive for EGFR, KRAS, ALK, or HER2 mutations. Quadruple-negative tumors were associated with a lack of GGN growth, whereas EGFR mutation-positive tumors were correlated with growth. These results provide insight into the genetic features of GGNs and help establish reasonable follow-up algorithms to reduce radiation exposure and avoid unnecessary surgeries.

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disclosure
The authors have declared no conflicts of interest.
Small-cell lung cancer detection in never-smokers: clinical characteristics and multigene mutation profiling using targeted next-generation sequencing

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Background: Once regarded as a smoker’s disease, small-cell lung cancer (SCLC) has been occasionally detected in never-smokers as smoking rates decrease worldwide. We investigated the clinical and genetic characteristics of SCLC in never-smokers.

Patients and methods: Patients diagnosed with SCLC were grouped into smokers and never-smokers. The clinical outcomes of the two groups were compared. For SCLC in never-smokers, somatic mutation profiling was carried out using the AmpliSeq™ Cancer Hotspot Panel v2 and semiconductor sequencing technology. Epidermal growth factor receptor (EGFR) mutation was confirmed by PNAClamp™.

Results: In total, 391 SCLC patients treated over a 5-year period were analyzed. Fifty patients (13%) were never-smokers. The median overall survival was 18.2 months in never-smokers and 13.1 months in smokers (P = 0.054). Never-smoking history was independently a good prognostic factor [hazard ratio = 0.645, 95% confidence interval (CI) 0.456–0.914], as were limited disease (HR = 0.372, 95% CI 0.294–0.471), and lower age (HR = 0.709, 95% CI 0.566–0.888). The objective response rates to first-line etoposide/cisplatin therapy were similar between never-smokers and smokers (75% versus 81%). Of 28 genetically evaluable never-smokers, EGFR mutations were detected in four cases (two L858R, one deletion in exon 19, and one G719A). Other mutations were in TP53 (n = 26), RB1 (n = 7), PTEN (n = 5), MET (n = 4), and SMAD4 (n = 3).

Conclusions: Never-smokers with SCLC are increasingly prevalent and have a better prognosis than smokers with SCLC in Korea. Our study warrants further investigation in this group.

Key words: small-cell lung cancer, never-smoker, survival, next-generation sequencing, epidermal growth factor receptor mutation

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