Significant accumulation of *KRAS* mutations in bronchiolar metaplasia-associated honeycomb lesions of interstitial pneumonia

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Abstract. Interstitial pneumonia (IP) is a major risk factor for lung adenocarcinoma (LADC). IP-related LADC predominantly develops in the bronchiolar metaplasia lining in honeycomb lesions. Kirsten rat sarcoma virus (*KRAS*) is the most common oncogene mutated in IP-related LADC. The present study examined the metaplastic epithelia in honeycomb lesions for *KRAS* mutations using digital droplet polymerase chain reaction (ddPCR), a sensitive method used to detect infrequent mutations. Significantly higher *KRAS* mutation variant allele frequencies (VAFs) were detected in the metaplastic lung epithelia from 13 patients with IP compared with those in 46 non-lesioned lung samples from patients without IP (G12V, P=0.0004, G12C, P=0.0181, and G12A, P=0.0234; Mann Whitney U test). Multivariate analyses revealed that higher *KRAS* G12V (logistic regression model; P=0.0133, odds ratio=7.11) and G12C (P=0.0191, odds ratio=5.81) VAFs in patients with IP were independent of confounding variables, such as smoking and age. In patients with IP, metaplastic epithelia exhibited significantly higher *KRAS* G12V and G12C VAFs compared with the non-lesioned counterparts (paired t-test; G12V, P=0.0158, G12C, P=0.0465). These results suggested that IP could increase *KRAS* mutations and supported the hypothesis that bronchiolar metaplasia could be a precursor for IP-related LADC.

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Abbreviations: KRAS, Kirsten rat sarcoma virus; PCR, polymerase chain reaction; IP, interstitial pneumonia; LADC, lung adenocarcinoma; VAF, variant allele frequency

Key words: interstitial pneumonia, bronchiolar metaplasia, adenocarcinoma, lung, carcinogenesis, *KRAS*

Introduction

Interstitial pneumonia (IP) is a group of inflammatory disorders that affect the alveolar septa. They are often progressive and intractable, and may cause airspaces to collapse, leading to honeycombing (1). This may be caused by exposure to hazardous chemicals, certain medications, and exogenous antigens. In most cases, the etiology is unknown.

IP is a strong risk factor for lung cancer (14.1%) (2). The most common histological type of IP-related lung cancer is adenocarcinoma (3). Our recent studies demonstrated that IP-related lung adenocarcinomas (LADCs) mostly comprise the non-terminal respiratory unit (TRU) subtype (4) and that IP-related non-TRU LADCs often develop from bronchiolar metaplasia lining in honeycomb lesions (5). These findings suggest that metaplastic epithelia could be a precursor for IP-related non-TRU LADC.

Generally, tissue injury could cause DNA damage (6,7). Repair of honeycomb lesions resulting from severe chronic lung injury has been described (8). Genetic mutations accumulate in the metaplastic epithelia lining in honeycomb lesions. Similar to IP, patients with chronic gastritis (9), hepatitis (10), pancreatitis (11), and inflammatory bowel disease (12) are also frequently affected by cancers of the affected organs (13,14). These observations support the idea that tissue remodeling lesions could be precursors for cancer.

Kirsten rat sarcoma virus (*KRAS*) is the most common oncogene that is mutated in IP-related LADCs (4). In the present study, we examined metaplastic epithelia lining in honeycomb lesions for the putative accumulation of *KRAS* mutations using digital droplet polymerase chain reaction (ddPCR). This is quantitative and the most sensitive method for detecting highly infrequent mutations (~0.01%) (15). The findings support the hypothesis that metaplastic epithelia could be a precursor for IP-related LADCs.

Materials and methods

Patients. Patients who underwent surgical lung resection (18 cases of surgical lung biopsy for IP, 16 cases of partial

lung resection for pneumothorax/bulla, and 35 cases of lobectomy for LADCs) in Kanagawa Prefectural Cardiovascular and Respiratory Center Hospital (Yokohama, Japan) between January 2014 and December 2020, were included in the analyses.

Tissue samples and DNA extraction. Tissues were fixed with buffered 10% formaldehyde solution for 48 h and embedded in paraffin wax. Sections were cut and stained lightly with hematoxylin. Metaplastic epithelia lining in honeycomb lesions were microscopically collected (Fig. 1) using a PALM microdissection system (Carl Zeiss). Non-lesioned tissues from patients with LADC and pneumothorax, and healthy tissue of the lung tissue from patients with IP were collected by macroscopic dissection. DNA was extracted using a NucleoSpin® Tissue kit (Macherey-Nagel) according to the protocol of the manufacturer. For the successful quantification of infrequent mutations of the *KRAS* gene by ddPCR, >1 μg total material (10 ng/μl) was required.

Quantification of KRAS mutations with ddPCR. Seven types of *KRAS* mutations (G12C, G12V, G12R, G12A, G12S, G12D, and G13D) were detected using the QX200 Droplet Digital PCR system (Bio-Rad). Commercial primers and probes were used ddPCR KRAS Screening Multiplex Kit (Bio-Rad). Briefly, up to 20,000 droplets were made from the master mix solution containing the probe, primers, template DNA, dNTPs, and DNA polymerase with the Cl000 Touch Thermal Cycler with a 96-Deep Well Reaction Module (Bio-Rad). PCR was performed using the following cycling conditions: polymerase activating step (10 min at 95˚C), followed by 40 reaction cycles (30 sec at 95˚C for denaturation and 1 min at 55˚C for annealing and extension), and DNA polymerase deactivation step (10 min at 98˚C). The PCR products were loaded onto a QX200™ Droplet Reader (Bio-Rad). The number of droplets containing mutant and wild-type alleles was counted using the QuantaSoft v.l.7.4 software (Bio-Rad). The variant allele frequency (VAF) was calculated as:

$$KRAS\ VAF\ (\%) = \frac{KRAS\ mutated\ allele\ (copies) \times 100}{KRAS\ mutated\ allele\ (copies) + KRAS\ wild\ type\ allele\ (copies)}.$$

Statistical analyses. Differences in *KRAS* VAFs between the groups of various parameters, including IP, age, smoking, and sex, were analyzed using the Mann Whitney U test. Multivariate analyses were performed using logistic regression analysis. Differences in *KRAS* G12V and G12C VAFs between metaplastic epithelia and non-lesioned bronchiolar epithelia in same patients with IP were analyzed using the paired t-test. Statistical significance was set at P<0.05. All analyses were performed using JMP Pro 15.0.0 (SAS Institute Inc.).

Results

Baseline characteristics of patients. The baseline characteristics of the 59 patients examined in this study are shown in Table I. Of the 13 patients in the IP group, nine suffered from idiopathic pulmonary fibrosis, three from collagen vascular disease interstitial lung disease (CVD-ILD; two cases of systemic sclerosis, one case of polymyositis), and one from non-specific IP (NSIP). One of the five LADCs in the IP group had a *KRAS* mutation (G12V). In the non-IP group, 16 patients had bulla-pneumothorax, and 35 had LADC. Of the 30 LADCs in the non-IP group, a *KRAS* mutation (G12C) was present in one patient.

Table I. Baseline characteristics of all the cases examined.

Characteristic	IP (n=13)	Non-IP (n=46)
Age, years		
≥65	9	18
<65	4	28
Smoking		
Smoker	8	14
Non-smoker	5	32
Gender		
Male	10	28
Female	3	18
Type of IPs		
IPF	9	-
NSIP	1	-
CVD-ILD	3	-
LADC		
+	5	30
-	8	16
KRAS mutations in LADCs	1[a]	1[b]

IP, interstitial pneumonia; IPF, idiopathic pulmonary fibrosis; NSIP, non-specific interstitial pneumonia; CVD-ILD, collagen vascular disease-related interstitial lung disease; LADC, lung adenocarcinoma. [a]The mutation type is *KRAS* G12V; [b]the mutation type is *KRAS* G12C.

Representative histological appearance of IP-related non-TRU LADC. A non-TRU LADC developed in the IP is shown in Fig. 1. The LADC cells and metaplastic epithelia lining in honeycomb lesions were collected separately by microdissection.

Quantification of KRAS mutations with ddPCR. Seven types of *KRAS* mutations (G12C, G12V, G12R, G12A, G12S, G12D, and G13D) were examined by ddPCR. Representative results from the IP and non-IP groups are shown in Fig. 2.

Difference in VAFs of KRAS mutation between IP and non-IP groups. The IP group showed a significantly higher prevalence of *KRAS* mutation VAFs of G12V (P=0.0004), G12C (P=0.0181), and G12A (P=0.0234) than in the non-IP group (Fig. 3A-C). No significant difference between the groups was seen in the other VAF types of *KRAS* mutations and the total VAFs (Fig. 3D-H). We focused on G12V, G12C, and G12A mutations and analyzed their relationships with the baseline characteristics.

Relationships between VAFs of KRAS G12V, G12C, and G12A mutations and baseline characteristics. The G12V VAF was significantly higher in elderly patients (P=0.0169; Fig. 4A). There was no statistically significant relationship between the G12C and G12A VAFs and age, although it tended to be higher in elderly patients (Fig. 4B and C). Smokers displayed

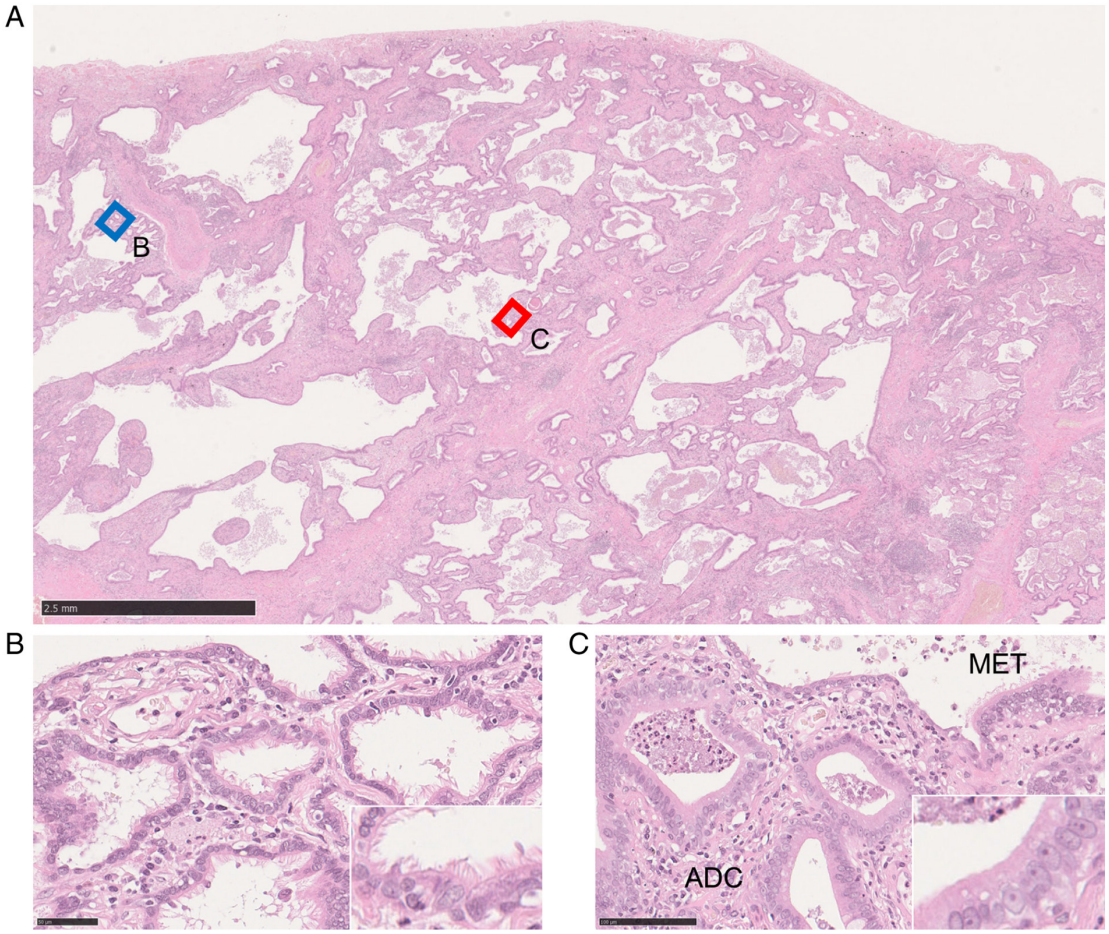

Figure 1. Representative photographs showing the histological appearance of a non-TRU LADC that developed in idiopathic pulmonary fibrosis. (A) Alveolar collapse and fibrosis are predominantly seen in the peripheral area of lobules. (B) A closer view of the blue square in (A) shows the bronchiolar metaplasia lining on collapsed alveoli. (C) A closer view of the red square in (A) shows non-TRU LADC cells (ADC) growing adjacent to metaplastic epithelia (MET). Metaplasia cells have cilia (B, inset), whereas LADC cells lack these structures and exhibit nuclear atypism indicated by enlarged nuclei with conspicuous nucleoli (C, inset). (B and C) Scale bars, 0.1 mm. TRU, terminal respiratory unit; LADC, lung adenocarcinoma.

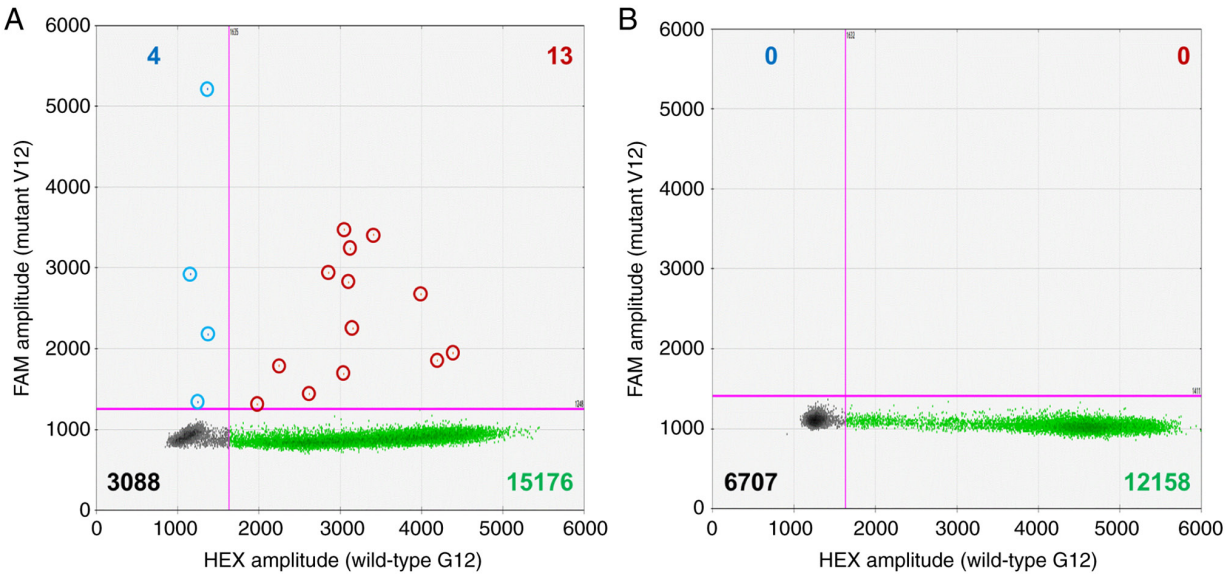

Figure 2. Representative results of ddPCR from the *KRAS* G12V mutations in (A) IP and (B) non-IP cases. Fluorescent amplitudes from the HEX-labeled-wild-type G12 PCR product (horizontal axis) and FAM-labeled-mutant V12 PCR product (vertical axis) are plotted. In both examinations, the number of droplets is sufficient for analysis (>12,000). (A) In the IP case, 17 (4+13) mutation droplets (blue and red dots) and 15,189 (13+15,176) wild-type droplets (green dots) were detected. According to the number and signal levels of the dots, mutant and wild-type alleles comprised 22 and 41,820 copies, respectively. Thus, the VAF was 0.052% [22/(22+41,820)]. (B) In contrast, no mutation- and 12,158 wild-type-droplets (green dots) were detected. The VAF was 0%. IP, interstitial pneumonia; VAF, variant allele frequency.

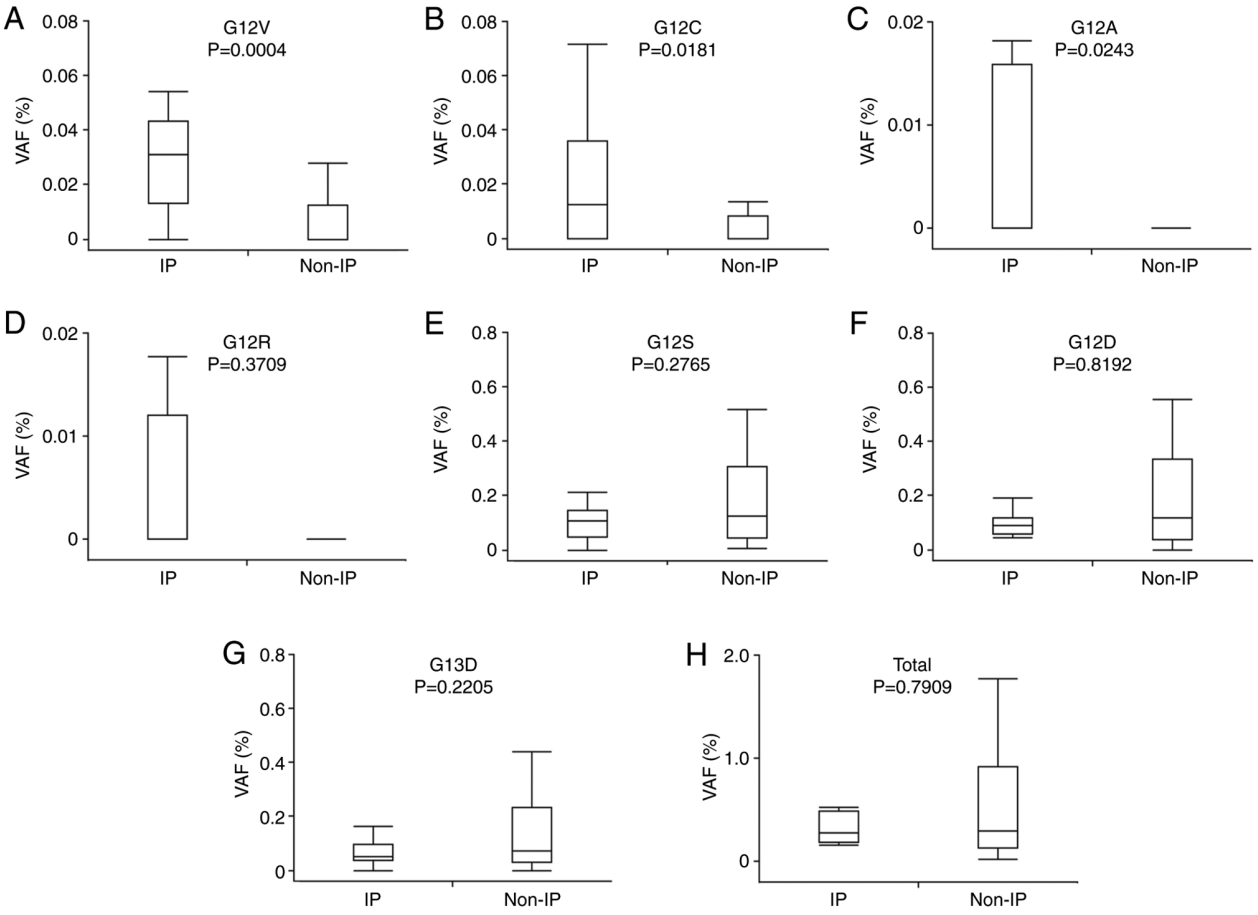

Figure 3. VAFs of seven *KRAS* mutations in IP (n=13) and non-IP (n=46) groups are shown as box-and-whisker plots. Differences between the two groups were analyzed by the Mann Whitney U test. The P-values are shown. The IP group displayed significantly higher VAFs than the non-IP group in (A) G12V, (B) G12C and (C) G12A mutants. No significant difference was evident between the groups in (D) G12R, (E) G12S, (F) G12D, (G) G13D and (H) total VAFs. 'Total' refers to the sum of all the VAFs of the mutant types. VAF, variant allele frequency; IP, interstitial pneumonia.

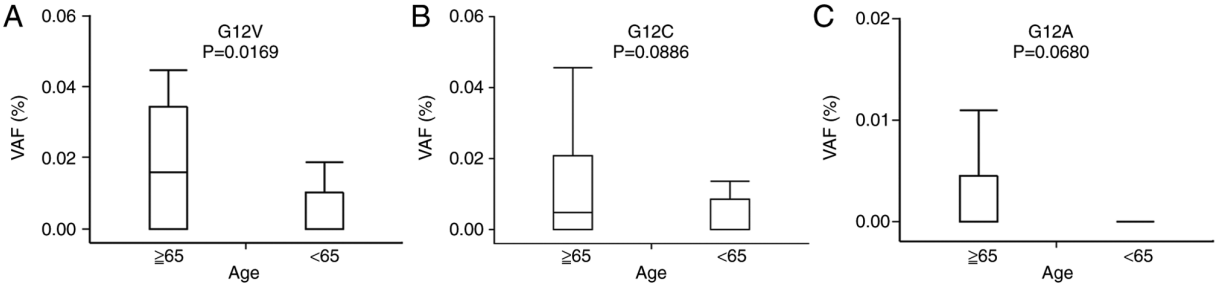

Figure 4. *KRAS* G12V, G12C, and G12A VAFs in elderly individuals (≥65 years old, n=27) and younger individuals (<65 years old, n=32) are shown as box-and-whisker plots. Differences between the two groups were analyzed by the Mann Whitney U test. P-values are shown. (A) Elderly individuals displayed significantly higher VAFs than younger individuals for G12V mutants. No significant inter-group difference in (B) G12C and (C) G12A mutants were evident. VAF, variant allele frequency.

significantly higher VAFs of G12V (P=0.0065), G12C (P=0.0049), and G12A (P=0.0158) than non-smokers (Fig. 5). There was no significant relationship between the G12V, G12C, and G12A VAFs and sex (Fig. 6) or LADC carcinogenesis (Fig. 7). In addition, there was no relationship between the VAFs of *KRAS* mutations and types of IP or oncogenic mutations in the LADCs (data not shown).

Multivariate analyses to confirm the independent relationships between VAFs of KRAS mutations and IP. As described above, age and smoking could be confounding factors associated with the higher VAFs of G12V, G12C, and G12A mutations in the IP group. Multivariate analyses with logistic regression revealed that IP was an exploratory variable for G12V and G12C VAFs (Table II), while smoking status and age were not. The IP group displayed a significantly higher G12V [odds ratio (OR), 7.11; 95% confidence interval (CI), 1.47-52.6] and G12C [odds ratio (OR), 5.81; 95% confidence interval (CI), 1.33-26.9] VAFs than the non-IP group (Table II). IP was an independent factor that caused the accumulation of G12V mutations.

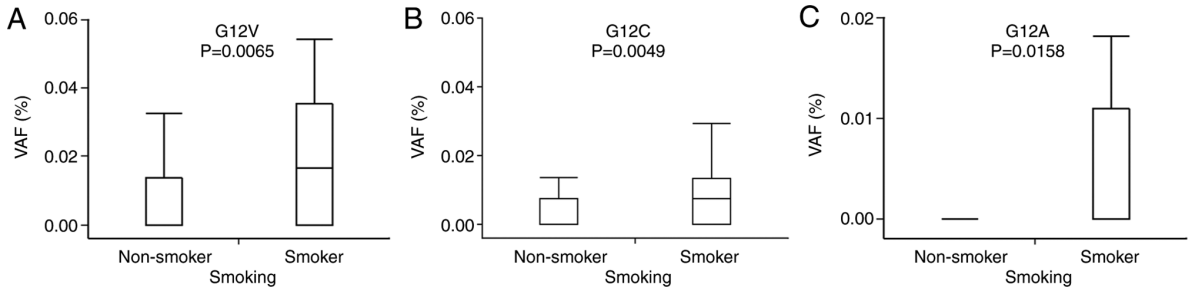

Figure 5. *KRAS* G12V, G12C, and G12A VAFs in smokers (n=23) and non-smokers (n=36) are shown as box-and-whisker plots. Differences between the two groups were analyzed by the Mann Whitney U test. P-values are shown. Smokers displayed significantly higher VAFs than non-smokers for all (A) G12V, (B) G12C and (C) G12A mutants. VAF, variant allele frequency.

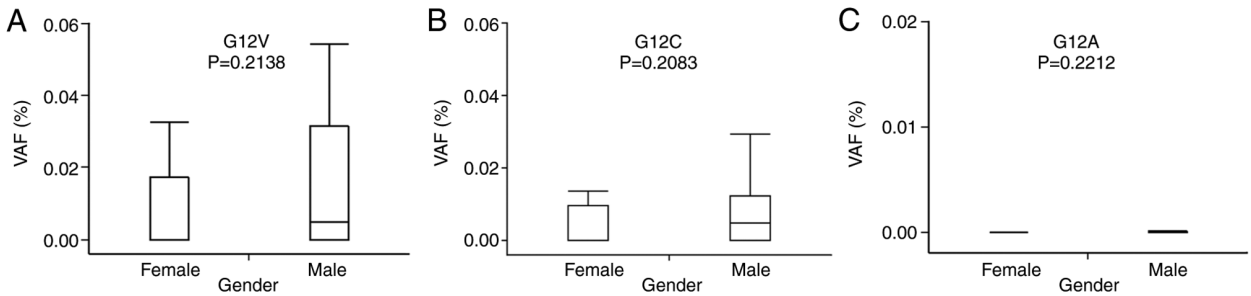

Figure 6. *KRAS* (A) G12V, (B) G12C and (C) G12A VAFs in men (n=38) and women (n=21) are shown as box-and-whisker plots. Differences between the two groups were analyzed by the Mann Whitney U test. P-values are shown. No significant between-group difference was evident. VAF, variant allele frequency.

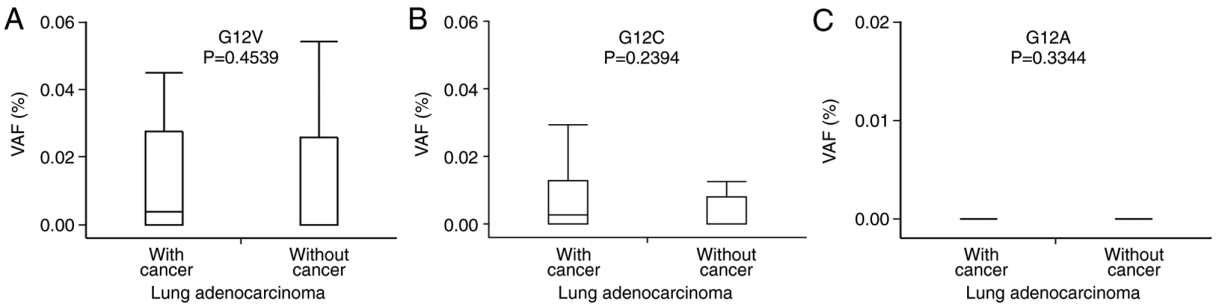

Figure 7. *KRAS* (A) G12V, (B) G12C and (C) G12A (C) VAFs in groups with (n=35) and without (n=24) LADC are shown as box-and-whisker plots. Differences between the two groups were analyzed by the Mann Whitney U test. P-values are shown. No significant between-group difference was evident. VAF, variant allele frequency; LADC, lung adenocarcinoma.

Table II. Multivariate analyses on relationship between G12V, G12C, and G12A VAFs and IP, smoking, and age.

VAF	Risk factor	P-value	Odds ratio (95% CI)
G12V	IP/Non-IP	0.0133[a]	7.11 (1.47-52.6)
	Smoker/non-smoker	0.1117	2.88 (0.78-10.9)
	Age ≥65/<65	0.2640	2.06 (0.57-7.39)
G12C	IP/Non-IP	0.0191[a]	5.81 (1.33-26.9)
	Smoker/non-smoker	0.6537	1.44 (0.28-7.11)
	Age ≥65/<65	0.3390	2.15 (0.44-11.1)
G12A	IP/Non-IP	0.1469	3.24 (0.65-15.9)
	Smoker/non-smoker	0.2461	2.76 (0.49-17.3)
	Age ≥65/<65	0.5684	1.65 (0.29-10.1)

VAF, variant allele frequency; IP, interstitial pneumonia; CI, confidence interval; P-values, and odds ratios are calculated with logistic regression. [a]P<0.05 is considered statistically significant.

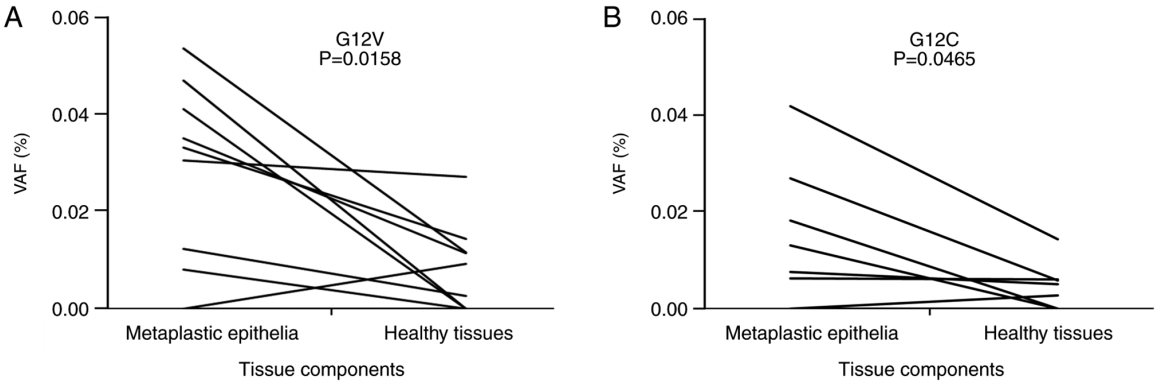

Figure 8. *KRAS* (A) G12V and (B) G12C VAFs in metaplastic and non-lesioned bronchiolar epithelia in 10 patients with IP are shown as a pair plot. Differences between the two groups were analyzed by the paired t-test. P-values are shown. The metaplastic epithelia group displayed significantly higher VAFs. VAF, variant allele frequency.

Table III. Baseline characteristics of the ten IP cases subjected to the paired analysis.

Characteristic	IP (n=10)
Age	
≥65	6
<65	4
Smoking	
Smoker	7
Non-smoker	3
Gender	
Male	8
Female	2
Type of IPs	
IPF	7
CVD-ILD	2
Radiation pneumonitis	1

IP, interstitial pneumonia; IPF, idiopathic pulmonary fibrosis; CVD-ILD, collagen vascular disease-related interstitial lung disease.

Comparison of KRAS G12V VAFs between metaplastic epithelia and non-lesioned bronchiolar epithelia. In the 10 IP cases (Table III), metaplastic epithelia and non-lesioned bronchiolar epithelia were separately collected by microdissection and examined for *KRAS* G12V and G12C VAFs. The metaplastic epithelia showed significantly higher *KRAS* G12V (P=0.0153) and G12C (P=0.0465) VAFs than the non-lesioned bronchiolar epithelia when compared in the same cases (Fig. 8). These observations suggest that metaplastic epithelia could accumulate more mutations.

Discussion

Metaplasia is an abnormal regeneration that results from severe chronic tissue injury and repair. Therefore, it is conceivable that many genetic mutations that are due to DNA damage may accumulate in metaplastic cells. Metaplasia is a precancerous condition in certain types of cancers. For example, gastric adenocarcinoma develops from gastric intestinal metaplasia (16), and squamous cell carcinoma develops from squamous cell metaplasia in different organs, such as the bronchus (17) and esophagus (18). Recently, we proposed the theory that bronchiolar metaplasia can be a precancerous condition for IP-related LADC, as most LADCs develop from metaplasia-lining honeycomb lesions (4,5). The present study detected a significant accumulation of some *KRAS* mutations (G12V, G12C, and G12A) in metaplastic epithelia. These findings further support our theory.

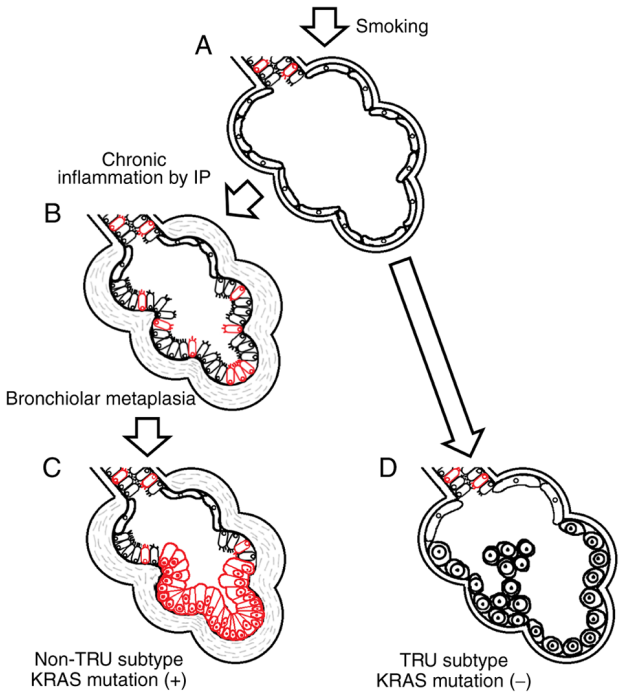

Figure 9. (A) Smoking induces *KRAS* mutations mainly in bronchial epithelial cells (red cells). (B) IP-related tissue damage promotes accumulation of *KRAS* mutations in metaplastic cells, (C) and one of them may transform to become non-TRU subtype LADC. (D) Instead, in non-IP lung tissue without *KRAS* mutations, TRU subtype LADC may develop.

Among the types of *KRAS* mutations, *KRAS* G12V and G12C mutations are common in LADCs, in which guanine is replaced by thymine. These mutations are caused by benzo[a]pyrene in cigarette smoke (19). On the other hand, *KRAS* G12A

mutation, in which guanine is replaced by cytosine, is also common in LADCs; however, their association with smoking is not understood (19). Interestingly, multivariate analyses in this study revealed that G12V and G12C VAFs, but not G12A VAF, were independently related to IP, suggesting that chronic tissue injury and repair due to IPs could play potential roles in the fixation and accumulation of smoking-related *KRAS* G12V and G12C mutations. The observations are schematically illustrated in Fig. 9.

A previous study comprehensively analyzed infrequent somatic mutations by a deep sequencing method that can detect mutations with frequencies of 0.1-1.0%. The findings demonstrated that the mutations accumulated even in non-cancerous esophageal epithelia. Their mutational burden was significantly more prominent in elderly individuals and heavy drinkers (20). The study also demonstrated a positive correlation between the mutational burden and risk of esophageal cancer, suggesting that tissues bearing a large mutational burden could be precancerous lesions (20). This finding supports our theory that metaplastic epithelia lining in honeycomb lesions, in which higher *KRAS* VAFs were detected, could be a precursor for IP-related LADC.

In the present study, there was no statistically significant relationship between *KRAS* mutations and VAFs, and the incidence of LADC. However, the number of patients involved in the study may have been limited. Therefore, future large-scale studies are needed to determine such potential relationship.

Another confounding result was that the types of *KRAS* mutations and higher VAFs, and those who developed LADCs were not necessarily consistent (Table SI). Similarly, a previous study on esophageal epithelia failed to identify any specific mutations in non-cancerous lesions, which could be drivers in related esophageal cancers (20). Non-cancerous lesions consist of heterogeneous cells that may have different random mutations. Only one or few cells transformed into cancerous cells, and this may explain the observed discrepancies.

In contrast, adenocarcinoma, squamous cell carcinoma and small cell carcinoma frequently develop in IP, where honeycombing lower lobes were preferentially affected (21-23). Similar to bronchiolar metaplasia, abnormal regeneration such as squamous cell metaplasia (21) and neuroendocrine cell hyperplasia (24) are thought to be involved in carcinogenesis. It would be interesting to investigate the mutational burden of these lesions.

In summary, metaplastic epithelia lining in honeycomb lesions showed a greater prevalence of *KRAS* mutation VAFs, suggesting that metaplasia could be a precursor for IP-related LADC. In addition, IP can be a potential cause for the fixation and accumulation of specific types of *KRAS* mutations. The observations are intriguing and deepen the understanding of the IP-related LADC carcinogenesis.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TK and KOk wrote most parts of the manuscript. TB, HA, HK, TI and TO collected patient information and compiled a clinical database. TK was responsible for the statistical analyses. KOk and KOh designed the study and suggested the contents of the manuscript. KOk, MM, CK and TT examined all the tissue sections and gave pathological diagnoses. TS, HM, MSu and MSe cut tissue sections and stained them with hematoxylin and eosin. TK performed microdissection and dd PCR analyses. All authors read and approved the final manuscript. TK and KOk confirmed the authenticity of all the raw data.

Ethics approval and consent to participate

The study was approved by the Ethics Committees of Yokohama City University (approval no. A200900001) and the Kanagawa Cardiovascular and Respiratory Center (approval no. KCRC-20-0020). Written informed consent was obtained from all patients according to the principles of the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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