Original Article

Characteristics and prognostic analysis of patients with detected *KRAS* mutations in resected lung adenocarcinomas by peptide nucleic acid-locked nucleic acid polymerase chain reaction (PNA-LNA PCR) clamp method

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Background: *Kirsten rat sarcoma virus* (*KRAS*) gene mutations are a type of driver mutation discovered in the 1980s, but for a long time no molecular targeted drugs were available for them. Recently, sotorasib was developed as a molecular targeted drug for *KRAS* mutations. It is therefore necessary to identify the characteristics of patients with *KRAS* mutations.

Methods: This was the single-institution retrospective study. Surgically resected tumors from lung adenocarcinoma patients were collected at a single institution from June 2016 to September 2019. Peptide nucleic acid-locked nucleic acid polymerase chain reaction (PNA-LNA PCR) clamp analysis of *KRAS G12X* mutations was compared with analysis by therascreen KRAS RGQ kit. The association between *KRAS* mutation status and patient characteristics and prognosis was assessed.

Results: Among 499 lung adenocarcinomas, *KRAS* mutations were evaluated in 197 cases, excluding stage IV lung cancer and tumors with epidermal *growth factor receptor* (*EGFR*) and *anaplastic lymphoma kinase (ALK*) mutations. *KRAS G12X* mutations were detected in 59 cases (29.9%). The highest frequency by gene mutation subtype was *G12V* in 23 cases (39.0%), followed by *G12C* in 16 cases (27.1%), *G12D* in 12 cases (20.3%), *G12S* in 4 cases (6.8%) and *G12A* in 2 cases. For the *G12C* mutation, the PNA-LNA PCR clamp and therascreen methods were consistent, but for the *G12D* and *G12S* mutations, the PNA-LNA PCR clamp method showed higher detection rates. In operable tumors, *G12C* mutations were more frequent in males, smokers, and patients with high expression of programmed death-ligand 1 (PD-L1), and had no correlation with prognosis.

Conclusions: By the PNA-LNA PCR clamp method, *G12C* mutation of surgical specimens was detected successfully. The PNA-LNA PCR clamp method is expected to be applied to the detection of druggable *G12C* mutations.

Keywords: *KRAS* mutation; non-small cell lung cancer (NSCLC); surgical specimen; disease-free survival (DFS)

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Introduction

The discovery of driver genes and the development of molecular targeted drugs against them have gradually improved the course of poor prognosis lung cancer (1-7). *Kirsten rat sarcoma virus* (*KRAS*) mutations were first reported in 1985 as driver mutations on the human chromosome (8,9). Various drugs targeting *KRAS* mutations have been developed over the past few decades but have failed to achieve sufficient efficacy (10).

Sotorasib has recently been launched as a molecular targeted therapy for *KRAS G12C* mutations. This development of an effective molecular targeted therapy for *KRAS* mutations has enhanced the importance of the frequency, distribution characteristics and detection methods of *KRAS* mutations.

RAS is a guanosine triphosphate (GTP) binding protein including v-Ha-ras Harvey rat sarcoma viral oncogene homolog (HRAS), KRAS, and neuroblastoma RAS viral oncogene homolog (NRAS). In humans, *KRAS* mutations account for 85% of *RAS* mutations (11) and are most common in pancreatic cancer, colorectal cancer, and lung cancer, in that order (12). The frequency of *KRAS* subtypes varies by organ cancer, with *G12D* being more common in pancreatic cancer (13) and *G12C* being more common in lung cancer (14).

The mechanism of carcinogenesis by *KRAS* mutations is as follows. KRAS binding to guanosine diphosphate (GDP) results in an inactivated state, while binding to GTP results in an activated state. By upstream signals such epidermal growth factor receptor (EGFR), GDP is exchanged for GTP through guanine nucleotide exchange factors (GEF). Mutations in the *KRAS* gene cause constant activation by sustained GTP binding to mutant KRAS and lead to signal transduction to the intracellular phosphoinositide 3-kinases (PI3K), mitogen-activated protein kinase (MAPK), and Ral guanine nucleotide exchange factors (RAL-GEFs) pathways, leading to tumor formation. This sustainability of GTP binding to mutated KRAS varies among *KRAS* mutation subtypes (15,16).

KRAS mutations are frequently present in lung adenocarcinoma. The presence of *KRAS* mutations in adenocarcinoma is the most common driver mutation for Caucasians (about 20–30%) (17-19), whereas the rate is about 10–15% in East Asians (20-22). Recently, a Japanese report showed 14% of Japanese patients to have *KRAS* mutations. Analysis of subtypes of *KRAS* mutations in the Japanese data showed that *G12C*, *G12D*, *G12V*, and *G12A* were the most common, in that order. *KRAS G12C* mutations are found in 4.0% of all lung adenocarcinoma patients (23). There have been few recent publications on the frequency and distribution of *KRAS*, but its background factors are becoming more important with the launch of molecular targeted drugs for *KRAS G12C*.

In phase I trials of sotorasib in non-small cell lung cancer (NSCLC) cases and phase II trials in previously treated NSCLC patients, objective response rate (ORR) was 32.2% and 37.1%, respectively, disease control rate (DCR) was 88.1% and 80.6%, and median progression-free survival (PFS) was 6.3 and 6.8 months (24,25). With these results, sotorasib has been approved by the Food and Drug Administration (FDA) and the Japanese Pharmaceuticals and Medical Devices Agency (PMDA) for patients with *KRAS G12C*.

Highlight box

Key findings

- Kirsten rat sarcoma virus (*KRAS*) mutations were detectable with high sensitivity by the peptide nucleic acid-locked nucleic acid polymerase chain reaction (PNA-LNA PCR) clamp method.

What is known and what is new?

- *KRAS* mutations are frequently present in lung adenocarcinoma. The presence of *KRAS* mutations in adenocarcinoma is the most common driver mutation for Caucasians (about 20–30%), whereas the rate is about 10–15% in East Asians.
- The frequency of *KRAS* mutations and their subtypes in surgically resected tumors in Japan was determined.
- The prognosis of patients with surgically resected tumors with *KRAS* mutations was comparable to that of patients with epidermal growth factor receptor (*EGFR*) and without *KRAS* mutations.
- *KRAS* mutations were detectable with high sensitivity by the PNA-LNA PCR clamp method.

What is the implication, and what should change now?

- The PNA-LNA PCR clamp method was more sensitive and may apply to multiple situations in detecting *KRAS* mutations in the future.

The QIAGEN therascreen KRAS RGQ PCR kit (for tissue) and Guardant360 CDX (for plasma) are approved as companion diagnostics for sotorasib (26). However, Guardant360 CDX is not covered by health insurance in many countries, including Japan, especially due to its cost and long turnaround time. On the other hand, although the therascreen PCR kit has the potential to overcome the two drawbacks of the Guardant360 CDX, a more sensitive test method is needed to detect *KRAS* gene mutations in plasma in the future. In this regard, the peptide nucleic acid-locked nucleic acid polymerase chain reaction (PNA-LNA PCR) clamp has been used to measure *EGFR* mutations in plasma as well as tumor tissue with high sensitivity (27). A comparison between the QIAGEN therascreen KRAS RGQ PCR kit and the PNA-LNA clamp method for *KRAS*, which is considered more sensitive, is needed.

In the present study, *KRAS* mutations in resected tumor tissue were evaluated by both the PNA-LNA PCR clamp method and the therascreen kit. We expected that the PNA-LNA PCR clamp method was superior to the therascreen in detection of *KRAS* mutations. The frequency of *KRAS* mutation subtypes and characteristics of patients with *KRAS* mutations were analyzed. We present this article in accordance with the STROBE reporting checklist (available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-23-15/rc).

Methods

Study design

This was a single-institution retrospective study. Surgically resected tumors from lung adenocarcinoma patients were collected. The PNA-LNA PCR clamp analysis of *KRAS G12X* mutations was compared with analysis by the therascreen KRAS RGQ kit. The association between *KRAS* mutation status and patient characteristics and prognosis was evaluated.

Eligible patients

Patients who underwent surgical procedures between June 1, 2016 and September 30, 2019 at the Department of Respiratory Surgery, Iwate Medical University, were eligible for *KRAS* gene mutation testing if they satisfied the following conditions. (I) Stage I–III disease according to Tumor-Node-Metastasis (TNM) classification 8th edition and histopathological diagnosis of lung adenocarcinoma. (II) It was possible to prepare 5 μm × 5 unstained thin section specimens from paraffin block specimens. Patients with stage IV disease were excluded due to its negative impact on disease-free survival (DFS) and overall survival (OS), and patients with squamous cell lung cancer were excluded due to the extremely small number of *KRAS* mutations (28,29). *KRAS* mutations are typically present as a single-driver mutation (30-33). Cases in which driver mutations such as *EGFR* mutations and *anaplastic lymphoma kinase* (*ALK*) mutations were confirmed to be mutually exclusive with *KRAS* mutations were excluded. However, patients with *EGFR* mutations who underwent surgery at the same time were used as comparators for *KRAS* patients for characteristics, DFS, and OS.

Testing methods

Five unstained thin sections of 5 μm × 5 μm were prepared from paraffin block specimens of surgical specimens of the subject patients, and DNA was extracted from sections with QIAamp DNA formalin fixed paraffin embedded (FFPE) Tissue (QIAGEN, Hilden, Germany). The PNA-LNA PCR clamp method was used to measure the *KRAS* exon *G12X* point mutation. Although this PCR system is as well established as the method used for *EGFR* gene mutations (34). This PCR system achieved a detection rate of less than 0.1% by using smaller PCR products and by increasing the number of cycles from 45 to 50. The therascreen® *KRAS* mutation detection kit was also used to evaluate the concordance with the companion diagnostic agent. DNA extraction and gene mutation testing were performed by LSI Medience Corporation (Tokyo, Japan). This method is also able to detect only the *G12X* point mutation. All discordant samples required confirmation by Sanger sequencing.

Collection of clinical data

Clinical information on patients who underwent *KRAS* gene testing, including age, gender, underlying disease, smoking history, programmed death-ligand 1 (PD-L1) expression, clinical stage, postoperative chemotherapy, relapse date, and death date, was collected from electronic medical records. PD-L1 expression was assessed in formalin-fixed, paraffin-embedded specimens at the department of pathology in Iwate Medical University School of Medicine, using the commercially available PD-L1 IHC 22C3 pharmDx assay (Dako North America). The last day of the data collection

period was September 30, 2022. As a control group, information was also collected on patients with *EGFR* mutations who had undergone surgery and were in stages I-III during the period of study. In the investigation of prognosis among *KRAS*, *EGFR*, and *KRAS* wild groups, data on the patients' sex, age, smoking status, pathological stage, complications, and adjuvant chemotherapies to check imbalance in them.

Endpoints

Endpoints included the frequency of *KRAS* mutation-positive patients, background factors of *KRAS* mutation-positive patients, impact of *KRAS* mutation positivity on DFS and OS, and comparison of *KRAS* mutation detection between the PNA-LNA PCR clamp method and the therascreen method.

Statistical analysis

All statistical analyses were performed using EZR, a statistical software program that extends the capabilities of R and R Commander. For DFS and OS, Kaplan-Meier curves were created and analyzed using the log-rank test and Cox proportional hazards model, with a two-sided 5% significance level. Bonferroni correction was used when statistical analysis required multigroup comparisons.

Ethical statement

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and was approved by the institutional ethics committee of Iwate Medical University (No. HG2021-002). The study is a retrospective observational study, with no new invasive procedures, and consent was obtained in an opt-out fashion.

Results

From June 1, 2016 to September 30, 2020, 630 lung cancer patients were treated at our institution (*Figure 1*). There were 499 patients with lung adenocarcinoma, including 219 patients with *EGFR* mutation, 12 with *ALK* fusion gene, 33 with stage IV disease, and 38 whose specimens could not be secured, all of whom were excluded from *KRAS* measurement. Excluding these patients, 197 patients were tested for *KRAS* mutations.

Among the 197 stage I–III adenocarcinomas, excluding those with *EGFR* mutations and *ALK* fusion, *KRAS G12X* mutations were detected in 59 cases (29.9%). Among 466 cases including those with *EGFR* and *ALK* mutations, the frequency of *KRAS* mutations was 12.7%. A rare oncogenic mutation, *KRAS A11V*, was detected only by the whole adenocarcinoma PNA-LNA PCR clamp method. The *KRAS G12C* mutation, an indication for treatment with molecular-targeted drugs, was detected in 16 (8.1%) of the adenocarcinomas tested, and in 3.4% of the adenocarcinomas including other driver gene mutations. The highest frequency by gene mutation subtype was 23 (39.0%) cases of *G12V*, followed by 16 (27.1%) cases of *G12C*, 12 (20.3%) cases of *G12D*, 4 (6.8%) cases of *G12S*, and 2 cases of *G12A* (*Figure 2*).

Both PNA-LNA PCR clamp and therascreen were performed, and the concordance rate was 95.9% (189/197) (*Table 1*). All discordant cases were confirmed by Sanger sequencing (*Table 2*). For the *G12C*, *G12V*, and *G12A* mutations, both the PNA-LNA PCR clamp and therascreen methods were consistent, but for the *G12D* and *G12S* mutations, the PNA-LNA PCR clamp method showed higher detection rates. There was one specimen with discrepant results between *G12V* by therascreen kit and *G12F* by PNA-LNA PCR clamp method, and the sequencing result was *G12F*. The sensitivity and specificity of the PNA-LNA PCR clamp method were 1.00 [95% confidence interval (CI): 1.00–1.00] and 0.95 (95% CI: 0.91–0.98), respectively. The negative predictive value was 1.00 (95% CI: 1.00–1.00).

We evaluated the background factors among the *KRAS* mutation-positive group and *KRAS* mutation-negative group and the *EGFR* mutation group without *KRAS* gene testing (*Table 3*). *KRAS G12C*-positive patients were more often male, smokers, and had higher expression rates of PD-L1. In terms of smoking status, there was a trend toward more *KRAS G12C* mutations among heavy smokers. By stage, *KRAS* mutations were equally frequent in both early and advanced stages of disease. There was also a tendency for *KRAS* mutations to be more prevalent in patients with high PD-L1 expression, which may be associated with smoking status. In terms of smoking status, *KRAS G12C* mutations were frequently found in heavy smokers. By staging, *KRAS* mutations were equally frequent in both early and advanced stages of disease. A tendency for *KRAS* mutations to be more prevalent in patients with high PD-L1 expression might be associated with smoking status.

In this study, there were no statistically significant differences in DFS or OS between patients with and

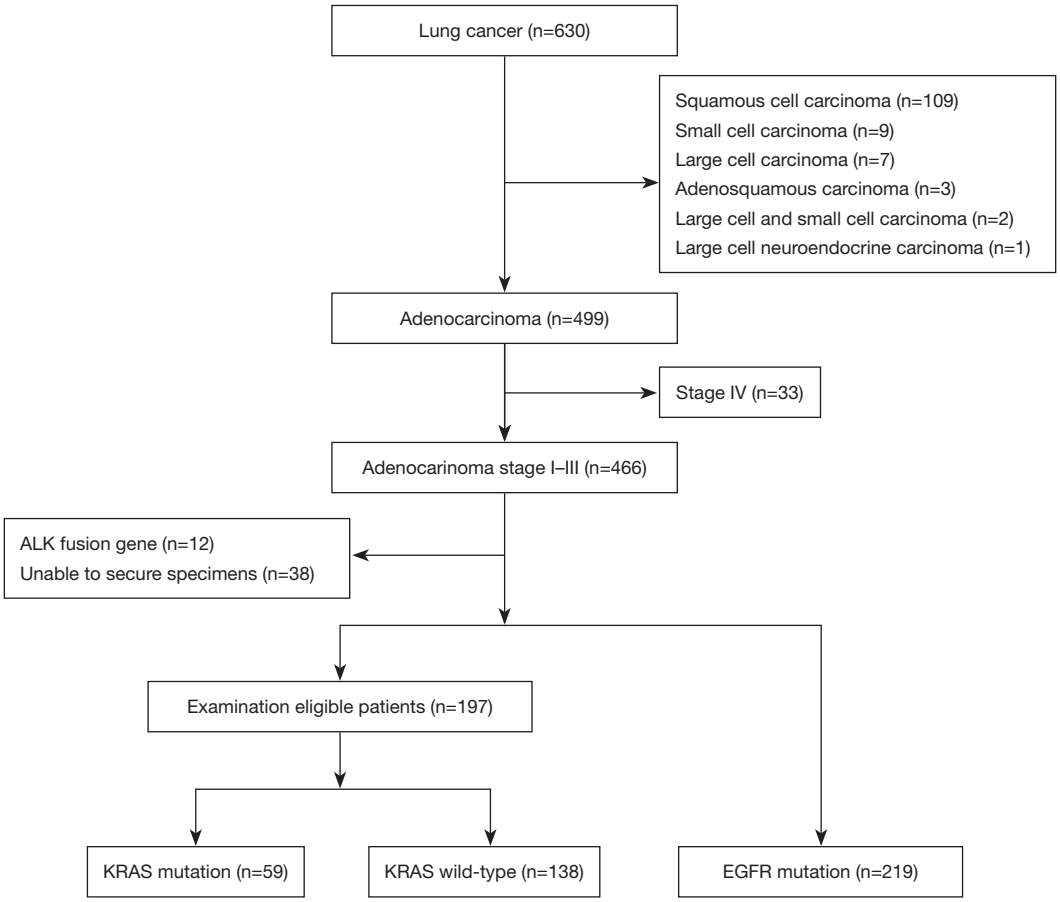

Figure 1 Consort diagram. ALK, anaplastic lymphoma kinase; KRAS, Kirsten rat sarcoma virus; EGFR, epidermal growth factor receptor.

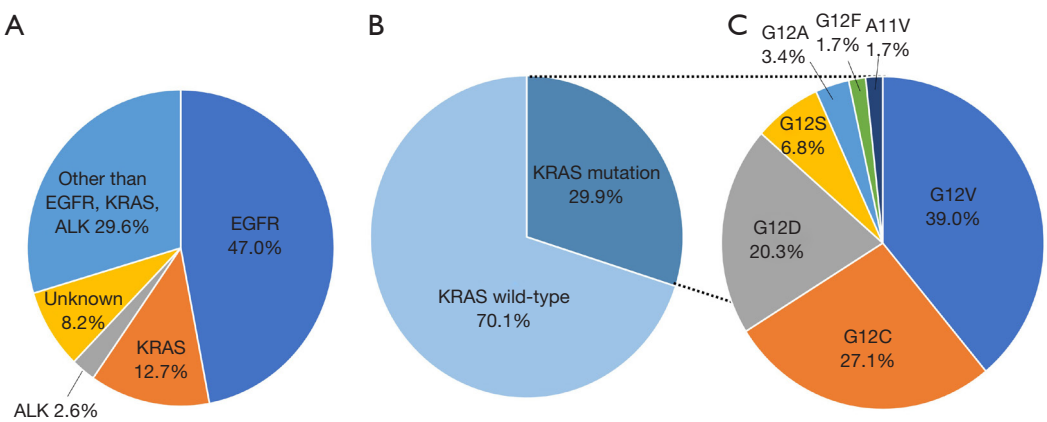

Figure 2 Distribution of mutations. (A) The pie chart shows the distribution of each gene mutation in 466 patients with operable stage I-III adenocarcinoma. (B) *KRAS* mutations among those tested for *KRAS* mutations (n=197). (C) Distribution of *KRAS* mutation subtypes in *KRAS* mutations is shown (n=59). ALK, anaplastic lymphoma kinase; KRAS, Kirsten rat sarcoma virus; EGFR, epidermal growth factor receptor.

Table 1 Results of *KRAS* gene mutation testing for PNA-LNA PCR clamp method and therascreen

Therascreen	PNA-LNA PCR clamp							
	Wild-type	*A11V*	*G12A*	*G12C*	*G12D*	*G12F*	*G12S*	*G12V*
Wild-type	138	1[†]			5[‡]		1[‡]	
A11V								
G12A			2					
G12C				16				
G12D					7			
G12F								
G12S							3	
G12V						1[†]		23

[†], genetic mutation testing not covered by therascreen; [‡], positive signal confirmed by therascreen, but cutoff not met and test is negative. Concordance rate: 95.9% (189/197). KRAS, Kirsten rat sarcoma virus; PNA-LNA PCR, peptide nucleic acid-locked nucleic acid polymerase chain reaction.

Table 2 Results of the Sanger method for cases in which test results did not correspond with the PNA-LNA PCR clamp method and therascreen

PNA-LNA PCR clamp	Therascreen KRAS mutation detection kit	Sanger sequencing
KRAS G12D	*KRAS* wild-type	*KRAS G12D*
KRAS G12D	*KRAS* wild-type	*KRAS G12D*
KRAS G12D	*KRAS* wild-type	*KRAS G12D*
KRAS A11V	*KRAS* wild-type	*KRAS A11V*[†]
KRAS G12S	*KRAS* wild-type	*KRAS G12S*
KRAS G12D	*KRAS* wild-type	*KRAS G12D*
KRAS G12D	*KRAS* wild-type	*KRAS G12D*
KRAS G12F	*KRAS G12V*	*KRAS G12F*[†]

[†], gene mutation not analyzed by therascreen. PNA-LNA PCR, peptide nucleic acid-locked nucleic acid polymerase chain reaction; KRAS, Kirsten rat sarcoma virus.

without genetic mutations (*Figure 3*, *Table 4*, *Table 5*). However, when divided by each stage, patients with stage II *EGFR* mutations tended to have shorter DFS (Figures S1-S3), possibly due to the small number of cases. There were no significant differences in DFS and OS between patients with *KRAS G12* and other subtypes of *KRAS* mutations (Figure S4).

DFS showed significant differences in univariate analysis for stage, PD-L1 (1–49 *vs.* <1, ≥50 *vs.* <1), smoking history (current *vs.* never, current *vs.* previous), adjuvant chemotherapy, and history of chronic obstructive pulmonary disease (COPD), but only stage II *vs.* I [hazard ratios (HR) =2.07, 95% CI: 1.01–4.24, P value =0.047], III *vs.* I (HR =4.47, 95% CI: 2.30–8.69, P value <0.001), III *vs.* II (HR =2.16, 95% CI: 1.07–4.37, P value =0.032) and PD-L1 ≥50 *vs.* <1 (HR =2.06, 95% CI: 1.06–4.02, P value =0.033) in multivariate analysis (*Table 4*).

For OS, univariate analysis showed significant differences in stage III *vs.* I, III *vs.* II, PD-L1 ≥50 *vs.* <1, and diabetes mellitus, while multivariate analysis showed significant differences in stage III *vs.* I (HR =7.31, 95% CI: 3.18–16.8, P value <0.001), III *vs.* II (HR =3.73, 95% CI: 1.18–11.7, P value =0.025), and diabetes mellitus (HR =2.36, 95% CI: 1.08–5.16, P value =0.031) (*Table 5*).

Discussion

In this study, *KRAS* mutations in resected tumor specimens (n=197) were evaluated by both the PNA-LNA PCR clamp method and the therascreen PCR kit. Discrepant cases were confirmed by sequencing. The overall frequency of *KRAS* in adenocarcinoma without stage IV was 12.7%, with *KRAS G12C* at 3.4%. *G12V* was the most common subtype, followed by *G12C* and *G12D*.

In the two PCRs used in this study, *G12C* was matched in all cases, but other subtypes were detected more frequently by the PNA-LNA PCR clamp method compared to the therascreen PCR kit. This result may be related to the fact that the minimum detection sensitivity of the PNA-

Table 3 Background factors among the *KRAS* wild-type group, mutation group and the *EGFR* mutation group

Characteristics	*KRAS* wild-type	*KRAS* mutation						*EGFR* mutation			
	All (n=138)	All (n=59)	*G12C* (n=16)	G12V (n=23)	*G12D* (n=12)	*G12A* (n=2)	Others (n=6)	All (n=219)	*L858R* (n=120)	*del19* (n=64)	Others (n=35)
Median age, years [range]	70 [36–84]	72 [44–85]	71 [44–85]	71 [58–84]	78 [49–85]	69 [65–73]	71.5 [69–84]	70 [43–86]	70 [51–86]	71 [43–85]	72 [48–84]
Age group, n (%)											
≤60 years	27 (19.6)	5 (8.5)	3 (18.8)	1 (4.3)	1 (8.3)	0 (0)	0 (0)	29 (13.2)	12 (10.0)	14 (21.9)	3 (8.6)
61–74 years	68 (49.3)	28 (47.5)	10 (62.5)	17 (73.9)	3 (25.0)	2 (100.0)	1 (16.7)	122 (55.7)	69 (57.5)	33 (51.6)	20 (57.1)
≥75 years	43 (31.2)	20 (33.9)	3 (18.8)	5 (21.7)	8 (66.7)	0 (0)	5 (83.3)	68 (31.1)	39 (32.5)	17 (26.6)	12 (34.3)
Sex, n (%)											
Male	79 (57.2)	46 (78.0)	14 (87.5)	17 (73.9)	7 (58.3)	2 (100.0)	6 (100.0)	83 (37.9)	41 (34.2)	28 (43.8)	14 (40.0)
Female	59 (42.8)	13 (22.0)	2 (12.5)	6 (26.1)	5 (41.7)	0 (0)	0 (0)	136 (62.1)	79 (65.8)	36 (56.3)	21 (60.0)
Smoking history, n (%)											
Never	57 (41.3)	12 (20.3)	1 (6.3)	5 (21.7)	5 (41.7)	0 (0)	1 (16.7)	145 (66.2)	86 (71.7)	35 (54.7)	24 (68.6)
Previous	50 (36.2)	28 (47.5)	8 (50.0)	9 (39.1)	6 (50.0)	2 (100.0)	3 (50.0)	55 (25.1)	28 (23.3)	17 (26.6)	10 (28.6)
Current	31 (22.5)	19 (32.2)	7 (43.8)	9 (39.1)	1 (8.3)	0 (0)	2 (33.3)	19 (8.7)	6 (5.0)	12 (18.8)	1 (2.9)
Brinkman index, n (%)											
<600	81 (58.7)	26 (44.1)	3 (18.8)	13 (56.5)	8 (66.7)	1 (50.0)	1 (16.7)	192 (87.7)	109 (90.8)	53 (82.8)	30 (85.7)
600–1,200	46 (33.3	21 (35.6)	8 (50.0)	7 (30.4)	1 (8.3)	1 (50.0)	4 (66.7)	23 (10.5)	9 (7.5)	9 (14.1)	5 (14.3)
>1,200	11 (8.0)	12 (20.3)	5 (31.3)	3 (13.0)	3 (35.0)	0 (0)	1 (16.7)	4 (1.8)	2 (1.7)	2 (3.1)	0 (0)
Stage, n (%)											
I	102 (73.9)	38 (64.4)	11 (68.8)	14 (60.9)	8 (66.7)	1 (50.0)	4 (66.7)	182 (83.1)	105 (87.5)	48 (68.6)	29 (82.9)
II	20 (14.5)	15 (25.4)	3 (18.8)	8 (34.8)	3 (25.0)	0 (0)	1 (16.7)	13 (5.9)	7 (5.8)	5 (7.1)	1 (2.9)
III	16 (11.6)	6 (10.2)	2 (12.5)	1 (4.3)	1 (8.3)	1 (50.0)	1 (16.7)	24 (11.0)	8 (6.7)	11 (15.7)	5 (14.3)
PD-L1 status, n (%)											
Unknown	19 (13.8)	4 (6.8)	0 (0)	1 (4.3)	1 (8.3)	0 (0)	2 (33.3)	22 (10.0)	11 (9.2)	7 (10.9)	4 (11.4)
<1%	70 (50.7)	33 (55.9)	6 (37.5)	14 (60.9)	9 (75.0)	2 (100.0)	2 (33.3)	132 (60.3)	78 (65.0)	32 (50.0)	22 (62.9)
1–49%	39 (28.3)	8 (13.6)	3 (18.8)	3 (13.0)	1 (8.3)	0 (0)	1 (16.7)	50 (22.8)	26 (21.7)	18 (28.1)	6 (17.1)
≥50%	10 (7.2)	14 (23.7)	7 (43.8)	5 (21.7)	1 (8.3)	0 (0)	1 (16.7)	15 (6.8)	5 (4.2)	7 (10.9)	3 (8.6)
Adjuvant chemotherapy, n (%)	51 (37.0)	22 (37.2)	5 (31.3)	11 (47.8)	5 (41.7)	0 (0)	1 (16.7)	59 (26.9)	30 (25.0)	19 (29.7)	10 (28.6)
Medical history, n (%)											
COPD	23 (16.7)	18 (30.5)	6 (37.5)	6 (26.1)	1 (8.3)	1 (50.0)	4 (66.7)	19 (8.7)	9 (7.5)	9 (14.1)	1 (2.9)
Hypertension	68 (49.3)	36 (61.0)	9 (56.2)	13 (56.5)	9 (75.0)	0 (0)	5 (83.3)	107 (48.9)	53 (44.2)	33 (51.6)	21 (60.0)
Diabetes mellitus	33 (23.9)	15 (25.4)	7 (43.8)	8 (34.8)	3 (25.0)	0 (0)	0 (0)	35 (16.0)	18 (15.0)	9 (14.1)	8 (22.9)
Dyslipidemia	31 (22.5)	13 (22.0)	4 (25.0)	4 (17.4)	3 (25.0)	0 (0)	2 (33.3)	48 (21.9)	26 (21.7)	12 (18.8)	10 (28.6)

Table 3 (*continued*)

Table 3 (*continued*)

Characteristics	*KRAS* wild-type	*KRAS* mutation						*EGFR* mutation			
	All (n=138)	All (n=59)	*G12C* (n=16)	G12V (n=23)	*G12D* (n=12)	*G12A* (n=2)	Others (n=6)	All (n=219)	*L858R* (n=120)	*del19* (n=64)	Others (n=35)
Heart failure	0 (0)	4 (6.8)	1 (6.3)	0 (0)	2 (16.7)	1 (50.0)	0 (0)	3 (1.4)	0 (0)	2 (3.1)	1 (2.9)
Ischemic heart disease	0 (0)	4 (6.8)	2 (12.5)	0 (0)	1 (8.3)	0 (0)	1 (16.7)	6 (2.7)	6 (5.0)	0 (0)	0 (0)
Arrhythmia	11 (8.0)	10 (16.9)	0 (0)	5 (21.7)	2 (16.7)	1 (50.0)	2 (33.3)	22 (10.0)	10 (8.3)	7 (10.9)	5 (14.3)
Cerebrovascular disease	11 (8.0)	7 (11.9)	1 (6.3)	2 (8.7)	2 (16.7)	1 (50.0)	1 (16.7)	19 (8.7)	11 (9.2)	6 (9.4)	2 (5.7)
Autoimmune disorder	4 (2.9)	1 (1.7)	0 (0)	0 (0)	1 (8.3)	0 (0)	0 (0)	12 (5.5)	9 (7.5)	0 (0)	3 (8.6)
Malignant tumor	31 (22.5)	15 (25.4)	6 (37.5)	8 (34.8)	1 (8.3)	0 (0)	0 (0)	56 (25.6)	34 (28.3)	14 (21.9)	8 (22.9)

KRAS, Kirsten rat sarcoma virus; EGFR, epidermal growth factor receptor; PD-L1, programmed death ligand 1; COPD, chronic obstructive pulmonary disease.

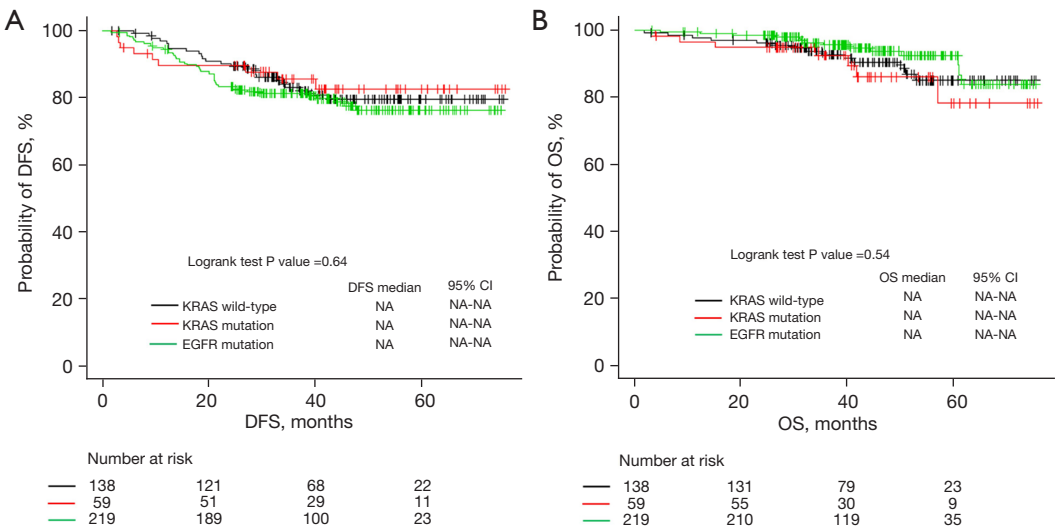

Figure 3 Kaplan-Meier curves for DFS (A) and OS (B) depending on mutation status. DFS, disease-free survival; KRAS, Kirsten rat sarcoma virus; EGFR, epidermal growth factor receptor; CI, confidence interval; NA, not available; OS, overall survival.

LNA PCR clamp method is about 0.1%. In comparison, the detection sensitivity of the therascreen PCR kit is only about 1%.

KRAS mutations are found in approximately 30% of lung adenocarcinomas and 4% of lung squamous cell carcinomas according to Western databases (12). In Japanese data, *KRAS* mutations are found in 9.7% of lung adenocarcinomas (22). This difference was attributed to the mutually exclusive nature of *KRAS* mutations with other driver mutations (30-33). *KRAS* mutations are less frequent in East Asia, where *EGFR* mutations are more common, and *KRAS* mutations are more common in Europe and the United States, where *EGFR* mutations are less frequent. In the present study, *KRAS* mutations were found in 12.7% of tumors, which is consistent with the frequency in Japanese patients.

Regarding *KRAS* mutation subtypes, a German prospective cohort study of advanced non-small cell lung

Table 4 Univariate and multivariate analysis on disease-free survival

Characteristics	Univariate analysis			Multivariate analysis		
	HR	95% CI	P value	HR	95% CI	P value
Age, years						
61–74 *vs.* ≤60	1.56	0.69–3.50	0.282			
≥75 *vs.* ≤60	1.60	0.68–3.75	0.279			
≥75 *vs.* 61–74	1.03	0.61–1.74	0.921			
Sex						
Male *vs.* female	1.62	0.99–2.65	0.054			
Stage						
II *vs.* I	3.13	1.65–5.95	<0.001	2.07	1.01–4.24	0.047
III *vs.* I	7.16	4.10–12.5	<0.001	4.47	2.30–8.69	<0.001
III *vs.* II	2.29	1.14–4.60	0.021	2.16	1.07–4.37	0.032
PD-L1 status						
1–49% *vs.* <1%	2.12	1.23–3.66	0.007	1.55	0.88–2.72	0.131
≥50% *vs.* <1%	3.48	1.84–6.58	<0.001	2.06	1.06–4.02	0.033
≥50% *vs.* 1–49%	1.64	0.84–3.19	0.144			
Smoking history						
Previous *vs.* never	0.97	0.53–1.75	0.910			
Current *vs.* never	2.23	1.25–3.96	0.006	1.40	0.77–2.55	0.227
Current *vs.* previous	2.31	1.20–4.43	0.012	1.82	0.94–3.54	0.076
Brinkman index						
600–1,200 *vs.* <600	1.47	0.85–2.52	0.165			
>1,200 *vs.* <600	1.23	0.44–3.42	0.695			
>1,200 *vs.* 600–1,200	0.84	0.28–2.46	0.746			
Mutation						
KRAS wild-type *vs. EGFR* mutation	1.02	0.60–1.74	0.936			
KRAS wild-type *vs. KRAS* mutation	1.08	0.50–2.34	0.849			
KRAS mutation *vs. EGFR* mutation	0.95	0.46–1.97	0.887			
Medical history						
Adjuvant chemotherapy	3.34	2.05–5.44	<0.001	1.68	0.93–3.04	0.084
COPD	1.98	1.11–3.52	0.020	1.20	0.64–2.25	0.574
Hypertension	0.80	0.49–1.30	0.376			
Diabetes mellitus	0.89	0.48–1.67	0.719			
Dyslipidemia	0.83	0.45–1.52	0.543			
Heart failure	0.78	0.11–5.62	0.805			
Ischemic heart disease	0.35	0.05–2.52	0.296			
Arrhythmia	1.08	0.49–2.36	0.855			
Cerebrovascular disease	0.98	0.40–2.45	0.974			
Autoimmune disorder	0.32	0.04–2.31	0.260			
Malignant tumor	0.80	0.45–1.45	0.468			

PD-L1, programmed death ligand 1; KRAS, Kirsten rat sarcoma virus; EGFR, epidermal growth factor receptor; COPD, chronic obstructive pulmonary disease; HR, hazard ratio; CI, confidence interval.

Table 5 Univariate and multivariate analysis on overall survival

Characteristics	Univariate analysis			Multivariate analysis		
	HR	95% CI	P value	HR	95% CI	P value
Age, years						
61–74 *vs.* ≤60	1.48	0.43–5.17	0.535			
≥75 *vs.* ≤60	1.90	0.53–6.81	0.325			
≥75 *vs.* 61–74	1.28	0.58–2.82	0.543			
Sex						
Male *vs.* female	1.55	0.73–3.29	0.249			
Stage						
II *vs.* I	2.15	0.70–6.61	0.180			
III *vs.* I	8.20	3.67–18.3	<0.001	7.31	3.18–16.8	<0.001
III *vs.* II	3.80	3.67–18.3	0.022	3.73	1.18–11.7	0.025
PD-L1 status						
1–49% *vs.* <1%	1.79	0.76–4.19	0.180			
≥50% *vs.* <1%	2.77	1.05–7.31	0.039	1.44	0.51–4.06	0.493
≥50% *vs.* 1–49%	1.55	0.55–4.36	0.406			
Smoking history						
Previous *vs.* never	1.54	0.65–3.63	0.323			
Current *vs.* never	2.21	0.86–5.71	0.101			
Current *vs.* previous	1.44	0.55–3.78	0.463			
Brinkman index						
600–1,200 *vs.* <600	1.34	0.59–3.07	0.485			
>1,200 *vs.* <600	0.86	0.11–6.44	0.883			
>1,200 *vs.* 600–1,200	0.64	0.08–5.14	0.675			
Mutation						
KRAS wild-type *vs. EGFR* mutation	1.46	0.62–3.43	0.389			
KRAS wild-type *vs. KRAS* mutation	0.61	0.23–1.60	0.311			
KRAS mutation *vs. EGFR* mutation	2.40	0.93–6.20	0.070			
Medical history						
Adjuvant chemotherapy	1.35	0.62–2.92	0.451			
COPD	1.96	0.79–4.85	0.145			
Hypertension	1.26	0.59–2.66	0.552			
Diabetes mellitus	2.32	1.07–5.03	0.033	2.36	1.08–5.16	0.031
Dyslipidemia	1.63	0.74–3.61	0.227			
Heart failure		Inf				
Ischemic heart disease		Inf				
Arrhythmia	0.34	0.05–2.49	0.287			
Cerebrovascular disease	2.27	0.78–6.59	0.130			
Autoimmune disorder		Inf				
Malignant tumor	0.65	0.25–1.70	0.378			

PD-L1, programmed death ligand 1; KRAS, Kirsten rat sarcoma virus; EGFR, epidermal growth factor receptor; COPD, chronic obstructive pulmonary disease; HR, hazard ratio; CI, confidence interval; Inf, infinity.

cancer reported that among patients with *KRAS* mutations, 38.9% were positive for *KRAS G12C* mutation, 21.2% for *G12V* mutation, and 13.9% for *G12D* (35). In the present study, the frequency of *KRAS* mutations was consistent with that in previous Japanese reports (22,23). However, the frequency of subtypes was different. In previous reports, *KRAS G12C* was the most common, but in this study, *G12V* was the most common, followed by *G12C*.

In previous reports, *KRAS G12C* was the most common, but in this study, *G12V* was the most common. The reason for this was unclear, and might be related to the fact that this study was conducted in an area with a high rate of smoking. *KRAS* mutations, in contrast to *EGFR* mutations, were more frequent in smokers (36,37). Several studies have shown an association between smoking and *KRAS* mutations: the guanine to thymine transversions resulting in *KRAS G12C* and *KRAS G12V* are more commonly found in past or current smokers; the guanine to adenine transition resulting in *KRAS G12D* is more common in non-smokers (36,38,39). In this study, the *G12C* and *G12V* mutations were more prevalent in heavy smokers, while the *G12D* mutation was not.

In this study, DFS was affected by PD-L1 in multivariate analysis, but OS was not affected by PD-L1. In a previously reported meta-analysis of 15 studies and 3,790 patients on prognosis in early resected NSCLC, PD-L1 expression was associated with significantly shorter DFS (HR =1.56, 95% CI: 1.18–2.05, P value <0.01), and significantly worse OS (HR =1.68, 95% CI: 1.29–2.18, P value <0.01) (40). These results differed from those of the present study. Also, there were no statistically significant differences in DFS and OS by genetic variants in this study. However, previously reported meta-analyses have shown that surgically treated NSCLC patients with *EGFR* mutations tend to have prolonged DFS and OS. Others have shown that *KRAS* mutations predict worse DFS and OS in resected NSCLC patients (41). This difference could be attributed to the shorter observation period in this study, fewer patients who relapsed, or fewer patients who received chemotherapy after relapse.

The prognosis of patients with *KRAS* mutations has been the subject of various studies. There are several reports of *KRAS* mutations having poor prognosis (42,43). On the other hand, a number of reports have found that *KRAS* mutations are not a poor prognostic factor (35,44). Many of these studies are retrospective cohort studies or were conducted before the advent of immune checkpoint inhibitors (ICI). In this study, lung cancer patients with *KRAS* mutations had a high frequency of PD-L1 expression. Similarly, several studies have shown that patients with *KRAS* mutation-positive tumors have higher PD-L1 expression and tumor mutation burden compared to *KRAS* wild-type patients (23,35,45). *KRAS* mutated tumors co-mutated with *TP53* or *cyclin dependent kinase inhibitor 2B* (*CDKN2a/B*) mutations are more responsive to treatment with immune checkpoint inhibitors (46). Treatment strategies including ICI are promising candidates for *KRAS* mutated lung cancer. ICI and *KRAS G12C* inhibitors such as sotorasib may alter the prognosis of *KRAS*-positive patients in the future.

In this study, *KRAS* gene mutations were detected using the PNA-LNA PCR clamp method, a highly sensitive detection method, together with the companion diagnostic therascreen kit. The two tests were all consistent for the *G12C* mutation, which is an indication for molecular targeted therapy. For mutations other than *G12C* mutation, the PNA-LNA PCR clamp had a higher detection rate. The PNA-LNA PCR clamp is more sensitive than the therascreen kit and is currently being considered for application to plasma gene mutation analysis. We have utilized the high sensitivity of the PNA-LNA PCR clamp method to monitor plasma *EGFR* gene mutations as a biomarker search in several clinical trials (27,47). We are now investigating the use of this sensitive and inexpensive method for plasma analysis of *KRAS* as well.

A limitation of this study is that it is a single-center surgical specimen study and has a short observation period. Although novel molecular-targeted agents are indicated for stage IV NSCLC, the target population in this study had earlier stage cancer. In addition, postoperative and recurrence-free survival was used as one of the outcomes, rather than PFS. Further studies will be needed for advanced stage lung cancer.

Conclusions

This study examined *KRAS* frequency and its background factors in Japan using surgical specimens, and found a trend toward higher frequency in smokers and males; *KRAS* mutations did not affect DFS and OS.

Both the PNA-LNA PCR clamp and therascreen kit were consistent in detecting *G12C* mutations; the PNA-LNA PCR clamp method was more sensitive and may be applicable to multiple situations in the detection of *KRAS* mutations in the future. Further studies in advanced lung cancer are warranted.

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Footnote

Reporting Checklist: The authors have completed the STROBE reporting checklist. Available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-23-15/rc

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-23-15/coif). HT is a current employee of LSI Medience, Inc., which is a for-profit company that deals with clinical testing and research equipment and reagents. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and was approved by the institutional ethics committee of Iwate Medical University (No. HG2021-002). The study is a retrospective observational study, with no new invasive procedures, and consent was obtained in an opt-out fashion.

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