Title: Analysis of intra-tumor heterogeneity of *EGFR* mutations in mixed-type lung adenocarcinoma

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Running title: Intra-tumor heterogeneity of EGFR in lung-cancer

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Micro abstract

Intra-tumor heterogeneity of epidermal growth factor receptor (*EGFR*) mutations in patients with mixed-type lung adenocarcinoma was analyzed according to histological patterns. Intra-tumor heterogeneity of *EGFR* mutations was detected in 9 of 38 tumors, and there was a significant correlation between heterogeneity of *EGFR* mutations and smoking history ($P<0.043$).

Clinical Practice Points

- *EGFR* mutations are factors predictive of response to EGFR-TKI treatment. However, lung cancer is thought to be the result of the accumulation of genetic alterations over a long course of exposure to a carcinogen, and the existence of intra-tumor heterogeneity of *EGFR* mutations remains unclear. Thus, the existence of intra-tumor heterogeneity was analyzed in patients with mixed-type lung adenocarcinoma according to histological patterns.
- Intra-tumor heterogeneity of *EGFR* mutations was detected in 9 of 38 tumors, and a significant correlation between heterogeneity of *EGFR*

mutations and smoking history was found.

- Response to EGFR-TKIs might be partial and insufficient in tumors having intra-tumor heterogeneity of *EGFR* mutations. New treatment strategies for patients with lung adenocarcinoma harboring heterogeneous *EGFR* mutations are needed.

Abstract

Background: Epidermal growth factor receptor *(EGFR)* mutations are predictive of the success of EGFR tyrosine kinase inhibitor (TKI) treatment in patients with advanced non-small cell lung cancer (NSCLC). As with other solid tumors, lung cancer is thought to be the result of an accumulation of genetic alterations following exposure to carcinogens. The aim of the present study was to clarify the relationship between multistep carcinogenesis and the accumulation of *EGFR* mutations. **Patients and Methods:** The intra-tumor heterogeneity of *EGFR* mutations was analyzed in 38 patients with resected mixed-type lung adenocarcinoma according to histological patterns, and the clinical features of the patients harboring intra-tumor heterogeneity of *EGFR* mutations were evaluated. **Results:** Intra-tumor heterogeneity of *EGFR* mutations was detected in 9 of 38 tumors. *EGFR* mutations were more common in the bronchioloalveolar (lepidic) carcinoma pattern than in the papillary and acinar patterns, although this difference was not significant. However, there was a significant correlation between intra-tumor heterogeneity of *EGFR* mutations and smoking history ($P<0.043$). **Conclusion:** Intra-tumor heterogeneity of *EGFR* mutations

correlates with the distribution of histological subtype in mixed-type adenocarcinoma and is associated with smoking history.

1. Introduction

Lung cancer is the most common cause of cancer-related deaths worldwide. Despite recent advances in the management of advanced non-small-cell lung cancer (NSCLC), the cure rate remains low.[1,2] Further molecular investigation of lung cancer is required to develop new treatment strategies and improve patients' prognoses.

Activation or proliferation of NSCLC is regulated by growth factors and receptors of the epidermal growth factor receptor (EGFR) subfamily.[3,4] Given this phenomenon, the first growth factor receptor to be proposed as a target in NSCLC treatment was EGFR and its signal transduction pathway. Gefitinib and erlotinib are EGFR tyrosine kinase inhibitors (TKIs) that are used in patients with advanced NSCLC. Recently, several studies have shown that *EGFR* mutations are factors predictive of response to EGFR-TKI treatment.[5-7] The most common mutations are a deletion in exon 19 and L858R point mutation.[8] Recently, two randomized, phase III trials were conducted in Japan to evaluate the efficacy of gefitinib in patients with chemotherapy-naïve, advanced NSCLC harboring *EGFR* mutations. In both trials, the patients treated with gefitinib had better progression-free survival

than those treated with platinum-doublet chemotherapy.[9,10] Gefitinib was subsequently approved in Japan as a first-line treatment option for patients with advanced NSCLC harboring *EGFR* mutations.

We previously reported a case of a patient with mixed-type lung adenocarcinoma (corresponding to "invasive adenocarcinoma" in the IASLC/ATA/ERS Classification of Lung Adenocarcinoma in Resection Specimens[11]) comprising papillary, acinar, and bronchioloalveolar carcinoma (BAC) (corresponding to "lepidic" in the IASLC/ATA/ERS Classification of Lung Adenocarcinoma in Resection Speimens[11]) patterns that responded partially to gefitinib after developing multiple metastases. In this patient, each pathological subtype contained a different type of *EGFR* mutation.[12]

As with other solid tumors, lung cancer is thought to be the result of the accumulation of genetic alterations over a long course of exposure to a carcinogen.[13] However, the genetic mechanism of this development is unclear in NSCLC, because there is no adequate means of monitoring one or a few genes; furthermore, the complexity of lung cancer cells limits pangenomic decipherment. In this study, intra-tumor heterogeneity of *EGFR* mutations was analyzed in patients with resected mixed-type lung adenocarcinoma

according to the histological patterns. Then, to clarify the relationship between multistep carcinogenesis and the accumulation of *EGFR* mutations, the clinical features of patients harboring intra-tumor heterogeneity of *EGFR* mutations were evaluated.

2. Methods

2.1. Patients and samples

Patients were recruited according to the following criteria: stage IA and IB mixed-type lung adenocarcinoma without poorly differentiated lesions (mixed-type lung adenocarcinoma and its histological pattern were defined in accordance with the 2004 World Health Organization pathologic (WHO) criteria[14]); underwent lobectomy or bilobectomy; age 85 years or younger; and without interstitial pulmonary fibrosis or occupational lung disease, such as asbestosis or silicosis. Cases with a poorly differentiated pattern were excluded because only clearly developed mixed type adenocarcinomas were evaluated in this study.

A total of 241 stage IA or IB lung cancer patients underwent surgery at Nagasaki University Hospital between 2002 and 2005. Of these, 181 had

adenocarcinoma, and a further 36 patients were excluded because they had been treated with segmentectomy or wedge resection. Of the 145 patients treated with lobectomy, 54 had mixed-type adenocarcinoma: 10 of these were excluded (3 were over 85 years old, 2 had asbestosis, 2 had adenocarcinoma with poorly differentiated lesions and 3 had pulmonary metastasis in the same lobe as the primary tumor on pathology). Of the remaining 44 cases, 6 were excluded because adequate genomic DNA could not be obtained, probably due to the long formalin-fixed time (Fig. 1). Thus, there were 38 samples of mixed adenocarcinoma with which to evaluate the intra-tumor heterogeneity of *EGFR* mutations. The clinical information was collected from the medical records of each patient. Disease-free survival (DFS) was defined as the time between the date of operation and disease progression, death from any cause, or last known follow-up.

Informed consent for use of the tumor specimens was obtained from all patients, and the Ethics Review Board on the Human Genome and Gene Analysis at Nagasaki University approved the study protocol.

2.2. Laser microdissection and DNA extraction

Each part of each specimen was classified into its lung adenocarcinoma pattern (papillary, acinar, or BAC) by a pathologist according to the 2004 WHO criteria.[14] Figures 2A, 2B, and 2C demonstrate the pathological features of an adenocarcinoma eligible for this study, which are papillary, acinar, and BAC, respectively. Approximately 5000-10000 tumor cells were microdissected according to the pathological patterns using a Leica application solutions laser micro dissection (AS LMD) system. Genomic DNA was extracted from tumor cells obtained from each microdissected specimen using MagExtractor™ –Genome (TOYOBO, Osaka, Japan) according to the manufacturer's protocol.

2.3. EGFR mutation analysis

The mutations of EGFR, deletions in exon 19 and point mutation of L858R in exon 21, were analyzed using mutant-enriched polymerase chain reaction (PCR) to increase the sensitivity of these mutations.[15] In brief, the procedure was as follows. The deletion region in exon 19 of *EGFR* was amplified by PCR with a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) from 10 ng of genomic DNA in a 25-μl reaction

mixture containing 0.5×GoTaq Green master mix (Promega, Madison, WI, USA), forward primer 5′-ATCCCAGAAGGTGAGAAAGATAAAATTC-3′ and reverse primer 5′-CCTGAGGTTCAGAGCCATGGA-3′. The amplification protocol comprised an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 5 min. The first PCR products were digested by restriction enzyme *Mse I* (New England BioLabs Inc., Beverly, MA, USA). The second PCR was carried out by 17 cycles using forward primer 5′-AAAATTCCCGTCGCTATC-3′ and reverse primer 5′-AAGCAGAAACTCACATCG-3′. The second PCR products were separated using 6% polyacrylamide gel electrophoresis (PAGE) (Nacalai Tesque, Kyoto, Japan) and visualized with an ultraviolet transilluminator (Alpha Innotech Co., San Leandro, CA, USA) after ethidium bromide (Nacalai Tesque) staining. Separately, the point mutation region in exon 21 of *EGFR* was amplified by PCR using 15 pmol each of forward primer 5′-CGCAGCATGTCAAGATCACAGAT-3′ and reverse primer 5′-TCCTGGTGTCAGGAAAATGCT-3′. The other constituents of the PCR mixture and the amplification protocol were as described above. The first

PCR products were digested by *Msc I* (New England BioLabs Inc.). After the digests were amplified using forward primer 5′-AGATCACAGATTTTGGGC-3′and reverse primer 5′-ATTCTTTCTCTTCCGCAC-3′, the second PCR products were digested by *Asu I* (Fermentas International Inc., Burlington, Canada). The digests were then separated on 8% PAGE and visualized by ethidium bromide staining.

2.4. Statistical analysis

The survival curves were calculated by the Kaplan-Meier method, and their difference was determined by the log-rank test. Pearson's χ^2 test and Fisher's exact test were performed to analyze variables. A two-tailed $P < 0.05$ was considered significant.

3. Results

3.1. Patients' characteristics and survival

The patients' characteristics are listed in Table 1. The median age was 68 years. Of these 38 patients, 22 (56%) were women, 30 (79%) were stage IA, and 24 (63%) were light (fewer than 10 packs per year) and never smokers.

The median DFS was 65.4 months. Four patients had recurrent disease. One had bronchial stump recurrence that was treated with stereotactic irradiation (STI). Two patients had new pulmonary nodules and were diagnosed with lung cancer recurrence; they were also treated with STI, and one of them died of radiation pneumonitis. One patient was diagnosed with a malignant pleural effusion and was treated with EGFR-TKIs because the patient was harboring the exon 19 deletion of *EGFR*.

The median overall survival from surgery was 65.8 months. Two patients have since died, one of pancreatic cancer and the other of sepsis after pacemaker implantation.

3.2. Histological subtypes and EGFR mutation analysis

Of the 38 tumors, 24 contained papillary, acinar, and BAC (lepidic) patterns, 11 had papillary and BAC (lepidic) patterns, and 3 had papillary and acinar patterns. The deletion in exon 19 and L858R mutation in exon 21 of *EGFR* were detected in 19 tumors (50%). There were no significant correlations between the presence or absence of *EGFR* mutations and age, sex, stage, or smoking status.

Intra-tumor heterogeneity of *EGFR* mutation was detected in 9 of the 38 tumors (Table 2). In five of these nine tumors, the papillary pattern contained wild-type *EGFR*; three of six tumors contained wild-type *EGFR* in the acinar pattern; and two of eight tumors contained wild-type *EGFR* in the BAC (lepidic) patterns. *EGFR* mutations appeared more common in the BAC subtype group; this observation was not significant (data not shown), and the same tendency was seen in all 38 tumors (data not shown). Two of the nine tumors contained both mutations, and one tumor consisted of mutation area only. Statistical analysis was not conducted due to the small sample size.

The characteristics of the nine patients with tumors showing intra-tumor heterogeneity are listed in Table 3. Of these 9 patients, 5 (56%) were men, 7 (78%) were stage IA, and 6 (67%) were smokers. Heterogeneity was found more frequently in smokers ($P<0.043$), but its relationships to age, sex, and disease stage were not significant (Table 4). In addition, the micropapillary pattern was not detected in any of the nine tumors.

3.3. Disease-free survival

DFS was stratified by sex, stage, age, smoking status, *EGFR* mutation, and

genetic heterogeneity of *EGFR*. The only significantly better DFS was seen in stage IA patients compared with stage IB ($P<0.001$) (data not shown).

4. Discussion

The results of the present study show that intra-tumor heterogeneity of a deletion of exon 19 and an L858R point mutation exists in accordance with the histological pattern in mixed-type adenocarcinoma. *EGFR* mutations were more common in the BAC (lepidic) pattern than in the papillary and acinar patterns, though the difference was not significant. A significant association was seen between intra-tumor heterogeneity of *EGFR* mutations and smoking history. These findings suggest that *EGFR* mutations might accumulate during tumor progression and occur more frequently in smokers.

EGFR status was examined in 38 cases of mixed-type lung adenocarcinoma, and mutations were detected in 19 cases (50%). It has previously been reported that approximately 50% of Japanese patients with lung adenocarcinoma harbors *EGFR* mutations.[16] Furthermore, Sugio et al. demonstrated that there is no significant relationship between stage and the presence of *EGFR* mutations.[17] Thus, the present result is comparable with

previous reports. Conversely, *EGFR* mutations have been reported to be significantly associated with never-smoking status,[18, 19] although several studies have shown that some Japanese patients with a smoking history harbor *EGFR* mutations. In two randomized, prospective, phase III trials in patients with advanced NSCLC harboring *EGFR* mutations, 141 of 400 (35.3%) patients had a smoking history.[9,10] In the present study, 7 of 19 (36.7%) patients with *EGFR* mutations had a smoking history. Thus, the incidence of *EGFR* mutations in the smoking population in the present study was also comparable with recent results.

It has been proposed that lung adenocarcinoma develops in a stepwise fashion from atypical adenomatous hyperplasia (AAH) through BAC (lepidic) to invasive adenocarcinoma.[20, 21] While the pathogenesis remains unknown, several studies have demonstrated *EGFR* abnormalities in the progression of lung adenocarcinoma.[22-24] Tang et al. performed a precise mapping analysis of *EGFR* mutations, gene copy number, and total and phosphorylated EGFR protein expressions for the same tissue sites. They examined normal bronchial and bronchiolar epithelium (NBE), tumor tissues, and some corresponding lymph-node metastases and showed that *EGFR* mutations

precede gene copy number abnormalities in the pathogenesis of these tumors, and that expressions of EGFR and pEGFR immunohistochemical protein are common in NBE.[21] However, Ohashi et al. reported that transgenic mice expressing the delE748-A752 mutant version of mouse *EGFR* driven by the SP-C promoter developed multifocal lung adenocarcinomas with the AAH and BAC (lepidic) pattern at 3-5 weeks of age.[25] Others also concluded that the mutations are early, pre-invasive changes, whereas copy number gains are later events associated with the invasive phenotype.[23,24] In the present study, the micro-dissected BAC (lepidic) pattern was thought to correspond to a pre-invasive tumor lesion: *EGFR* mutations were more common, though not significantly, in the BAC (lepidic) pattern. The hypothesis that AAH progresses to BAC (lepidic) might be adoptable in the tumor having BAC (lepidic) pattern subtype and harboring *EGFR* mutations homogeneously. The response of these tumors to EGFR-TKIs is expected to be good and homogeneous.

EGFR mutations were seen more often in the BAC (lepidic) pattern, which is thought to develop in the acinar or papillary invasive component, than in the papillary and acinar patterns, though the difference was not

significant in this study. Taniguchi et al. showed that intra-tumor heterogeneity existed in proximity to the same pathological patterns.[26] Since *EGFR* mutations were analyzed according to the pathological patterns in this study, the mutation-positive and -negative BAC (lepidic) patterns could also be examined at the same time. All patients analyzed in this trial had early stage lung adenocarcinoma, and it is possible that the *EGFR* mutation-positive BAC (lepidic) pattern may have developed to the *EGFR* mutation-positive invasive component, such as acinar or papillary patterns, if this adenocarcinoma had not been resected. Most studies evaluating the intra-tumor heterogeneity of *EGFR* mutations used the resection specimens obtained from resectable stage lung adenocarcinoma, including the present study. In further investigations, physicians will try to use the pathological specimens obtained from advanced lung adenocarcinoma patients so as to then clinically treat with EGFR-TKIs.

Recently, it has been reported that intra-tumor heterogeneity of *EGFR* mutation status is extremely rare.[27,28] Yatabe et al. reported that pseudo-heterogeneity with less sensitive *EGFR* mutation detection methods is associated with unbalanced mutation signals due to *EGFR* amplification.[27]

In the present study, the sensitivity of the mutant-enriched PCR method was not inferior to the PNA-LNA PCR and PCR clamp methods.[29] They also analyzed intra-tumor heterogeneity without considering the pathological patterns. We considered that the intra-tumor heterogeneity could be demonstrated by an analysis in accordance with the pathological pattern of mixed-type adenocarcinoma in the present study. On the other hand, the possibility of intra-tumor heterogeneity has also been reported.[30,31] In these papers, the heterogeneity pattern was the combination of subclones with and without *EGFR* mutations. The present data are also comparable with these results. Further examinations need to clarify whether there is intra-tumor heterogeneity of *EGFR* mutation status in lung adenocarcinoma.

In conclusion, intra-tumor heterogeneity of a deletion of exon 19 and an L858R point mutation correlates with the histological patterns in mixed adenocarcinoma. This heterogeneity was significantly associated with smoking history. With heterogeneity in advanced lung adenocarcinoma, the response to EGFR-TKIs would be partial and insufficient. New treatment strategies, such as the combination of EGFR-TKIs and cytotoxic agents in chemotherapy, are needed to improve the prognosis of patients with lung

adenocarcinoma harboring heterogeneous *EGFR* mutations.

Conflict of Interest Statement

No financial support was received for this publication, and none of the authors has any conflicts of interest to declare.

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Figure legends

Figure 1. Study profile.

Figure 2. Pathological features of the different areas of a mixed type lung adenocarcinoma according to 2004 WHO criteria. (A) papillary adenocarcinoma {100×magnification, Hematoxylin and Eosin (H&E) staining}, (B) acinar adenocarcinoma (100×magnification, H&E staining), (C) bronchioloalveolar carcinoma (100×magnification, H&E staining).

Table 1. Patients Characteristics (n=38)

	No. of Patients
Age (years)	
Median (range)	68 (39-83)
Gender	
Male	16
Female	22
Disease stage	
IA	30
IB	8
Smoking history	
Never/light smoker	24
Smoker	14

Table 2. *EGFR* mutation status in mixed adenocarcinoma with genetic heterogeneity (n=9)

Case ID	Papillary subtypes	Acinar subtypes	BAC subtypes
1	L858R	-	wild type
2	ex19 del	wild type	-
3	L858R	ex19 del, L858R	L858R
4	wild type	ex19 del	wild type
5	wild type	-	ex19 del, L858R
6	wild type	wild type	ex19 del
7	wild type	-	ex19 del
8	wild type	ex19 del	ex19 del
9	L858R	wild type	L858R

Table 3. Patients Characteristics of mixed adenocarcinoma with *EGFR* genetic heterogeneity (n=9)

	No. of Patients
Age (years)	
Median (range)	67 (53-81)
Gender	
Male	5
Female	4
Disease stage	
IA	7
IB	2
Smoking history	
Never/light smoker	3
Smoker	6

Table 4. Correlation of patients profiles and intra- tumor heterogeneity of *EGFR* mutation

Factors	Odd Ratio	p value
Age, <68 years	1.429	0.6398
Sex, Male	1.875	0.4091
Stage, IA	1.065	0.7027
Smoking	4.667	0.0474

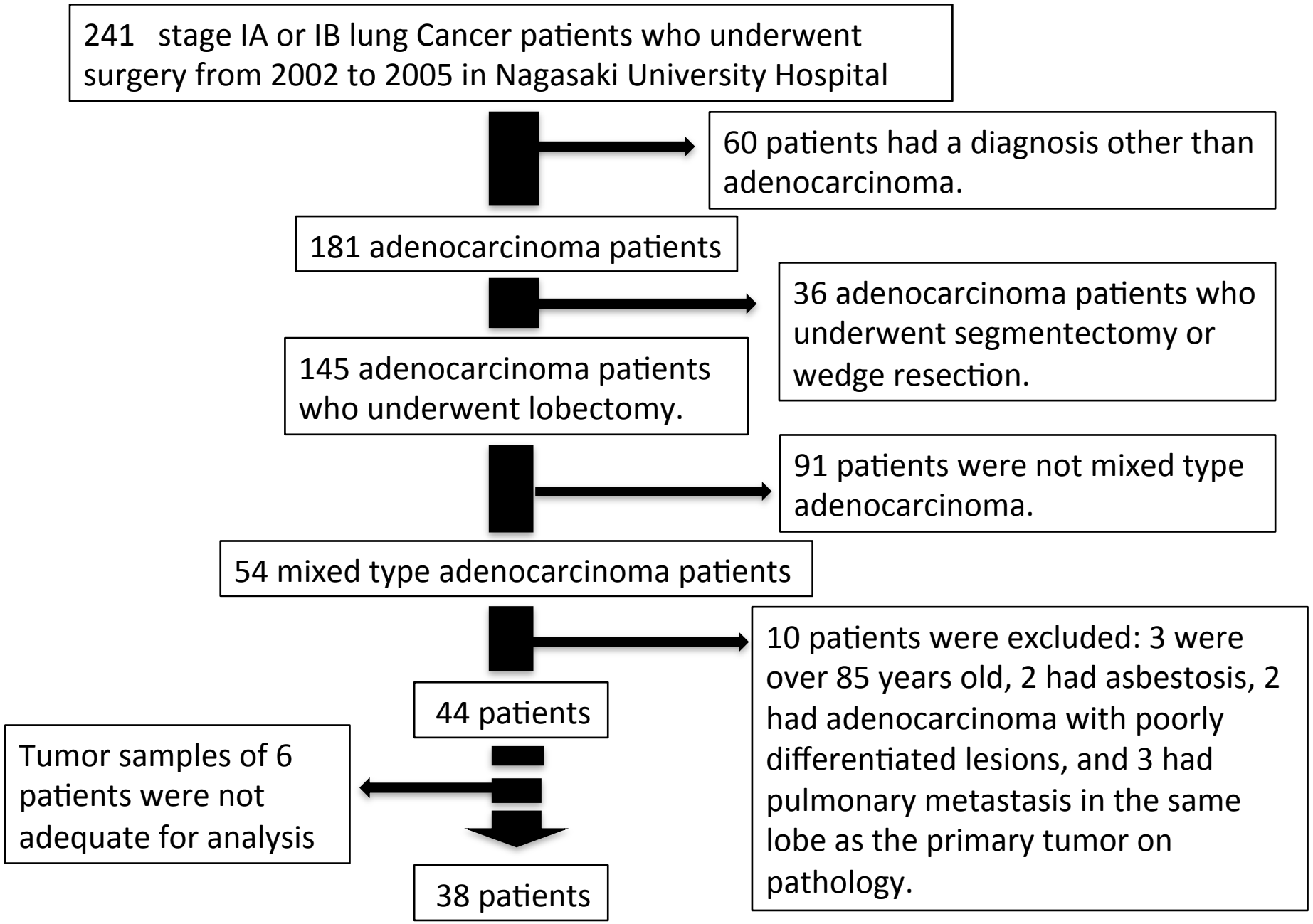
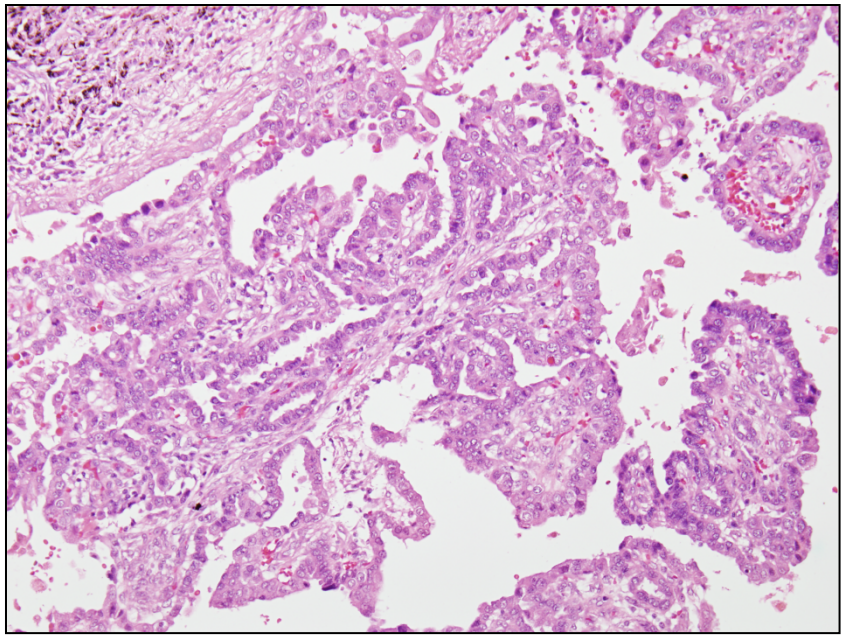

241 stage IA or IB lung Cancer patients who underwent surgery from 2002 to 2005 in Nagasaki University Hospital
60 patients had a diagnosis other than adenocarcinoma.
181 adenocarcinoma patients
36 adenocarcinoma patients who underwent segmentectomy or wedge resection.
145 adenocarcinoma patients who underwent lobectomy.
91 patients were not mixed type adenocarcinoma.
54 mixed type adenocarcinoma patients
10 patients were excluded: 3 were over 85 years old, 2 had asbestosis, 2 had adenocarcinoma with poorly differentiated lesions, and 3 had pulmonary metastasis in the same lobe as the primary tumor on pathology.
44 patients
Tumor samples of 6 patients were not adequate for analysis
38 patients

Figure 1

A



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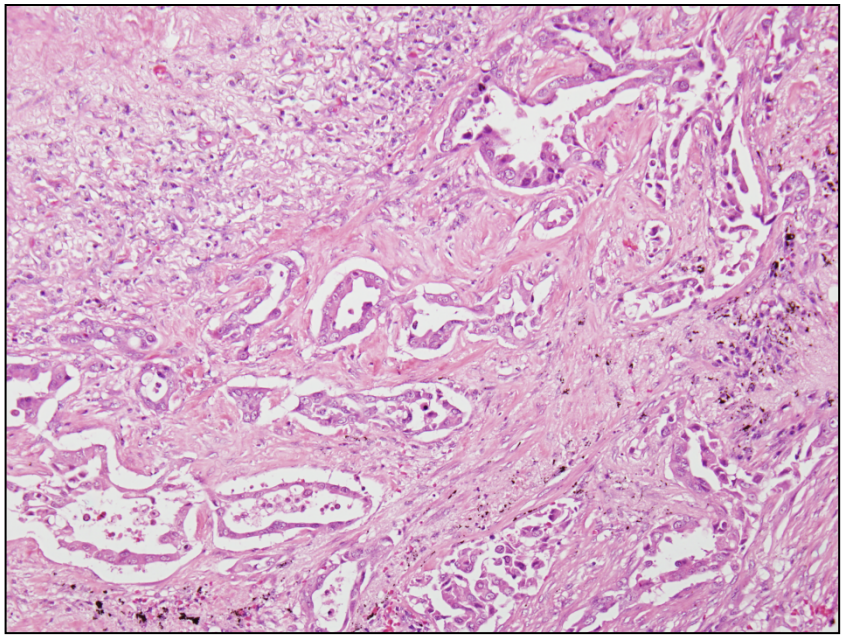

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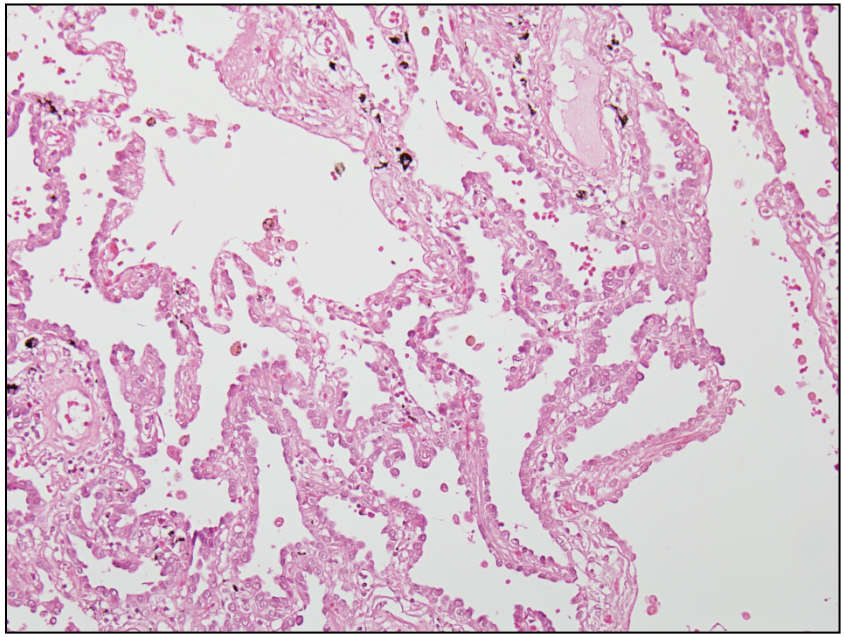

Figure 2