Table 2 Incidence of pneumonia hospitalisations and pneumonia-associated mortality among people aged ≥18 years before and after the 2011 Tohoku earthquake and tsunami, Kesennuma City, Miyagi, Japan

	Pre-disaster period (1 March 2010–10 March 2011)*			Post-disaster period (11 March 2011–30 June 2011)*			
	Pop.†	N‡	Weekly incidence rate§ (95% CI)	Pop.†	N‡	Weekly incidence rate§ (95% CI)	Rate ratio (95% CI)¶
Pneumonia hospitalisations							
Total	63365	305	9.2 (8 to 10.4)	61104	208	38.3 (28.6 to 48)	5.7 (3.9 to 8.4)
Age category (years)							
18–49	23354	14	1 (0.4 to 1.5)	22291	6	3.6 (-0.4 to 7.7)	10 (1.9 to 54.3)
50–64	17590	24	2.5 (1.3 to 3.6)	17245	18	7.3 (0.6 to 14)	6.1 (1.5 to 24.7)
65–79	15803	85	10.6 (8.2 to 13.1)	15241	62	62.6 (37.5 to 87.7)	6.2 (3.3 to 11.5)
80+	6618	182	52.3 (43.8 to 60.8)	6327	122	193.3 (129.1 to 257.5)	5.2 (3.2 to 8.5)
Residence location							
Home	62239	262	8.1 (7 to 9.2)	54460	111	21 (12.9 to 29)	2.7 (1.7 to 4.4)
Nursing home	1126	43	57 (38.6 to 75.5)	796	38	882.8 (481.3 to 1284.3)	28.2 (11.7 to 68)
Evacuation shelter	–	–	–	5848	59	328.7 (190.8 to 466.7)	10.2 (6.2 to 16.9)
Pneumonia-associated deaths							
Total	63365	55	1.6 (1.2 to 2.1)	61104	49	12.8 (7.5 to 18.1)	8.9 (4.4 to 17.8)
Age category (years)							
18–79	56747	13	0.4 (0.2 to 0.7)	54777	12	8.7 (3 to 14.4)	18.6 (5.3 to 64.9)
80-	6618	42	12 (8.5 to 15.5)	6327	37	66.3 (32.8 to 99.8)	6.7 (3 to 14.8)
Residence location							
Home	62239	46	1.4 (1 to 1.8)	54460	27	7.1 (2.7 to 11.5)	4.8 (2 to 11.2)
Nursing home	1126	9	12.4 (4.5 to 20.3)	796	17	555.2 (216.6 to 893.7)	40.6 (9.1 to 180.8)
Evacuation shelter	–	–	–	5848	5	80.6 (0.2 to 160.9)	11.6 (3.7 to 36.2)

*The pre-disaster and post-disaster cases were categorised according to the date of onset. The near-drowning-related cases were excluded.
†Population in 28 February 2011 for the pre-disaster period and in 31 May 2011 for the post-disaster period. The population in each residential category reflects the period average. Data provided by Kesennuma City Hall.
‡Number of patients living in Kesennuma.
§Per 100 000 people. Weekly incidence rates were estimated using segmented generalised linear Poisson regression models allowing for time trends and the change in the population size.
¶Rate ratios were estimated using segmented generalised linear Poisson regression models. Rate ratios for evacuation shelter residents were estimated using the overall pre-disaster incidence as a reference.

disasters on pneumonia incidence was overlooked in developing countries with relatively young populations.

A comparable event may have been observed in Japan after the Hanshin-Awaji earthquake that occurred in Hyogo Prefecture (where 15% of the population were aged ≥65 years) in January 1995. Among 1948 patients admitted for illness during the first 15 days after the earthquake, 418 (21%) had pneumonia. Their average age was 66 years,[24] although population-based impact estimates were unavailable. In contrast, no pneumonia outbreak was documented after Hurricane Katrina, which occurred during the summer.[25 26] Freezing temperatures may be a critical factor in pneumonia outbreaks after a disaster.

In our study, eight cases of near-drowning-related pneumonia were identified. Pneumonia associated with the aspiration of tsunami water drew global attention after a series of melioidosis cases among the Indian Ocean tsunami survivors was reported.[8–10] This condition has been sometimes referred to as 'tsunami lung', which is defined as pneumonia caused by the aspiration of tsunami water containing soil, oil and sewage.[27 28] However, there is no evidence that this condition is distinct from seawater drownings unrelated to tsunami disasters. Furthermore, the clinical characteristics of victims of the Indian Ocean tsunami may not be comparable to those of patients in settings where *Burkholderia pseudomallei* is not endemic, as in our case. Natural disasters do not cause new diseases that are not endemic to the affected area.[29–31] The term 'tsunami lung' must be used with caution to avoid media sensationalism.

The limitations of our study arise from the nature of hospital-based data collection. In Japan, 70% of the medical costs for people aged <70 years and 80–90% of the medical costs for people aged ≥70 years are covered by insurance,[32] and all medical fees for the disaster-affected people were waived after 11 March.[33] The cost was not a barrier to hospitalisation throughout the study period. Non-pneumonia diseases, such as heart failure, might have been misdiagnosed as pneumonia during the post-disaster period especially among older patients. However, the cases in this study were confirmed by experts using a standardised case definition, and the microbiological confirmation rate was similar between the pre-disaster and post-disaster period. Thus, the impact of misclassification and potential changes in admission criteria on our incidence estimates must be minimal. However, due to the limited microbiological data, the aetiology of our cases was not fully established.

Pneumonia and pneumonia-related deaths among older people have been overlooked in emergency preparedness and humanitarian responses, most likely because both are common events in this population. The key findings of our study are: disaster-affected people, especially those exposed to stressful living conditions, are at high risk of developing pneumonia and pneumonia-related death during the emergency phase of a disaster; and the pneumonia burden becomes substantial in areas with an aging population. This situation may arise in low-income and middle-income countries, as their populations are rapidly aging.[34] In addition to using the PPV23 or pneumococcal conjugate vaccine for disaster-affected populations, the provision of optimal living conditions, medical check-ups and oral hygiene care must be a priority for older people after natural disasters.[35]

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Review Article

Importance of the cytological samples for the epidermal growth factor receptor gene mutation test for non-small cell lung cancer

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Mutations in the epidermal growth factor receptor (*EGFR*) gene confer it with cancer driver gene functions in non-small cell lung cancer (NSCLC). Epidermal growth factor receptor -tyrosine kinase inhibitors are effective agents against NSCLC with a mutated *EGFR* gene. Accordingly, many guidelines recommend the use of an *EGFR* mutation test in NSCLC. However, not all patients are tested in most countries where tissue samples are mainly used for the test. As of 2011, most of the patients with advanced NSCLC are tested in Japan, and the use of cytological samples has significantly contributed to this success. A portion of samples used to determine a definite diagnosis of NSCLC, either tissue samples or cytological samples, is ensured to contain cancer cells, and is then investigated by an *EGFR* mutation test that is applicable to both tissue samples and cytological samples. Cytological samples now account for one-third of all the samples investigated. *EGFR* mutation is detected in cytological samples at a similar rate with tissue samples. The criterion ensuring an *EGFR* mutation test to have satisfactory sensitivity and specificity for use in both tissue and cytological samples is presented. Cytological samples are valuable clinical sources being collected less invasively than tissue samples, and should therefore be extensively used in *EGFR* mutation testing. (*Cancer Sci* 2013; 104: 291–297)

Cancer driver genes are mutated genes that confer a significant growth advantages on cells and play key roles in the cancer development.[1,2] Therapies targeting cancer driver genes have presented dramatic responses in many malignancies, including lung cancer,[3–6] leukemia,[7] and melanoma.[8] Information on cancer driver gene is indispensable for selecting an appropriate treatment for particular cancers.

Somatic mutations in the epidermal growth factor receptor (*EGFR*) gene are frequently observed in non-small cell lung cancer (NSCLC).[9–11] The mutated *EGFR* gene is a cancer driver gene and NSCLCs harboring it responds well to treatment with EGFR-tyrosine kinase inhibitors (EGFR-TKIs) such as gefitinib and erlotinib.[3–6] Many therapeutic guidelines recommend the use of EGFR-TKIs for the treatment of NSCLC with mutated *EGFR*.[12–14] Accordingly, an increasing number of patients with NSCLC have been tested for *EGFR* mutations. The procedures for testing have been discussed.[15–17] However, a significant proportion of patients are still untested in many counties, simply because tissue samples are not available. In contrast, almost all patients have been tested in Japan, where either tissue samples or cytological samples are used for the mutation test. Cytological samples have advantages over tissue samples: the former is collected using less-invasive procedures than the latter, while the former is suited to *EGFR* mutation test similarly to the latter. Here, we summarize the sampling and testing scheme enabling *EGFR* mutation test in cytological samples. The scheme may be useful worldwide and applicable to many solid tumors other than NSCLC.

Importance of cytological samples for *EGFR* mutation test in NSCLC

Figure 1 shows the sequence of events in NSCLC diagnosis and treatment in clinical practice. First, lung cancer is provisionally diagnosed by the imaging studies. Next, samples are collected from the legion suspicious of cancer. Pathologists examine the sample and determine a definite diagnosis. Treatment is started thereafter.

By dividing the samples submitted for pathological examination into aliquots [Fig. 1(B)], the mutation test can be performed for all patients without the need to collect additional samples. Moreover, information on the mutation status is readily applicable to the determination of the treatment regimens. Determination of *EGFR* mutation status at this timing is the most practical and useful.

Either a tissue sample or a cytological sample is submitted to the pathologists. Tissue samples include surgically resected samples and biopsy samples. Cytological samples include sputum, bronchoscopy samples (obtained by brushing or washing), pleural effusion, and samples obtained by fine needle aspiration. Tissue samples are collected from only a portion of patients, while cytological samples are collected from almost all patients. For example, a cytological sample (i.e. pleural effusion) is easily aspirated from patients with malignant pleural effusion, while a tissue sample is very difficult to obtain from such patients. Moreover, the invasive procedures required to collect tissue samples are often contraindicated in patients with a poor performance status.[18] *EGFR* mutation tests that are applicable only to tissue samples exclude the patients described above and thus are unacceptable.

Contamination of the normal cells

Collecting samples that solely contain cancer cells is almost impossible. Stromal cells and blood cells are normal cells that inevitably contaminate cancer samples. Normal *EGFR* gene sequence in the genomic DNA derived from normal cells obscures the somatic mutations carried in cancer cells. Figure 2 shows the percentage of the cancer cells to the total

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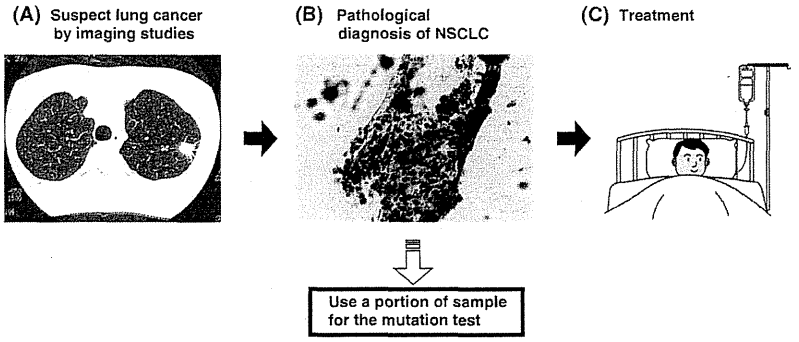

Fig. 1. Flow chart showing the routine clinical practice for non-small cell lung cancer (NSCLC) diagnosis and treatment. (A) Lung cancer is suspected by imaging studies. (B) A definite diagnosis of NSCLC is determined by pathological examination. Because a definite diagnosis is mandatory before initiating cancer treatment, all patients provide either tissue samples or cytological samples containing cancer cells. At this point, access to the cancer cells is available and we are able to perform the mutation test. (C) Treatment is initiated after a definite diagnosis is determined.

number of cells in clinical samples.[19] Tissue samples contained many normal cells, and cytological samples contain more of these cells. Empirically, the lowest percentage of cancer cells in pathologically cancer-positive samples is 1%. Samples with a percentage of <1% may also exist. However, Figure 2 suggests that pathologists hesitate to determine a definite diagnosis using such samples and thus request re-sampling. Therefore, 1% is a good estimate of the detection limit of pathological examination, and thus is a detection limit obligatory for an *EGFR* mutation test to be applicable to all pathologically cancer-positive samples. This is the theoretical consensus in our country and constitutes qualification criterion for *EGFR* mutation tests.[19,20]

Procedure ensuring the presence of cancer cells

Figure 3 illustrates sample submission procedures. The presence of cancer cells should be confirmed before performing *EGFR* mutation test, otherwise false-negative results are obtained. For tissue samples (Fig. 3A), serial thin sections are made: the presence of cancer cells is confirmed in one section, and the test is performed with the other sections. For cytological samples (Fig. 3B), the cells are suspended and mixed well in a saline buffer. The suspension is then divided into two aliquots. The presence of cancer cells is confirmed in one aliquot, and the other is kept frozen or stored in a DNA-isolation solution (e.g. AL buffer; Qiagen, Hilden, Germany) until the pathological examination is complete. Tissue samples may be treated in the same manner as cytological samples (Fig. 3C). In the last procedure, formalin fixation, which fragments DNA into small pieces, is avoided, as well as quick penetration of the DNA-isolation solution into the cells is enabled. Tissues processed as shown in Figure 3C thus yields more definitive results in the test than those treated as shown in Figure 3A.

The procedure shown in Figure 3B,C also applies when cytological samples are subjected to clinical tests based on reverse transcriptase-PCR (RT-PCR) reaction, for example, detection of the fusion genes such as *EML4-ALK*.[21] In such case, the following modifications should be made: cells in the aliquot for the mutation test should be collected by centrifugation (1300*g*, 5 min) at the earliest convenience after the sample collection (e.g. 20 min) and stored in a RNA protect reagent (e.g. RNAprotect Cell Reagent; Qiagen). Many RNA protect reagents allow us to isolate both DNA and RNA, and thus to perform both PCR- and RT-PCR-based investigations.

Recently, liquid-based cytology is often used for the diagnosis of NSCLC. It has been reported that *EGFR* mutation test is reliably performed for liquid-based cytology samples when combined with high sensitivity detection methods.[22] The procedure shown in Figure 3B,C is applicable to liquid-based cytology samples, and should be strictly observed.

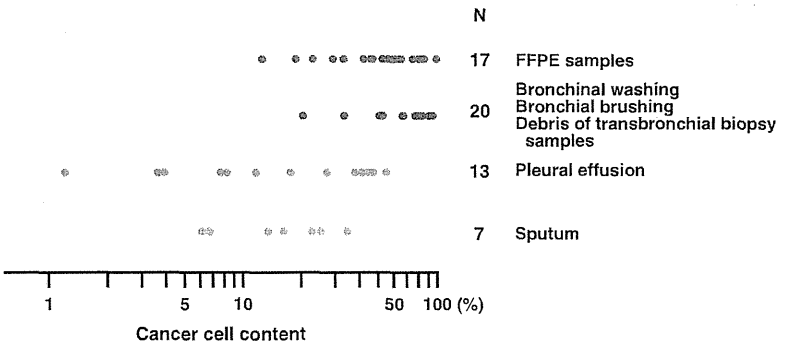

Fig. 2. Ratios of cancer cells to normal cells in pathologically cancer-positive samples. The ratios of the number of cancer cells to the total number of cells in a variety of samples are shown (modified from Tanaka *et al.*[19]) Archival slides that had enabled a definite cancer diagnosis were randomly chosen, and the numbers of cancer cells and normal cells were counted. Tissue samples (i.e. formalin-fixed, paraffin-embedded [FFPE] samples) are indicated by a warm color and cytological samples (i.e. the others) are indicated by cold colors.

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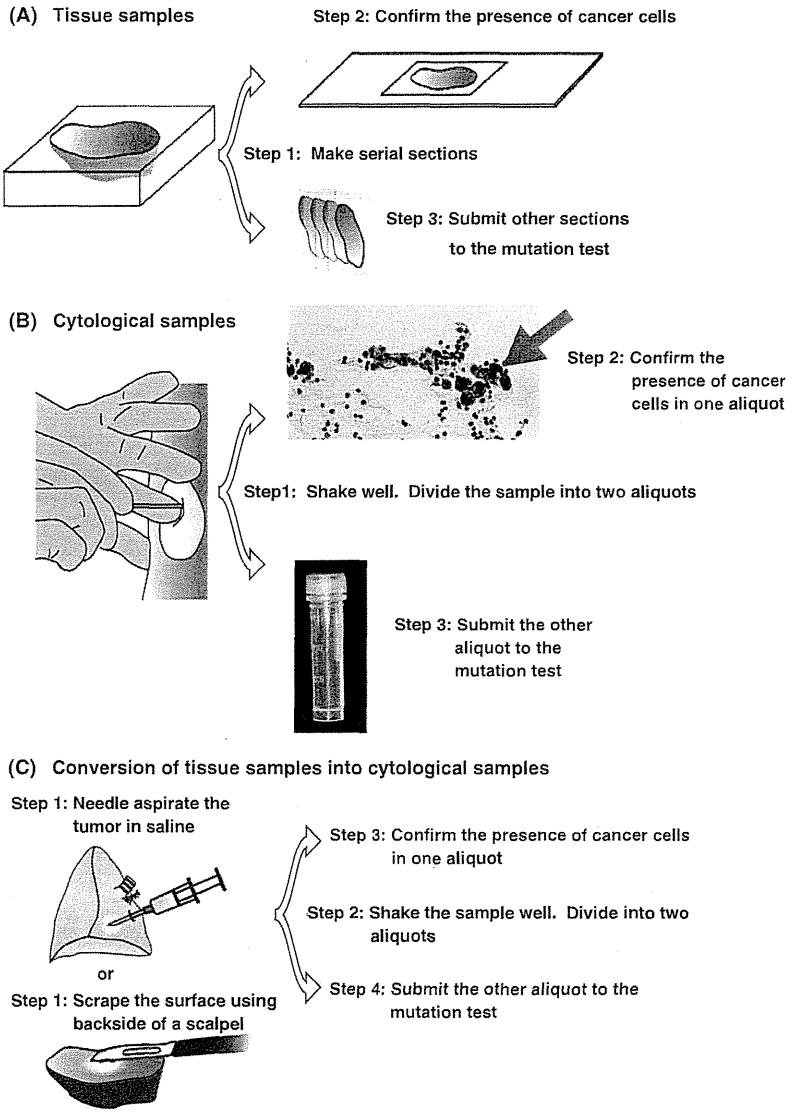

Fig. 3. Sample preparation procedures. (A) Tissue samples. Step 1: Serial sectioning. Step 2: The presence of cancer cells is confirmed in 1 section. Step 3: The *EGFR* mutation is investigated using other sections. Macro-dissection may be required to remove normal tissue before step 1. (B) Cytological samples. Step 1: Suspend the cells in saline. Divide the samples into two aliquots. Step 2: Confirm the presence of cancer cells in one aliquot. Step 3: Investigate the *EGFR* mutation using the other aliquot. (C) Preparation of cytological samples from tissue. Step 1: Scrape the surface of the tissue. Suspend the cells in saline. Step 2: Divide the samples into two aliquots. Step 3: Confirm the presence of cancer cells in one aliquot. Step 4: Investigate the *EGFR* mutation using the other aliquot.

EGFR mutation test statistics in Japan

Figure 4A shows the cumulative number of *EGFR* mutation tests performed in the three major, commercial Japanese laboratories. An additional 2000 or more samples are tested in university or hospital laboratories. The cost of the test was reimbursed by the National Health Insurance on an once-in-a-lifetime basis until March 2012. Currently, it is reimbursed on an every-exacerbation basis. Therefore, the numbers before March 2012 are almost equal to the numbers of the patients tested. The number of patients newly diagnosed with advanced NSCLC is estimated to be 50 000/year.[23,24] Altogether, most of the patients with an advanced disease, and thus are the targets of EGFR-TKIs, were tested in 2011.

The percentage of the samples with a mutated *EGFR* gene decreased as the number of the tests approached the number of patients with advanced NSCLC. This is likely because of an increase in the number of samples with fewer mutations; that is, samples with non-adenocarcinoma histology, or samples collected from aged male patients.

Figure 4B shows the fraction of samples submitted to the test according to category. Almost 40% were cytological samples. This demonstrates that the use of cytological samples is indispensable for testing all advanced NSCLC patients. Accordingly, almost all *EGFR* mutation tests have been performed by one of three highly sensitive, PCR-based methods that include the PNA–LNA PCR clamp,[4,18,19,25–27] the Cycleave method,[5] and the PCR invader,[20] all of which detect *EGFR* mutations in samples with a ratio of cancer cells of 1%.[20]

Figure 4C shows the rates of *EGFR* mutations according to sample categories. The mutation rates for tissue samples and cytological samples were similar. A direct comparison between each category may be inappropriate because an inherent difference should exist in the mutation rate between the categories. For example, the mutation rate for pleural effusion is likely to be high because malignant pleural effusion is mostly caused by adenocarcinoma. The rate of samples in which DNA failed to be amplified by PCR is high in formalin-fixed, paraffin-embedded (FFPE) samples, probably because of the fragmentation of DNA by formalin.[28,29]

Clinical studies and cytological samples

The use of cytological samples enables a rapid accrual of patients for a variety of clinical trials. Clinical studies in which

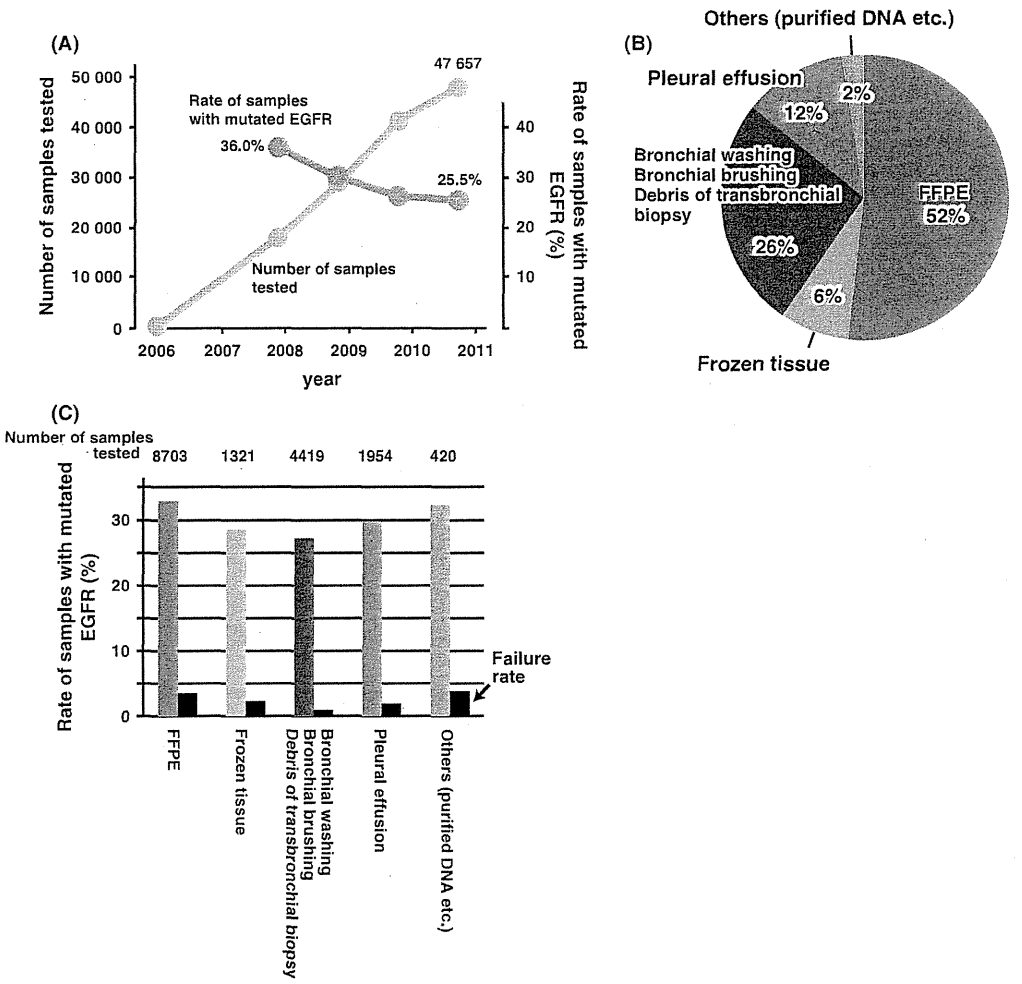

Fig. 4. The *EGFR* mutation test in Japan. (A) The number of the *EGFR* mutation tests performed in three major commercial laboratories in Japan. The rate of *EGFR* mutation-positive samples, which was curated from the database of one of the laboratories, is also shown. (B) The sample categories, which were summarized from approximately 17 000 samples submitted to one of the laboratories in 2009.[36] Tissue samples (i.e. formalin-fixed, paraffin-embedded [FFPE] and frozen tissue) are indicated in warm colors, while cytological samples (i.e. bronchoscopy specimens and pleural effusion) are indicated in cold colors. (C) The rate of *EGFR* mutations according to sample category summarized from the data for approximately 17 000 samples.[36] The failure rate represents the proportion of samples for which polymerase chain reaction (PCR) fails to amplify the target DNA. Tissue samples are indicated in warm colors, and cytological samples are indicated in cold colors.

mutation in the *EGFR* gene have mostly been tested in cytological samples include a phase II study,[30] a randomized phase III study,[4] and a phase II study for patients with poor performance status.[18] The last study is particularly important because cytological samples were the only samples available for many of the patients.

Criterion required for the kits testing *EGFR* mutation

After years of clinical investigations and discussions, Japanese clinicians treating NSCLC have reached a consensus that comprises the following elements: (i) cytological samples are valuable clinical specimens for testing *EGFR* mutations; (ii) a complete review of all patients with advanced NSCLC for *EGFR* mutations is very difficult to achieve without employing cytological samples; and (iii) in order to test both tissue and cytological samples, the *EGFR* mutation test should be able to detect mutations in samples with a ratio of cancer cells of 1%.

To attain the consensus above, we describe our provisional criterion that the kit used for *EGFR* mutation test is required to satisfy (Table 1).

Issues associated with the DNA-based mutation test

We discuss some of the issues frequently raised in relation to *EGFR* mutation test. Detection of somatic mutations in organs other than the lung may share common issues.

DNA amount. When cells are sampled from a mixture of cancer cells and normal cells, the number of cancer cells conforms to a binomial distribution. When 100 cells (~650 pg DNA) are sampled from a cell mixture in which the ratio of cancer cells is 1%, there is a 37% chance that no cancer cells are sampled. When 800 cells (5 ng DNA) are sampled, there is more than a 96% chance that the ratio of cancer cells in the sample is more than 0.4%, and there is more than a 90% chance that the ratio is more than 0.6% (Fig. 5). Considering sampling errors, the mutation test should be performed using more than 5 ng DNA.

Use of serum samples for mutation detection. Several studies have reported the detection of mutated genes in serum.[31–33] The use of serum is attractive because serum collection is less invasive than many other sampling procedures. However, a serious concern arises when mutated

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Table 1. Specifications for the EGFR mutation tests that can be used in the clinical practice

Criterion

Kits used for *EGFR* mutation test are required to detect the type of mutations described in the *Mutations* section (see below) from the samples with a ratio of the cancer cells of 1%. To attain this, the kits are required to pass the assay described in the *Assay* section.

Mutations

Mandatory†

- E746-A750del (2235–2249delGGAATTAAGAGAAGC)
- E746-A750del (2236–2250delGAATTAAGAGAAGCA)
- L858R
- G719S
- T790M

Recommended†

- L747-S752del P753S (2240–2257delTAAGAGAAGCAACATCTC)
- L747-E749del A750P (2239–2247delTTAAGAGAA, 2248G > C)
- G719A
- G719C
- L861Q

Assay

Mutations that occur at the same position are usually detected at similar sensitivity. Therefore, only a single exon 19 deletion is included in the assay. The assay uses plasmid constructs each containing Del E746–A750 (2235–2249delGGAATTAAGAGAAGC), L858R, G719S, or T790M. Each plasmid DNA is mixed with normal human genomic DNA (10 ng/μL) to make the Assay Samples by achieving a copy number ratio of 1–200 of mutant *EGFR* sequence to normal *EGFR* sequence (Fig. S1). This simulates the test conditions in which the ratio of cancer cells to normal cells is 1–100 (Fig. 2). For the assay, 100 Assay Samples comprising 20 samples for each of the four mutants, and 20 Assay Samples containing only the normal human genomic DNA (10 ng/μL), are set up. There are randomized, and then investigated. This test is expected to correctly identify both presence and type of mutations in 95% of the samples (Fig. S2). Use of more than 5 ng of DNA from each Assay Sample is mandatory. Because the copy number of the mutant *EGFR* gene sequence conforms to a binomial distribution, use of <5 ng DNA causes significant sampling errors (see Fig. 5).

†These mutations except for T790M confer sensitivity to the EGFR-TKIs and account for the mutations occurring in 77% of the patients.[11,19] T790M confers resistance to the EGFR-TKIs. †These mutations account for the mutations occurring in 10% of the patients.[11,19]

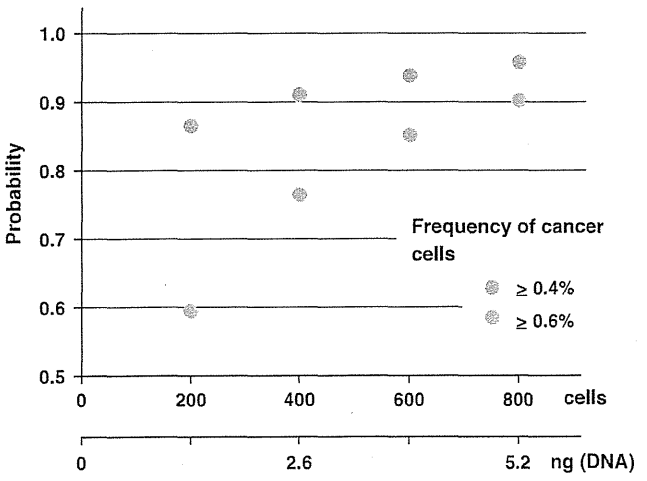

Fig. 5. Sampling errors. The number of cancer cells sampled from a mixture of cancer cells and normal cells conforms to a binomial distribution. It is assumed that the ratio of cancer cells to normal cells in the cell suspension is 1:100. When 800 cells are collected from the suspension, there is a 96% chance that the ratio of cancer cells in the collection is more than 0.4% (i.e. 32 cells) and a 90% chance that the ratio is more than 0.6% (i.e. 48 cells).

genes are not detected in serum, because the reason for this is difficult to ascertain. Possible explanations include (i) the serum does not contain sufficient cancer-derived DNA; and (ii) the cancer cells do not contain the mutated gene. The rate at which serum is shown to contain an insufficient amount of cancer-derived DNA is significant,[34] which inflates the false-negative rate. The mutation test for detecting mutated gene in serum is currently unacceptable for clinical practice.

Use of circulating tumor cells for the detection of mutations. Circulating tumor cells (CTCs) are the cells detached from the tumor, enter the blood stream, and circulate throughout the body. Circulating tumor cells are a very attractive target for the mutation testing because they may be readily collected from peripheral blood.[35] However, a simple calculation casts doubt on their clinical utility. The pulmonary capillaries have a diameter of 5 μm and trap particles with a size of 10–60 μm, which is the size of the ^{99m}Tc-macro-aggregated albumin that is used to embolize and image the pulmonary capillaries in pulmonary perfusion scintigraphy. The diameter of NSCLC cells is usually much larger than 5 μm, and they are thus considered unable to pass through the pulmonary capillaries. Rather, they are likely to be trapped at the entrance of the capillaries and subsequently eliminated. It is thus assumed that CTCs are eliminated during a single passage through the pulmonary circulation. Therefore, for 10 CTCs to be detected in 1 mL of blood, 10 (CTCs)/mL × 5000 (mL/min: cardiac output) × 1440 (min/day) = 7.2×10^7 CTCs/day (i.e. almost a gram of cells) are required to enter into circulation. Considering that cancer cells have a doubling time of more than 24 h, this formula indicates that a gram of cancer tissue should be present in the patients that doubles in 24 h and release half of the descendant cells into the circulation. This suggests that the patient has a large tumor burden, and thus is in a very advanced stage of the disease. Circulating tumor cells are considered difficult to isolate from patients in the early stages of NSCLC and thus may have limited clinical utility.

A detection system with a higher sensitivity. A mutation test may detect mutations in a sample in which the ratio of cancer cell is 0.1%. However, because the copy number of genomic DNA conforms to a binomial distribution, more than 50 ng of genomic DNA (DNA from 8000 cells) should be used for a

successful test. The requirement for a large amount of DNA may increase the stress associated with sample collection on patients. An increase in the sensitivity of the test may not parallel an increase in its clinical utility.

Clinical samples in which the ratio of cancer cells is <1%. While ascertaining the presence of cancer cells in one aliquot of the sample (Fig. 3), pathologists may notice that the ratio of cancer cells in the sample may be <1%. On such occasions, the pathologists should notify clinicians that the sample may not be suitable for mutation testing and that re-sampling may be required. Cooperation of clinicians and pathologists is highly recommended for reducing the false-negative rate that stems from samples of unsatisfactory quality.

Future perspectives

Our ever-expanding understanding of cancer driver genes lengthens the list of the gene mutations to be tested. In contrast, patients desire clinical procedures to be less stressful by the collection of smaller or fewer samples. Mutation testing aims to select patients suitable for specific treatments. At the same time, it excludes patients not suitable for certain treatments. If negative for mutations, excluded patients may be disappointed recalling their undergoing stressful sampling procedures only to obtain negative results. A long list of genes, therefore, does not justify the collection procedures much more stressful than those currently used.

The sample quality should be determined at the time of sampling. If inappropriately handled, DNA or RNA may be degraded immediately after sampling. Clinician training is very important such that they are prepared to handle the samples for the mutation test. Currently, most clinicians are aware of sample-processing methods for pathological examinations. However, most of these procedures are inappropriate for DNA and RNA. For example, formalin fixation fragments DNA, while paraffin embedding makes DNA purification difficult. Following suitable procedures for DNA or RNA examination significantly reduces the amount of sample required for the test.

Next-generation sequencing is being introduced for mutation testing. We anticipate an increase in the number of genes tested and a reduction in the cost of testing. However, whatever method is used, sensitivity of the test is limited by the amount of DNA available (Fig. 5), and the amount of DNA is limited by the size and type of cancer lesion and sampling procedures. As a result, sensitivity of the mutation tests stays, at least for the time being, around the current level. Development of sampling procedures that is far less invasive to the patient than those currently used and thus, able to collect more cancer-derived DNA, is wanted to overcome the limitation of sensitivity, and will contribute greatly to future mutation testing.

Disclosure statement

Koichi Hagiwara holds a patent on the PNA-LNA PCR clamp method and received royalties from the Mitsubishi Chemical Medience. Koichi Hagiwara and Kunihiko Kobayashi received lecture fees from AstraZeneca.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Preparation of assay samples.

Fig. S2. Assay.

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REVIEW

Epidermal growth factor receptor (*EGFR*) mutation and personalized therapy in advanced nonsmall cell lung cancer (NSCLC)

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Abstract Before 2009, nonsmall cell lung cancer (NSCLC) was one disease entity treated by cytotoxic chemotherapy that provided a response rate of 20–35 % and a median survival time (MST) of 10–12 months. In 2004, it was found that activated mutations of the *epidermal growth factor receptor* (*EGFR*) gene were present in a subset of NSCLC and that tumors with *EGFR* mutations were highly sensitive to EGFR tyrosine kinase inhibitors (TKI). Four phase III studies (North East Japan (NEJ) 002, West Japan Thoracic Oncology Group (WJTOG) 3405, OPTIMAL, and EUROTAC) prospectively compared TKI (gefitinib or erlotinib) with cytotoxic chemotherapy as first-line therapy in *EGFR*-mutated NSCLC. These studies confirmed that progression-free survival (PFS) with TKIs (as the primary endpoint) was significantly longer than that with standard chemotherapy (hazard ratio [HR]=0.16–0.49) from 2009 to 2011. Although the NEJ 002 study showed identical overall survival (OS) between the arms (HR=0.89), quality of life (QoL) was maintained much longer in patients treated with gefitinib. In conclusion, TKI should be considered as the standard first-line therapy in advanced *EGFR*-mutated NSCLC. Since 2009, a new step has been introduced in the treatment algorithm for advanced NSCLC.

Keywords Nonsmall cell lung cancer (NSCLC) · *EGFR* mutation · EGFR-TKI · Gefitinib · Erlotinib

Introduction

Recent sequencing of DNA to identify polymorphisms has catalyzed the quest for protein kinase "driver" mutations, which contribute to the transformation of a normal cell to a proliferating cancerous cell. On the other hand, kinase "passenger" mutations are considered to reflect mutations that merely build up in the course of cancerous cell replication and proliferation. At present, there are driver mutations in nonsmall cell lung cancer (NSCLC), such as *epidermal growth factor receptor* (*EGFR*) mutations [1–3], a fusion gene between echinoderm microtubule-associated protein-like 4 (*EML4*) and the anaplastic lymphoma kinase (*ALK*) [4, 5], and fusion genes with RET proto-oncogene (*RET*) [6–8], for which specific agents have been developed. In this manuscript, a road to personalized therapy by *EGFR* mutations in advanced NSCLC, which was the first experience to treat advanced NSCLC patients individually, is reviewed.

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Personalized therapy by *EGFR* mutations in advanced NSCLC

Dysregulation of protein kinases is frequently observed in cancer cells; therefore, protein kinases are attractive targets in the development of anticancer drugs. Small molecule inhibitors that block binding of adenosine-5′-triphosphate (ATP) to the tyrosine kinase catalytic domain have been developed, and gefitinib and erlotinib are the first generation of such agents, which act as tyrosine kinase inhibitors (TKI) at the *EGFR*. In 2004, three groups of researchers reported that activating mutations of *EGFR* detected by direct sequencing were present in a subset of NSCLC and that tumors with *EGFR* mutations were highly sensitive to EGFR-TKI [1–3].

Although this knowledge is the first evidence for division of subpopulations in NSCLC and of the possibility of treating NSCLC patients individually, there have been two streams of clinical studies. Clinical efficacy of EGFR-TKIs such as gefitinib or erlotinib has been investigated initially

in unselected patients [9–13] and, subsequently, on the basis of clinical characteristics [14]. On the other hand, in order to develop personalized therapy in NSCLC, clinical efficacy of EGFR-TKIs has been indicated by molecular selection in phase 3 trials of NSCLC (Table 1) [15–19].

Unselected patients

In the BR.21 phase III comparative study [9], 731 previously treated NSCLC patients (unselected by *EGFR* mutations) were allocated randomly to the erlotinib or placebo groups at a ratio of 2:1. At the primary endpoints, erlotinib was significantly superior in terms of both progression-free survival (PFS) (2.2 months vs. 1.8 months, respectively, hazard ratio (HR)=0.61, $p<0.001$) and median survival time (MST) (6.7 months vs. 4.7 months, respectively, HR=0.70, $p<0.001$). On the basis of the results of BR.21, erlotinib has become a standard therapy for previously treated patients with advanced NSCLC and is now used in previously treated cases of NSCLC that may or may not have *EGFR* mutations.

In order to evaluate gefitinib, a phase III study (Iressa Survival Evaluation in Advanced Lung Cancer (ISEL)) was carried out [10]. A total of 1,692 patients refractory to or intolerant of their latest chemotherapy were randomized to receive either gefitinib (250 mg/day) or placebo plus best supportive care (BSC). The primary endpoint, MST, was 5.1 months in the placebo group and 5.6 months in the gefitinib group, with no significant differences between the two groups ($p=0.087$). Therefore, efficacy of gefitinib in NSCLC patients unselected by *EGFR* mutations was not indicated. Another randomized phase III study (INTEREST) [11] compared gefitinib with standard second-line chemotherapy using docetaxel in 1,433 previously treated NSCLC patients unselected by *EGFR* mutations. As to overall survival (OS), which was the primary endpoint of the study, the HR was 1.020 (95 % confidence interval [CI]: 0.905–1.150) and did not exceed the preset upper limit (1.154), thus endorsing the noninferiority of gefitinib to docetaxel. However, the V-15-32 randomized phase III study, which aimed to confirm the noninferiority of gefitinib to docetaxel in regard to OS [12], was carried out in Japan and involved 490 previously treated NSCLC patients unselected by *EGFR* mutations. MST were 14.0 and 11.5 months for the gefitinib and docetaxel groups, respectively, and the HR was 1.12 (95 % CI: 0.89–1.40). Thus, the study did not demonstrate noninferiority of gefitinib to docetaxel. The potency of gefitinib in unselected patients with NSCLC is considered to be controversial.

Table 1 Clinical studies using EGFR-TKI

	Second-line treatment	First-line treatment
Unselected patients	BR.21 ISEL INTEREST V-15-32	
Selection by background		IPASS
Selection by EGFR mutation		NEJ Gefitinib Study-02 WJTOG 3405 OPTIMAL (CTONG 0802) EURTAC-SLCG GECP06/01

Selection by background

In preplanned subgroup analyses of the ISEL trial mentioned above [20], gefitinib was shown to extend survival in Asian patients (MST: 9.5 months vs. 5.5 months, HR=0.66, $p=0.01$). In addition, covariate analyses of demographic subsets among patients of Asian origin treated with gefitinib showed a survival advantage (HR<1) across never-smokers (HR, 0.37; $p=0.0004$) and adenocarcinoma patients (HR, 0.54; $p=0.0028$). Therefore, in March 2006, the Iressa® Pan-Asia Study (IPASS) was initiated to investigate the effectiveness of first-line gefitinib in previously untreated patients in East Asia who had advanced pulmonary adenocarcinoma and who were light or nonsmokers [14]. The IPASS included 1,217 NSCLC patients selected by backgrounds and compared gefitinib therapy with carboplatin (CBDCA)+paclitaxel (PTX) therapy as a first-line treatment. As to PFS, which was the primary endpoint of this study, the HR was 0.741 (95 % CI: 0.651–0.845), and it was reported that the outcome was significantly better in the gefitinib group. However, since the survival curves for the two groups crossed each other, it was difficult to interpret the value of HR (Fig. 1a). Because Cox analysis should be used in cases having a constant relationship between HR and time [21], this could not be used when the curves crossed each other. For example, PFS of gefitinib was better, the same, or worse than that of CDBCA+PTX at 12, 6, or 3 months, respectively (Fig. 1a).

Although the result at the primary endpoint in the IPASS was inconclusive, the importance of the IPASS report is demonstrated in its subset analyses [14]. Among 1,217 patients enrolled, an *EGFR* mutation test (amplification mutation refractory system) was performed on tumor samples from 437 patients (36 %). In this analysis, the crossing of the survival curves seen in Fig. 1a disappeared (Fig. 1b, c). In the subgroup of 261 patients who were positive for *EGFR* mutation, PFS was significantly longer among those who received gefitinib than among those who received CBDCA–PTX (HR=0.48; $P<0.001$), whereas in the subgroup of 176 patients who were negative for the mutation, PFS was significantly longer among those who received CBDCA–PTX (HR=2.85; $P<0.001$). Thus, the critical

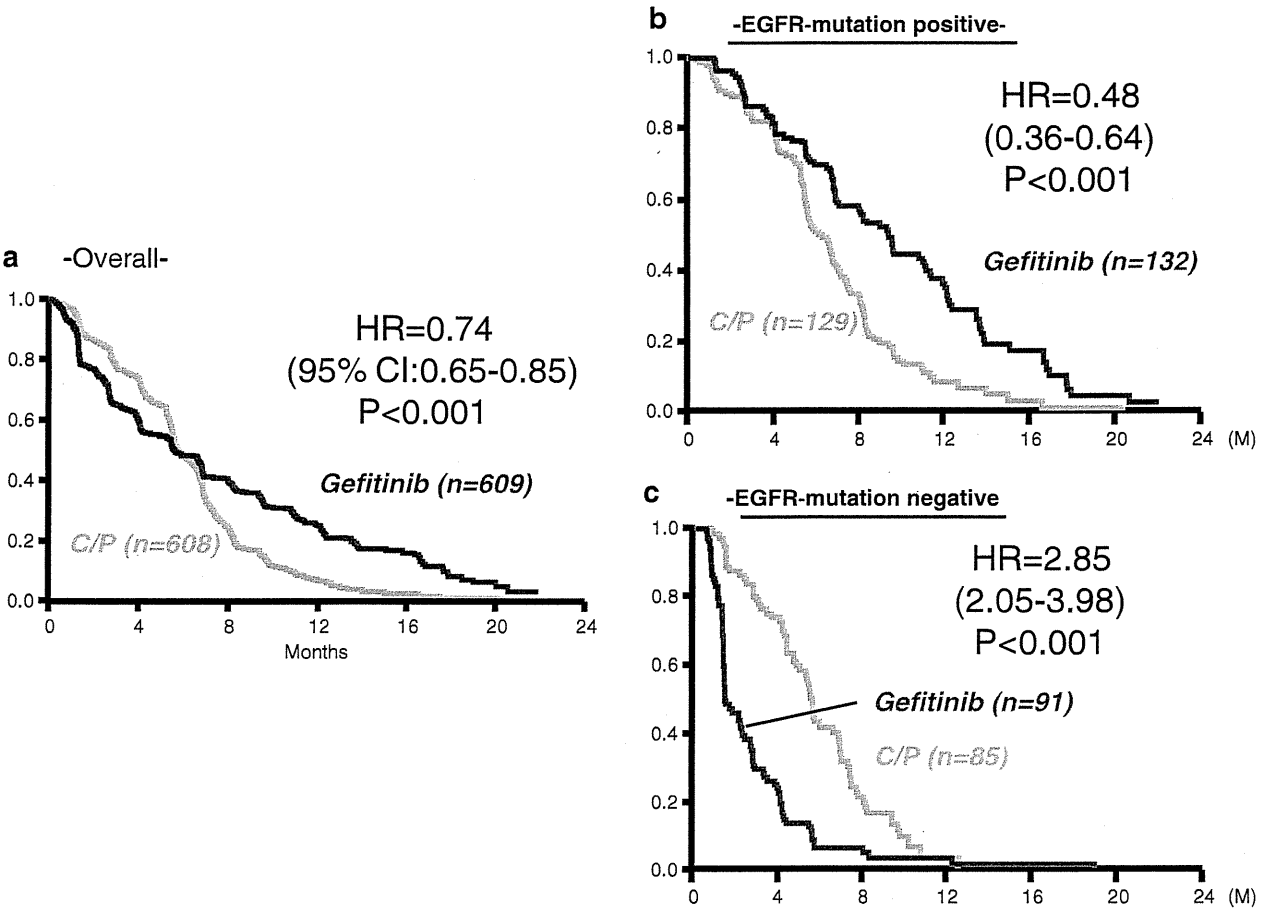

Fig. 1 Progression-free survival in IPASS. **a** Kaplan–Meier curves of PFS for Asian patients treated with gefitinib or carboplatin plus paclitaxel who had pulmonary adenocarcinoma and who were light or nonsmokers. **b** and **c** show PFS for patients with or without *EGFR* mutations treated with gefitinib or carboplatin plus paclitaxel, respectively, in subset analyses. [14]

message was that there was no indication for gefitinib in patients who were negative for the *EGFR* mutation.

In addition to the *EGFR* mutation test described above, the biomarkers analyzed in IPASS were *EGFR* gene copy number (fluorescent in situ hybridization (FISH)), and EGFR protein expression (immunohistochemistry) [22]. PFS was significantly longer with gefitinib in patients whose tumors had both high *EGFR* gene copy number and *EGFR* mutation (HR, 0.48) but was significantly shorter when a high *EGFR* gene copy number was not accompanied by *EGFR* mutation (HR, 3.85) (Fig. 2). Among the three biomarkers, *EGFR* mutations are the strongest predictive biomarker for PFS and tumor response to first-line gefitinib vs. CBDCA+PTX. Selection by backgrounds, Asian origin, adenocarcinoma histology, and light or nonsmoking resulted in an *EGFR* mutation-rich population at a rate of 60 % (261 EGFR-mutated patients/437 patients evaluated). Thus, if the strategy of selection by backgrounds is employed, there should be a 40 % risk associated with TKI treatment for patients without *EGFR* mutations.

Selection by *EGFR* mutations

Since 2004 when the pivotal studies reported on the relationship between EGFR mutations and TKI sensitivity, multiple phase II studies have confirmed a striking response to EGFR-TKIs in this population in Japan [23–29]. A combined analysis employing these phase II studies, named *I*RESSA *C*ombined *A*nalysis of the *M*utation *P*ositives (I-CAMP) study, indicated longer PFS with gefitinib than with standard chemotherapy [30]. In March 2006, at the same time that the IPASS study started, two phase III trials, the North East Japan (NEJ) 002 study and the West Japan Thoracic Oncology Group (WJTOG) 3405 [16, 17], were initiated, which compared gefitinib with standard chemotherapy in first-line treatment for *EGFR*-mutated NSCLC (Table 2). NEJ 002 first confirmed as the primary endpoint that PFS in the gefitinib group was significantly longer than that in the CBDCA plus PTX group (10.8 months vs. 5.4 months, HR=0.30, $P<0.001$) [15, 16]. In WJTOG3405, the gefitinib group also had significantly longer PFS compared with the cisplatin plus docetaxel

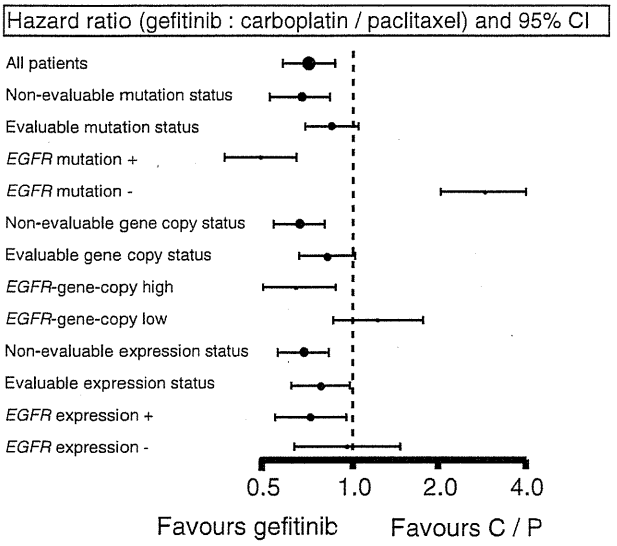

Fig. 2 Biomarker for gefitinib. In comparing *EGFR* mutation, *EGFR* gene copy number, and *EGFR* expression status, *EGFR* mutation is the best biomarker for gefitinib. [22]

goup, with a median PFS of 9.2 months vs. 6.3 months (HR 0.489, $p<0.0001$) [17]. In order to evaluate erlotinib further, the phase III OPTIMAL study [18] was initiated in August 2008. It compared the PFS of erlotinib with gemcitabine plus CBDCA in the first-line treatment of Chinese patients with advanced *EGFR* mutation-positive NSCLC. The median PFS was significantly longer in erlotinib-treated patients than in those on chemotherapy (13.1 vs. 4.6 months; HR=0.16; $p<0.0001$). In another phase III study, EURTAC [19], started in February 2007, PFS with erlotinib was compared with standard chemotherapy for first-line treatment of European patients with advanced *EGFR* mutation-positive NSCLC. The preplanned interim analysis showed that the median PFS was 9.7 months in the erlotinib group, compared with 5.2 months in the standard chemotherapy group (HR=0.37; $p<0.0001$).

OS was retrospectively compared between advanced NSCLC patients with sensitive *EGFR* mutations who began first-line systemic therapy before and after gefitinib approval in Japan (January 1999–July 2001 and July 2002–December 2004, respectively) [31]. In 136 (41 %) of the 330 patients treated at the National Cancer Center Hospital of Japan, although no significant survival improvement was observed in patients without *EGFR* mutations (MST: 13.2 vs. 10.4 months, respectively; $P=0.13$), OS was significantly longer among the *EGFR*-mutant patients treated after gefitinib approval compared with the OS of patients treated before gefitinib approval (MST: 27.2 vs. 13.6 months, respectively; $P<0.001$). However, a combined analysis of ICAMP and a post hoc analysis of IPASS suggested identical survival of patients on gefitinib and chemotherapy in first-line treatment for *EGFR*-mutated patients [30, 32]. Furthermore, a secondary endpoint of both NEJ 002 [33] and WJTOG3405 [34] prospectively showed identical OS between gefitinib and chemotherapy in first-line treatment of NSCLC patients harboring sensitive *EGFR* mutations (Table 2), although OS data from OPTIMAL and EURTAC are immature at the present time. It must be explained that in almost all of the patients who were treated with first-line chemotherapy in NEJ 002 and WJTOG 3405, a crossover treatment with gefitinib was undertaken. Therefore, from the viewpoint of OS, the effect of gefitinib is additive to that of chemotherapy, indicating that both first-line and second-line gefitinib are acceptable.

When OS is identical between two arms, improvement in quality of life (QoL) and disease-related symptoms are among the key goals in the treatment of NSCLC. IPASS reported better QoL in *EGFR*-mutated patients treated with gefitinib than in those treated with CBDCA+PTX, but this analysis was a post hoc estimation [35]. With the exception of WJTOG3405, the

Table 2 Phase III studies of TKI for *EGFR*-mutated patients

Trial	Arm	Number	RR	PFS	OS	Ref.
NEJ 002	Gefitinib	114	74 %	10.8 m	27.7 m	NEJM (2010)
	CbPXL	110	31 %	5.4 m	26.6 m	OS: Ann Oncol. (in press)
				HR=0.30*	HR=0.89	QOL: Oncologist (2012)
WJTOG 3405	Gefitinib	86	62 %	9.2 m	36 m	Lancet Oncol (2010)
	CisDTX	86	32 %	6.3 m	39 m	OS: ASCO (2012)
				HR=0.49*	HR=1.19	
OPTIMAL	Erlotinib	83	83 %	13.1 m	NR	Lancet Oncol (2011)
	CbGEM	82	36 %	4.6 m	NR	QOL: ASCO (2012)
				HR=0.16*		
EURTAC	Erlotinib	86	58 %	9.7 m	NR	Lancet Oncol (2012)
	Pt doublet	87	15 %	5.2 m	NR	
				HR=0.37*		

*shows a significant difference between arms

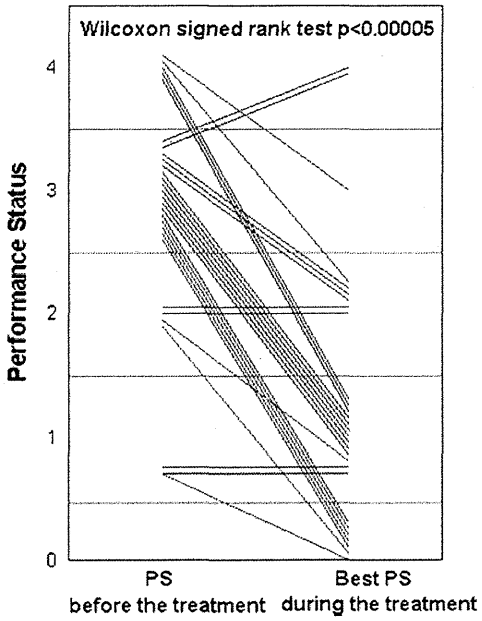

Fig. 3 Performance status (PS) improvement by gefitinib in the NEJ 001 Study. Each *line* shows changes of PS in a patient. [39]

other three trials listed in Table 2 prospectively investigated QoL of NSCLC patients with sensitive *EGFR* mutations who were treated with EGFR-TKI or standard chemotherapy, and NEJ 002 and OPTIMAL have presented the results [36, 37]. In NEJ 002, patients' QoL was assessed weekly using the Care Notebook [38], and the primary endpoint of the QoL analysis was time to deterioration from baseline on each of the physical, mental, and life well-being QoL scales. Kaplan–Meier probability curves and logrank tests showed that time to defined deterioration in physical and life well-being significantly favored gefitinib over chemotherapy (HR=0.34; $p<0.0001$ and HR, 0.43; $p<0.0001$, respectively); this indicated that QoL was maintained much longer in patients treated with gefitinib than in those treated with standard chemotherapy [36]. In OPTIMAL, the Functional Assessment of Cancer Therapy (FACT) measuring system showed that compared with the gemcitabine/CBDCA group, the erlotinib group had a clinically relevant improvement in QoL, as assessed by scores on the FACT-L (73 % vs. 29.6 %; odds ratio (OR)=6.9; $p<0.0001$), the LCSS (75.7 % vs. 31.5 %; OR=6.77; $p<0.0001$), and the TOI (71.6 % vs. 24.1 %; OR=7.79; $p<0.0001$) [37]. These QoL results conclusively indicate that EGFR-TKI should be considered as the standard first-line therapy for advanced EGFR-mutated NSCLC despite the lack of survival advantage.

EGFR-TKIs for *EGFR*-mutated patients with poor performance status and advanced age

The multicenter phase II NEJ 001 study was undertaken to investigate the efficacy and feasibility of gefitinib treatment for advanced NSCLC patients harboring *EGFR* mutations but who were ineligible for chemotherapy due to poor performance status (PS) [39]. The overall response rate was 66 %, and median PFS and MST were 6.5 months and 17.8 months, respectively. PS improvement rate was 79 % ($p<0.00005$); in particular, 68 % of the 22 patients improved from PS ≥3 at baseline to PS 0 or 1. (Fig. 3) Thus, the "Lazarus Response" was observed in treatment-naïve, poor PS patients with NSCLC and *EGFR* mutations [40]. In patients with sensitive *EGFR* mutations but with extremely poor PS (suspected MST less than 4 months with BSC), the difference in benefit with or without gefitinib treatment was so marked that a randomized phase III study to compare gefitinib to BSC alone may not be justified. This was the first occasion on which changes in treatment guidelines were provoked by a phase II study of NSCLC. Since previously there has been no standard treatment for these patients with short life expectancy other than BSC, examination of *EGFR* mutations as a biomarker is also strongly recommended in this patient population.

In regard to so-called "fit" elderly patients harboring *EGFR* mutations, the NEJ 003 phase II study [41] investigated patients with chemotherapy-naïve history, a median age of 80 years (range: 75–87 years), and PS 0–1, who were treated with gefitinib as a first-line treatment. The response rate was 74 %, and the median PFS and OS were 12.3 months and 33.8 months, respectively. Considering its strong antitumor activity and mild toxicity, first-line gefitinib may be preferable to standard chemotherapy in this population. However, a phase III study comparing gefitinib to standard chemotherapy may be needed to provide the final evidence of benefit in advanced *EGFR*-mutated "fit" elderly patients.

Tarceva Lung cancer Survival Treatment (TRUST) [42] was an open-label, phase IV study of unselected patients with advanced NSCLC. In a subpopulation of elderly patients (≥70 years) receiving first-line erlotinib ($n=485$) in TRUST [43], the disease control rate was 79 %, median PFS was 4.57 months, and MST was 7.29 months. A total of 87 subpopulation patients (18 %) had an erlotinib-related adverse event (AE); 4 % had a ≥3 grade erlotinib-related AE. Erlotinib was effective and well-tolerated and may be considered for unselected, elderly patients with advanced NSCLC who are unsuitable for standard first-line chemotherapy or radiotherapy. However, there have been few prospective studies of erlotinib in advanced, *EGFR*-mutated, "fit" elderly patients.

EGFR mutation tests

Direct sequencing of *EGFR* requires histology obtained by operation. The NEJ 001, NEJ 002, and NEJ 003 series all used the same *EGFR* mutation test, the peptide nucleic acid-locked nucleic acid polymerase chain reaction clamp (PNA LNA PCR clamp) [44–46]. This is

a technological innovation that can make not only tissue-based assessment but also cytology-based assessment of *EGFR* mutations. Briefly, genomic DNA fragments surrounding mutation hot spots of the *EGFR* gene are amplified by PCR in the presence of a clamp primer synthesized from PNA with a wild-type sequence. This leads to preferential amplification of the mutant sequence, which is detected by a fluorescent primer that incorporates LNA to increase specificity. As a result, a mutant *EGFR* sequence is detected in the presence of a 100-fold wild-type sequence. Thus, by the PNA LNA PCR clamp, a small number of *EGFR* mutation-positive cancer cells are detected within 3 h. The sensitivity and specificity of the PNA-LNA PCR clamp were 97 % and 100 %, respectively [46]. Therefore, *EGFR* testing by the PNA LNA PCR clamp was possible in patients with extremely poor PS and of advanced age.

In 2012, the performance, sensitivity, and concordance among five *EGFR* tests of PCR-Invader®, PNA LNA PCR clamp, direct sequencing, Cycleave™, and Scorpion Amplification Refractory Mutation System (ARMS)® were reported [47]. All tests, except direct sequencing, detected mutation types at ≥1 % mutant DNA. Analysis success rates were 91.4–100 %, and interassay concordance rates of successfully analyzed samples were 94.3–100 %. It was concluded that cytology-derived DNA is a viable alternative to formalin-fixed paraffin-embedded (FFPE) tissue samples for analyzing *EGFR* mutations.

It was clarified that frequencies of *EGFR*-mutated NSCLC patients are approximately 31 % and 16.6 % in Japan and Europe, respectively [46, 48]. In Japan, approximately 50,000 patients were newly diagnosed as NSCLC in 1 year. In 2011, approximately 48,000 tests for *EGFR* mutations were carried out under national health insurance, indicating that most patients with NSCLC were screened in Japan. Under circumstances where *EGFR* mutations, *EML4-ALK* fusion gene, and *RET* fusion genes should be tested, routine screening for all of these will be required when making diagnosis of NSCLC.

Conflict of interest Koichi Hagiwara received consulting fee or honorarium from AstraZeneca and received payment for patent for EGFR test method. Kunihiko Kobayashi has been paid for conducting lectures by Taiho, AstraZeneca, and Chugai.

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Effectiveness of Gefitinib against Non–Small-Cell Lung Cancer with the Uncommon EGFR Mutations G719X and L861Q

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Introduction: In non–small-cell lung cancer, an exon 19 deletion and an L858R point mutation in the epidermal growth factor receptor (EGFR) are predictors of a response to EGFR-tyrosine kinase inhibitors. However, it is uncertain whether other uncommon EGFR mutations are associated with sensitivity to EGFR-tyrosine kinase inhibitors.

Methods: A post-hoc analysis to assess prognostic factors was performed with the use of patients with EGFR mutations (exon 19 deletion, L858R, G719X, and L861Q) who were treated with gefitinib in the NEJ002 study, which compared gefitinib with carboplatin-paclitaxel as the first-line therapy.

Results: In the NEJ002 study, 225 patients with EGFR mutations received gefitinib at any treatment line. The Cox proportional hazards model indicated that performance status, response to chemotherapy, response to gefitinib, and mutation types were significant prognostic factors. Overall survival (OS) was significantly shorter among patients with uncommon EGFR mutations (G719X or L861Q) compared with OS of those with common EGFR mutations (12 versus 28.4 months; $p = 0.002$). In the gefitinib group (n = 114), patients with uncommon EGFR mutations had a significantly shorter OS (11.9 versus 29.3 months; $p < 0.001$). By contrast, OS was similar between patients with uncommon mutations and those with common mutations in the carboplatin-paclitaxel group (n = 111; 22.8 versus 28 months; $p = 0.358$).

Conclusions: The post-hoc analyses clearly demonstrated shorter survival for gefitinib-treated patients with uncommon EGFR mutations compared with the survival of those with common mutations and suggest that the first-line chemotherapy may be relatively effective for non–small-cell lung cancer with uncommon EGFR mutations.

Key Words: Gefitinib, G719X, L861Q, NEJ002, Uncommon epidermal growth factor receptor mutations.

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The clinical efficacy of epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs), such as gefitinib and erlotinib, has been demonstrated in non–small-cell lung cancer (NSCLC) patients in whom standard chemotherapy has failed.[1,2] Further studies have revealed that the presence of activating mutations in the EGFR kinase domain is strongly associated with the therapeutic efficacy of EGFR-TKIs.[3,4]

Randomized phase 3 trials have demonstrated that EGFR-TKIs significantly improve median progression-free survival (PFS) compared with platinum-doublet therapy in EGFR-mutated patients.[5–8] However, not all mutations in the EGFR kinase domain are responsive to EGFR-TKI treatment. These phase 3 trials have shown that EGFR-TKIs are effective for patients with common EGFR mutations, such as an exon 19 deletion or the L858R point mutation, which account for more than 90% of EGFR mutations. Retrospective studies and case reports suggest that some uncommon mutations are associated with sensitivity to EGFR-TKIs.[9–20] These mutations

include G719X in exon 18, which accounts for approximately 3% of EGFR mutations, and L861Q in exon 21, which represents approximately 2% of EGFR mutations. However, these uncommon EGFR mutations have not been clearly shown to be predictive markers for the efficacy of EGFR-TKIs because of their low frequency.

To investigate the efficacy of gefitinib in patients with uncommon mutations, we conducted a post-hoc analysis of the NEJ002, which compared gefitinib and carboplatin-paclitaxel as first-line therapies for advanced NSCLC with activating EGFR mutations.

PATIENTS AND METHODS

Patient Population

We retrospectively analyzed the data of 225 patients who received gefitinib treatment at any point in the NEJ002 study.[6] The eligibility criteria of the NEJ002 study included the presence of advanced NSCLC harboring an EGFR mutation (exon 19 deletion or L858R, G719X, or L861Q point mutation) without the resistant EGFR mutation T790M (identified using the peptide nucleic acid–locked nucleic acid polymerase chain reaction clamp method), no history of chemotherapy, an age of 75 years or younger, a performance status of 0 to 1, and appropriate organ function.[21,22] Patients provided a written informed consent. The study was conducted in accordance with the Helsinki Declaration of the World Medical Association. The protocol was approved by the institutional review board of each participating institution.

Treatment

Eligible patients were randomly assigned to receive either gefitinib (250 mg/day) or paclitaxel (200 mg/m^2)/carboplatin (area under the curve, 6.0) on day 1 every 3 weeks. Chemotherapy was continued for at least three cycles. Gefitinib was administered until the disease progressed, intolerable toxicities developed, or consent was withdrawn. The protocol recommended that the crossover regimen be used as a second-line treatment.

Clinical Assessments

The antitumor response to treatment was assessed using computed tomography every 2 months. Unidirectional measurements were adopted on the basis of the Response Evaluation Criteria in Solid Tumors (version 1.0).[23] PFS was evaluated from the date of randomization to the date when disease progression was first observed or death occurred. The treatment response and PFS were determined by an external review of computed tomography scans by experts who were not aware of the treatment assignments. Overall survival (OS) was evaluated from the date of randomization to the date of death.

Statistical Analysis

To assess prognostic factors for OS, we used univariate and multivariate Cox proportional hazards models. Kaplan–Meier survival curves were constructed for PFS and OS, and differences between groups were identified using the log-rank test. Differences in response rates were identified using Fisher's exact test. Each analysis was two sided, with a 5% significance level and a 95% confidence interval. All analyses were performed using SAS for Windows software (release 9.1; SAS Institute, Cary, NC).

RESULTS

Patient Population

A total of 230 chemonaive patients were enrolled in the NEJ002 study: 115 patients were assigned to receive gefitinib and 115 were assigned to receive carboplatin-paclitaxel (Fig. 1). To evaluate the efficacy of gefitinib in NSCLC patients with uncommon EGFR mutations, we analyzed the data of 114 patients in the gefitinib group and 111 patients in the carboplatin-paclitaxel group. We identified five patients who had uncommon EGFR mutations in each group. Two patients, who had common mutations and were treated with first-line chemotherapy consisting of carboplatin-paclitaxel, were excluded from the PFS analysis in the NEJ002 study. However, both were treated with gefitinib and were included in this post-hoc analysis. The demographic and disease characteristics of the patients with uncommon EGFR mutations were similar to those of patients with common EGFR mutations (Table 1). The characteristics of each patient with uncommon EGFR mutations are shown in supplementary Table S1 (Supplemental Digital Content 1, http://links.lww.com/JTO/A494).

Survival Factors

In the univariate analysis of 225 patients who received gefitinib at any point, uncommon EGFR mutations had a significant detrimental effect on survival (Table 2). We also identified performance statuses 1 and 2, distant metastasis, brain metastasis, stable disease, and progressive disease as significant predictors of worse prognosis for standard chemotherapy and stable disease and progressive disease as significant predictors of worse prognosis for gefitinib. When these variables were included in the Cox proportional hazards model, we found that uncommon EGFR mutations, performance statuses 1 and 2, stable disease and progressive disease for standard chemotherapy, and stable disease and progressive disease for gefitinib had significant hazard ratios (Table 2).

Uncommon EGFR Mutations and Survival

The Kaplan–Meier curve for OS for uncommon versus common EGFR mutations is shown in Figure 2*A*. The OS was significantly shorter among patients with uncommon EGFR mutations compared with OS of those with common EGFR mutations in the overall population (12 versus 28.4 months; $p = 0.002$). A significantly shorter survival time was observed in patients with uncommon EGFR mutations compared with survival time in those with common EGFR mutations in the gefitinib group (11.9 versus 29.3 months; $p < 0.001$) (Fig. 2*B*). However, a similar survival time was observed between the subgroups of uncommon and common EGFR mutations in the carboplatin-paclitaxel group (22.8 versus 28 months; $p = 0.358$) (Fig. 2*C*).

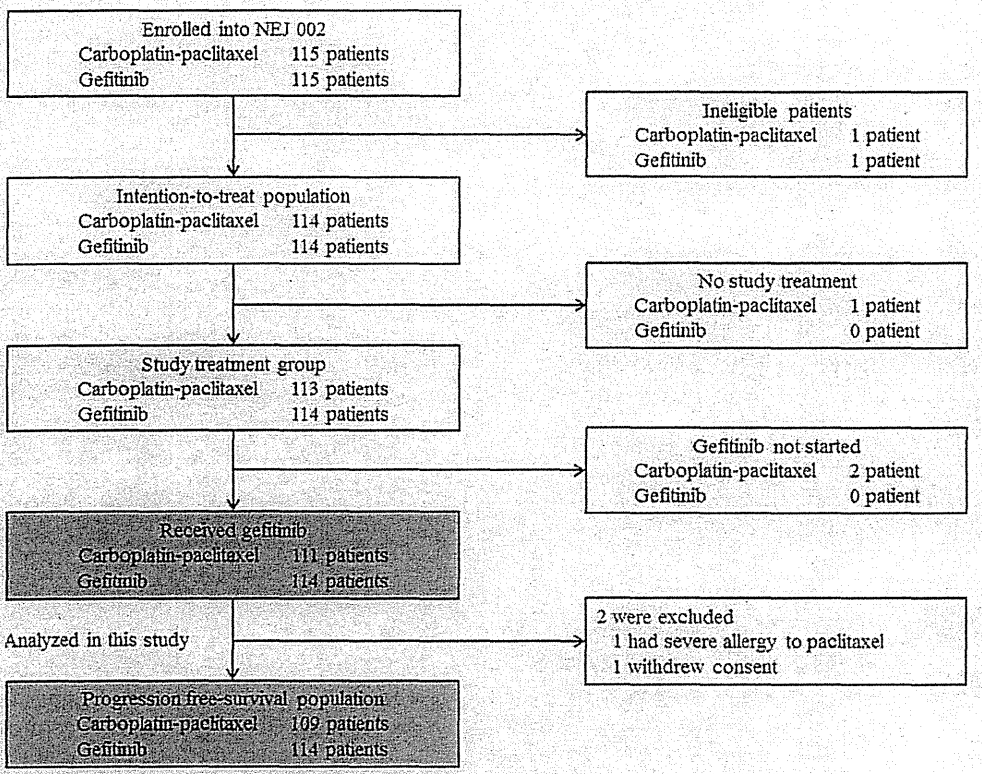

FIGURE 1. Enrollment, randomization, and follow-up of the study patients.

To examine whether the sequence of platinum doublet and gefitinib affected OS, we performed a further subgroup analysis. The survival time tended to be shorter among patients receiving first-line gefitinib compared with the survival time among those receiving first-line carboplatin-paclitaxel in the uncommon EGFR mutation group (11.9 versus 22.8 months; $p = 0.102$). Consistent with previous publications, a similar survival time was observed between patients receiving first-line gefitinib and those receiving first-line carboplatin-paclitaxel in the common EGFR mutation group (29.3 versus 28 months; $p = 0.378$).

Uncommon EGFR Mutations, PFS, and Response

In the gefitinib group, the median PFS was significantly shorter for patients with uncommon EGFR mutations compared with median PFS of those with common EGFR mutations (2.2 versus 11.4 months; $p < 0.001$) (Fig. 3*A*). By contrast, the median PFS did not differ significantly between patients with uncommon EGFR mutations and those with common EGFR mutations in the carboplatin-paclitaxel group (5.9 versus 5.4 months; $p = 0.847$) (Fig. 3*B*). The objective response rate was significantly lower in patients with uncommon EGFR mutations compared with the objective response rate in those with common EGFR mutations when treated with gefitinib (20% versus 76%; $p = 0.017$) (supplementary Table S2, Supplemental Digital Content 1, http://links.lww.com/JTO/A494). By contrast, similar objective response rates were observed for patients with uncommon EGFR mutations and those with common EGFR mutations in the carboplatin-paclitaxel group (20% versus 32%; $p = 0.336$) (supplementary Table S2, Supplemental Digital Content 1, http://links.lww.com/JTO/A494).

DISCUSSION

Recent studies suggest that NSCLC patients with uncommon EGFR mutations are less responsive to EGFR-TKIs compared with patients with L858R and exon 19 deletions.[9–20] However, the efficacy of EGFR-TKIs in NSCLC patients with uncommon mutations has not been fully elucidated.

We conducted a post-hoc analysis of the NEJ002 study to evaluate the effectiveness of gefitinib against NSCLC with G719X or L861Q. The NEJ002 study, comparing gefitinib and standard carboplatin-paclitaxel chemotherapy as the first-line treatment for patients with EGFR mutations, demonstrated no significant difference in OS between gefitinib and carboplatin-paclitaxel.[6] In contrast to other phase 3 trials investigating EGFR-TKIs for patients with common EGFR mutations of exon 19 deletion and L858R, the NEJ002 is the only study that included uncommon EGFR mutations of G719X and L861Q.

The current study clearly demonstrated that NSCLC patients with the uncommon EGFR mutations G719X and L861Q had shorter survival than the survival of those with an exon 19 deletion or L858R mutation (Fig. 2). Our results are consistent with other clinical studies on EGFR-TKIs in

TABLE 1. Patient Characteristics

Number of Patients	Uncommon Mutation 10	Common Mutation 215
Sex		
Female	4	139
Male	6	76
Age (yr)		
Median	63	65
Range	42–75	35–75
Smoking status		
Never smoked	5	134
Smoker	5	81
Performance status		
0/1/2	5/5/0	105/107/3
Histology		
Adenocarcinoma	9	202
Others	1	13
Clinical stage		
Stage IIIB	3	32
Stage IV	6	165
Postoperative	1	18
Type of EGFR mutation		
G719X	7	
L861Q	3	
Exon 19 deletion		115
L858R		97
19 deletion + L858R		3

EGFR, epidermal growth factor receptor.

patients with uncommon EGFR mutations (supplementary Table S3, Supplemental Digital Content 1, http://links.lww.com/JTO/A494). The overall response rate to EGFR-TKIs in patients with uncommon EGFR mutations was 41%, which is lower than the response rate to TKIs (62%–83%) of patients with common EGFR mutations.[7,8,24] In the NEJ002 study, G719X included G719C and G719S. No patients harbored G719A. To investigate the effectiveness of gefitinib on each uncommon EGFR mutations, we evaluated the difference in OS between patients with uncommon EGFR mutations (G719C versus G719S and G719X versus L861Q). There was no significant difference between these subgroups (data not shown).

This study showed that the PFS and OS tended to be shorter among patients treated with first-line gefitinib compared with PFS and OS among those treated with first-line carboplatin-paclitaxel in the uncommon EGFR mutation group (supplementary Table S2, Supplemental Digital Content 1, http://links.lww.com/JTO/A494). We also found poor disease control rate with gefitinib in patients with uncommon mutations. Three of five patients with uncommon mutations in the gefitinib group had progressive disease. By contrast, no patients with uncommon mutations had progressive disease in the carboplatin-paclitaxel group. Although the number of patients with uncommon mutations in each treatment group was small, platinum-doublet therapy might be a better choice than gefitinib for first-line therapy in patients with uncommon EGFR mutations. Because some of patients with uncommon mutations showed good clinical response to gefitinib in this study and they seemed to be heterogeneous in terms of response to gefitinib, administration of gefitinib should be considered for patients with uncommon mutations when disease progression was observed after first-line chemotherapy.

In vitro studies have indicated that the affinity of gefitinib for EGFR proteins with uncommon EGFR mutations is lower than the affinity of gefitinib for EGFR proteins with common EGFR mutations.[25] A sixfold or 14-fold higher concentration of gefitinib was required to inhibit the growth of cells expressing G719X or L861Q, respectively, compared with cells expressing L858R.[26] These results may explain the lack of response to gefitinib in patients with uncommon EGFR mutations. The authors also examined the sensitivity of G719X and L861Q mutations to erlotinib and irreversible TKIs.[27] Cells expressing G719X were less resistant to erlotinib than gefitinib in vitro; however, L861Q was resistant to both erlotinib and gefitinib. In contrast to erlotinib, irreversible TKIs inhibited the growth of cells with G719X or L861Q at a

TABLE 2. Univariate and Multivariate Analysis by Cox Proportional Hazards Model

	Univariate			Multivariate		
	HR	95% CI	*p*	HR	95% CI	*p*
Age (≥70/<70)	1.047	0.719–1.525	0.81			
Sex (female/male)	0.73	0.51–1.045	0.86			
Smoking status (+/−)	1.376	0.967–1.958	0.076			
Performance status (1, 2/0)	1.792	1.263–2.541	0.001	1.85	1.297–2.639	0.001
Histology (nonadeno/adeno)	0.647	0.302–1.387	0.263			
Types of EGFR-m (uncommon/common)	2.967	1.501–5.868	0.018	2.445	1.177–5.079	0.017
Distant metastasis (+/−)	4.914	1.113–5.741	0.027	2.849	1.241–6.54	0.135
Brain metastasis (+/−)	1.781	1.248–2.542	0.002	1.311	0.897–1.915	0.162
Response to Cb/TXL (SD, PD/CR, PR)	1.742	1.113–2.728	0.015	1.748	1.11–2.754	0.016
Response to G (SD, PD/CR, PR)	2.878	2.012–4.117	0.002	2.601	1.794–3.771	<0.001

HR, hazard ratio; CI, confidential interval; EGFR-m, epidermal growth factor receptor mutation; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; Cb/TXL, carboplatin plus paclitaxel; G, gefitinib.