



Analytical performance of the cobas EGFR mutation assay for Japanese non-small-cell lung cancer



Hideharu Kimura^a, Tatsuo Ohira^b, Osamu Uchida^c, Jun Matsubayashi^d, Shinichiro Shimizu^e, Toshitaka Nagao^d, Norihiko Ikeda^b, Kazuto Nishio^{a,*}

^a Department of Genome Biology, Kinki University Faculty of Medicine, 337-2 Ohno-Higashi, Osaka-Sayama, Osaka 589-8511 Japan

^b Department of Surgery, Tokyo Medical University, 6-7-1 Nishi-shinjuku, Shinjuku-ku, Tokyo 160-0023, Japan

^c Department of Respiratory Surgery, Funabashi Municipal Medical Center, 1-21-1 Kanasugi, Funabashi, Chiba 273-8588, Japan

^d Department of Anatomic Pathology, Tokyo Medical University, 6-7-1 Nishi-shinjuku, Shinjuku-ku, Tokyo 160-0023, Japan

^e Department of Pathology, Funabashi Municipal Medical Center, 1-21-1 Kanasugi, Funabashi, Chiba 273-8588, Japan

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abstract

Introduction: Clinical outcomes in non-small-cell lung cancer (NSCLC) patients with epidermal growth factor receptor (EGFR) mutations have been reported to be correlated with the use of EGFR-tyrosine kinase inhibitors (EGFR-TKIs). Therefore, it is essential to confirm the presence of EGFR mutations using highly sensitive testing methods. In this study, we compared the performance of the cobas[®] EGFR Mutation Test (cobas EGFR assay) and the therascreen[®] EGFR RQ PCR Kit (therascreen EGFR assay) for use as an in vitro diagnostic (IVD) product.

Methods: We extracted DNA from 150 formalin-fixed, paraffin-embedded tissue samples from 150 patients diagnosed with NSCLC, and performed a comparative study of the cobas EGFR and therascreen EGFR assay methods. All discordant results were re-analyzed by direct sequencing.

Results: The concordance rate between the cobas EGFR assay and the therascreen EGFR assay was 98.0% (145/148). EGFR mutations were detected at a frequency of 40.9% (61/149) in NSCLC specimens using the cobas EGFR assay and 40.2% (60/149) using the therascreen EGFR assay. Three discrepant results were found in this study. Two double mutations were detected by the cobas EGFR assay but only one in the therascreen EGFR assay. No invalid results resulted from sample analysis by the cobas EGFR assay.

Conclusions: Our results show a high concordance rate (98.0%) of cobas EGFR assay with an existing IVD product, the therascreen EGFR assay. Since they are IVD diagnostic products, both assays proved to be simple, validated methods in detecting the most common, clinically significant EGFR mutations and proved to be helpful for appropriate treatment guidance for NSCLC patients.

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1. Introduction

Non-small-cell lung cancer (NSCLC) patients frequently have activating EGFR mutations and respond well to treatment with small molecule EGFR-tyrosine kinase inhibitors (TKIs) such as gefitinib and erlotinib [1–4]. Both the American Society of Clinical Oncology and the Japan Lung Cancer Society recommend EGFR mutation testing in patients being considered for EGFR-TKI treatment as a first-line therapy [5]. Similar guidelines recommending testing for EGFR mutations were established by the College of American Pathologists, the International Association for the Study of Lung Cancer, and the Association for Molecular Pathology [6]. Patients' EGFR mutation status prior to the commencement of treatment impacts outcomes and, as a result, EGFR testing has been

developed as a companion diagnostic; this relationship between therapeutic and diagnostic agents contributes to personalized healthcare. Recently, it was reported that about half of patients who are initially sensitive to EGFR-TKIs may acquire resistance to EGFR-TKIs [7] following a period of therapy, mainly as a result of the appearance of EGFR mutations associated with resistance to treatment, such as T790M. Indeed, a recent study suggested that the T790M mutation may be present in a small proportion of tumor cells prior to treatment, with the proportion of mutant alleles increasing gradually during treatment [8]. Similar findings were observed for exon 20 insertions; that they are usually associated with primary or de novo resistance to EGFR-TKI therapy [9]. Thus, it is important to re-assess EGFR mutation status during treatment to determine the most appropriate treatment regimens for patients.

A number of PCR-based techniques are used in the clinic for the assessment of EGFR mutations. In Japan, the “Scorpion-ARMS” therascreen[®] EGFR Rotor-Gene Q (RGQ) PCR Kit (therascreen EGFR

* Corresponding author. Tel.: +81 72 366 0221; fax: +81 072 367 6369.

E-mail address: knishio@med.kindai.ac.jp (K. Nishio).

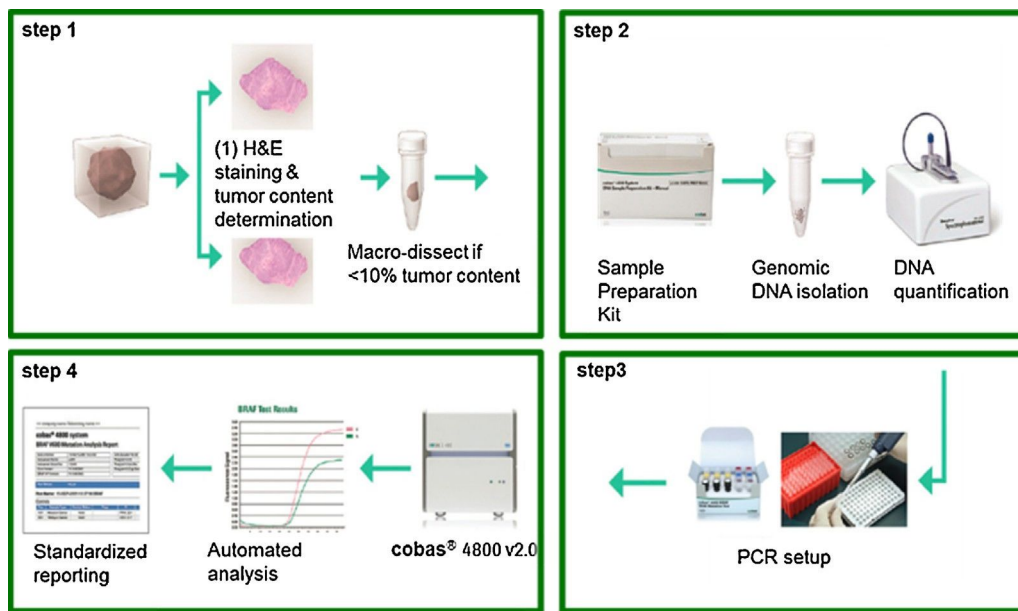


Fig. 1. Assay flow for the cobas EGFR mutation assay. The assay is composed of four steps. Step 1: 5- μ m sections are prepared from FFPE tissue. One section is used for H&E staining to assess tumor content and the other section is used for DNA isolation. Step 2: Genomic DNA is isolated using the cobas[®] DNA Sample Preparation Kit. Step 3: DNA is mixed with reagents after quantification. Step 4: DNA is amplified using the cobas z 480 system. Results are automatically reported.

assay; Qiagen, Hilden, Germany) is the only available in vitro diagnostic (IVD) test.

In this study, we compared the performance of the cobas EGFR assay and the theascreen EGFR assay using formalin-fixed, paraffin-embedded (FFPE) tissue specimens from NSCLC patients.

2. Materials and methods

2.1. Tissue samples

A series of archived 150 FFPE tissue samples which was surgically resected from 150 Japanese patients diagnosed with NSCLC, collected between March 2011 and December 2012, was obtained from Tokyo Medical University (Tokyo, Japan) and Funabashi Medical Hospital (Funabashi, Japan). All patients enrolled in the study provided informed consent for the use of resected tissue. The study was approved by the ethics committee of each participating institute and conducted according to Institutional Review Board guidelines.

2.2. cobas EGFR Mutation Test

The cobas EGFR assay is an allele-specific real-time PCR system (Figure, Supplemental Digital Content 1; Fig. 1) that qualitatively measures the amplification of DNA to identify 41 mutations in exons 18–21 of the EGFR gene from 50 ng of DNA derived from human FFPE NSCLC tissues (Table, Supplemental Digital Content 2). Within each reaction mixture, exon 28 was amplified as an internal control. DNA isolation, amplification/detection, and result reporting can be performed in less than 8 h with up to 30 specimens processed simultaneously. The cobas EGFR assay has fully automated results reporting.

2.3. Specimen preparation for cobas EGFR assay

Two FFPE tissue sections of 5 μ m thickness were prepared for this assay. One was used for DNA extraction and the other was used to confirm the presence of tumor content by hematoxylin and eosin

(H&E) staining, which was performed by a pathologist. Any specimen containing <10% tumor content by area was macrodissected.

2.4. DNA extraction

FFPE tissue specimens were deparaffinized and then DNA extraction was performed according to the standard procedure described in the cobas[®] DNA Sample Preparation Kit (Roche Molecular Systems, Inc., USA) package insert. Briefly, the sample was incubated for 1 h at 56 °C and then for additional one hour at 90 °C in the presence of a protease and chaotropic lysis/binding buffer that causes the release of nucleic acids but protects released genomic DNA from degradation by DNase. The amount of genomic DNA was spectrophotometrically determined and adjusted to a fixed concentration of 2 ng/ μ L.

2.5. PCR amplification and detection

A total of 150 ng of DNA is required for the cobas EGFR assay. Target DNA was amplified and detected using the cobas[®] z480 analyzer (Roche Molecular Systems Inc.) according to the instructions for the cobas[®] EGFR Mutation Test, which measures the fluorescence generated by specific PCR products. All results were automatically performed by cobas[®] 4800 software.

2.6. theascreen[®] EGFR RGQ PCR Kit

The theascreen assay is a real time-PCR assay that combines the Amplification Refractory Mutation System (ARMS) and Scorpions fluorescent primer/probe system. It can detect 29 somatic mutations in exons 18–21 of EGFR. A maximum of 7 results can be obtained from one run. The theascreen EGFR assay was performed according to the manufacturer's guidelines (Qiagen). Briefly, DNA was isolated from FFPE tissue samples and the total sample DNA assessed by amplifying a region of exon 2 from EGFR by PCR. Next, the DNA samples were tested for the presence or absence of EGFR mutations by real-time PCR using a Scorpion probe and primers specific for wild type and mutant EGFR DNA. The difference

Table 1

Clinical characteristics of the patients providing surgically resected FFPE samples in NSCLC.

	N = 149
Gender	
Male	75
Female	74
Age	
Younger than 65 years	42
Older than 65 years	107
Histology	
Adenocarcinoma or adeno-squamous cell carcinoma (Ad)	126
Squamous cell carcinoma (Sq)	17
Large cell carcinoma (Ia)	2
Other	4
Smoking history	
Smoker	18
Ever smoker	73
Never smoker	56
ND	2

ND, not determined; N, number.

between the mutation assay cycle threshold (C_T) and control assay C_T from the same sample was used to calculate sample ΔC_T values. Samples designated mutation positive if the ΔC_T was less than the cutoff ΔC_T value.

2.7. Sanger sequencing

DNA samples obtained from specimens that were discordant between cobas EGFR and therascreen EGFR assays were amplified using the following site-specific primers: Exon 18 Forward, 5'-TGGAGCCTCTTACACCCAGT-3', Reverse, 5'-ACAGCTTGCAAGGACTCTGG-3'; Exon 19 Forward, 5'-TCTGGA-TCCCAGAAGGTGAG-3', Reverse, 5'-CAGCTGCCAGACATGAGAAA-3'; Exon 20 Forward, 5'-CATTCATGCGTCTTCACCTG-3', Reverse, 5'-GTCTTTGTGTTCCCGGACAT-3'; Exon 21 Forward, 5'-GATCTGTCCCTCACAGCAGGGTC-3', Reverse, 5'-GGCTGACCTAAAGCCACCTCC-3'. The fragments were subcloned into the Zero Blunt TOPO vector (Zero Blunt TOPO PCR Cloning Kit; Life Technologies, USA). Direct sequencing was performed with 100 colonies from one specimen by ABI3100 Genetic Analyzer (ABI) using the BigDye® Terminators v3.1 Cycle Sequencing Kit (Life Technologies). One mutation detected in 100 results was classed as "Mutation Detected" in this study. This assay required 1 µg of genomic DNA from specimens. Sanger sequencing was performed with the specimen that resulted double mutation (L858R and M790M) from cobas EGFR assay but single mutation (L858R) from therascreen EGFR assay at Mitsubishi Chemical Medicine Corporation followed by daily routine. The sequencing result was used as Golden standard.

3. Results

3.1. Study population

A series of 150 FFPE tissue samples from patients diagnosed with NSCLC was examined. One specimen was excluded owing to a lack of a completed consent form, leaving 149 samples available for analysis. The clinical and pathological characteristics of the patients providing the evaluable specimens are summarized in Table 1.

3.2. EGFR mutation types

EGFR mutations were identified in 63 NSCLC specimens (42.3%) using the cobas EGFR assay and 61 specimens (40.9%) using the

Table 2

Methods correlation between mutation findings using the cobas EGFR and therascreen EGFR assays.

		therascreen			Total
		MD	MND	Invalid	
cobas	MD	59	2	0	61
	MND	1	86	1	88
	Invalid	0	0	0	0
	Total	60	88	1	149

MD, mutation detected; MND, mutation not detected.

Table 3

Detailed concordant rate between cobas EGFR and therascreen EGFR assays.

Mutation	MD concordance	MND concordance	Total concordance
G719X	100% (3/3)	100% (145/145)	100% (148/148)
exon19del	95.7% (22/23)	100% (125/125)	99.3% (146/147)
S768I	100% (1/1)	99.3% (146/147)	99.3% (147/148)
T790M	—	99.3% (147/148)	99.3% (147/148)
exon20ins	—	100% (148/148)	100% (148/148)
L858R	100% (34/34)	99.1% (113/114)	99.3% (147/148)

Del, deletion; Ins, insertion; MD, mutation detected; MND, mutation not detected.

therascreen EGFR assay (Table, Supplementary Digital Content 4). Exon 19 deletions (Ex19del) and a point mutation (L858R) accounted for 90.5% (57/63) and 93.4% (57/61) of all mutations identified using the cobas EGFR assay and therascreen EGFR assay, respectively (Table, Supplementary Digital Content 3). This confirms the findings of a previous study [10], which found that Ex19del and L858R mutations accounted for 90% of NSCLC EGFR activating mutations. The exon 20 insert mutation (Ex20Ins) was not observed in any of the samples tested in this study. A T790M point mutation was detected by the cobas EGFR assay (0.68%) but not by the therascreen EGFR assay.

3.3. Invalid test rate

Mutation analysis of exons 18–21 of the EGFR gene was successfully performed in all 149 specimens (100%) using the cobas EGFR assay. In contrast, in experiments using the therascreen EGFR assay, two test specimens initially gave invalid test results. In those cases, DNA was extracted from new FFPE tissue samples. However, because one sample gave an invalid result again, this case was excluded from the analysis, resulting in an invalid rate of 0.68% (1/148) for the therascreen EGFR assay (Table 2). In addition, one invalid control occurred in the therascreen EGFR assay (data not shown).

3.4. Method correlation agreement analysis

The correlation rate between cobas EGFR assay and therascreen EGFR assay was 98.0%. Of the 149 evaluable samples tested, only three discordants between the two EGFR mutation assays were observed (Table 3).

3.5. Re-analysis of discordants by direct sequencing

Test specimens that gave discordant results between the cobas EGFR and therascreen EGFR assays were retested using direct sequencing from sub-cloned samples (Table 4). A discordant MND by therascreen EGFR assay was observed by direct sequencing to be an L858R point mutation, confirming the MD result assessed by cobas EGFR assay. In addition, an Ex19del mutation identified as MD by therascreen EGFR assay was shown to be MND by direct sequencing, again confirming the cobas EGFR assay result. The cobas EGFR assay identified one case with a double mutation, L858R and T790M

Table 4

Re-analysis of discordants by direct sequencing.

	therascreen		Cobas		Sequencing (reanalysis)
Sample 1	MND	–	MD	L858R	MD
Sample 2	MD	EX19Del	MND	– S768I	MND
Sample 3	MND	–	MD	L858R,T790M	MND
Sample 4	MD	L858R	MD		L858R

Del, deletion; Ins, insertion; MD, mutation detected; MND, mutation not detected.

Table 5

Re-analysis: combined therascreen EGFR assay and Sanger sequencing for resolution of discordant results.

		therascreen and/or Sanger sequencing	
		MD	MND
cobas	MD	60	1
	MND	0	87

MD, mutation detected; MND, mutation not detected.

(Table 4). However, only the L858R mutation was identified by the therascreen EGFR assay and only the T790M mutation was detected by direct sequencing. We then performed a re-analysis using a combination of the therascreen EGFR assay and Sanger sequencing for resolution of the discordant results (Table 5). This demonstrated an MD concordance rate of 100% (60/60), an MND concordance rate of 98.9% (87/88) and a total concordance rate of 99.3% (147/148) between the tests.

4. Discussion

The overall correlation results of the cobas EGFR assay, an existing EGFR mutation screening method (the therascreen EGFR assay) plus direct sequencing was 99.3% (147/148) (Table 5). It also indicated that the cobas assay is at least as robust method to detect the most common clinically significant EGFR mutations as the existing therascreen EGFR assay.

Although we identified 3 discordant results among 149 (2.0%) specimens in this study, retesting by direct sanger sequencing confirmed that two of the three discordant results were in fact correctly called by the cobas EGFR assay. Although both assays share similar characteristics in terms of amplification methods and detection principles, the slight differences (e.g. probe and primer construction) between the two of them, influenced their sensitivities to the mutations. Also, the remaining discordant result analysis highlighted the importance of the purity of the extracted DNA for the PCR amplification. In fact, an Ex 20 S768I mutation identified as Mutation Detected (MD) by the cobas EGFR assay but not the therascreen EGFR assay, was not detected by direct sequencing, either. In this case, direct sequencing failed more than two times to detect the EGFR gene when using the extracted DNA from the QIAmp DNA FFPE Tissue extraction kit (Qiagen) suggesting that the quality of the DNA was not adequate for the testing (data not shown). This potential difference in DNA quality might be the reason why we have experienced discordant results in some cases.

One T790M mutation was detected together with L858R by the cobas EGFR assay in this study. As there is known heterogeneity with regard to the T790M mutation within tumor cells, it is difficult to mention that the extracted DNA was completely the same, even if we used serial sections. However the raw data from the cobas system showed high enough signals to robustly detect the mutation (data not shown). According to the package insert, cobas EGFR needs at least 3.13 ng DNA which includes 5% mutated DNA to detect the mutation. Therefore it appears that the cobas test might

be more sensitive than the therascreen test because, according to the therascreen package insert, it needs 7.02% mutated DNA within the input DNA [11,12].

About half of the patients who are initially sensitive to EGFR-TKIs may acquire resistance to EGFR-TKIs [7] following a period of therapy, mainly because of the selection for the cells with the T790M mutation in EGFR. In addition, the correlation between the presence of intrinsic T790M mutations and patient outcomes has been shown [8], and is probably related to the slow growth of tumors bearing the T790M mutation. Thus, it is important to re-assess EGFR mutation status during treatment in order to determine the most appropriate treatment regimens for patients.

For IVD products, it is important to have rapid and simple testing. The cobas EGFR assay has two advantages over the therascreen assay in this regard. One is that the process consists of easily performed and stable methods. Additionally, it takes only 8 h to go from tumor specimen to results using the semi-automated system. Thus, patients assessed using the cobas EGFR assay can begin the most appropriate treatment within a shorter time period. The other advantage is that only a very small amount of DNA (150 ng) is required to detect the tumor mutation status using the cobas EGFR assay. Moreover, it confirms the accuracy of the results by co-amplification of an internal control (i.e. exon 28). One of the issues associated with detecting EGFR mutations in advanced NSCLC patients is not obtaining a sufficient quantity of specimen to confirm the presence/absence of several biomarkers. It is important to be able to perform tests using just a small amount of DNA; thus, the cobas EGFR assay is suitable and reliable for the detection of targeted common EGFR mutations. In this study, we had high concordance with surgically resected specimens which had enough tumors. However, at clinical practice, minimal invisible samples such as pleural effusion or bronchial wash would be used from advanced NSCLC patients having difficulty of collecting tissue. To access this difficulty, even if the samples are small enough, at least confirming the amount of tumor cells by pathologist is required to have appropriate test result. Under the condition, it might be able to provide reliable result even if using either FFPE samples or cytology samples. It is important to accumulate the data with cytology samples which makes improvement of suitable testing for advanced NSCLC patients in the future.

5. Conclusion

In the near future, more mutations that can serve as predictive markers for molecular-targeted treatments will be discovered, and mutation detection tests will play an increasingly important role in the clinical setting. The benefits of treatment will be maximized only if used together with clinically validated and accurate companion diagnostics. The cobas system offers the possibility of detecting additional mutations, not only mutations of EGFR. The combination of the cobas system with molecular-targeted treatments represents an important tool for physicians, supporting their efforts to effectively treat tumors.

Conflict of interest

Hideharu Kimura, Tatsuo Ohira, Osamu Uchida, Jun Matsubayashi, Shinichirou Shimizu, Toshitaka Nagao, Kazuto Nishio, Norihiko Ikeda was funded by Roche Diagnostics K.K. (Tokyo, Japan). There was no other financial support for the investigators.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.lungcan.2013.12.012>.

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Detection of epidermal growth factor receptor T790M mutation in plasma DNA from patients refractory to epidermal growth factor receptor tyrosine kinase inhibitor

Kazuko Sakai,¹ Atsushi Horiike,² Darryl L. Irwin,³ Keita Kudo,² Yoshihiko Fujita,¹ Azusa Tanimoto,² Toshio Sakatani,² Ryota Saito,² Kyohei Kaburaki,² Noriko Yanagitani,² Fumiyoshi Ohyanagi,² Makoto Nishio² and Kazuto Nishio^{1,4}

¹Department of Genome Biology, Kinki University Faculty of Medicine, Osaka; ²Thoracic Oncology Center, Cancer Institute Hospital, Japanese Foundation for Cancer Research, Tokyo, Japan; ³Sequenom Inc, Brisbane, Queensland, Australia

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A secondary epidermal growth factor receptor (EGFR) mutation, the substitution of threonine 790 with methionine (T790M), leads to acquired resistance to reversible EGFR-tyrosine kinase inhibitors (EGFR-TKIs). A non-invasive method for detecting T790M mutation would be desirable to direct patient treatment strategy. Plasma DNA samples were obtained after discontinuation of gefitinib or erlotinib in 75 patients with non-small cell lung cancer (NSCLC). T790M mutation was amplified using the SABER (single allele base extension reaction) technique and analyzed using the Sequenom MassARRAY platform. We examined the T790M mutation status in plasma samples obtained after treatment with an EGFR-TKI. The SABER assay sensitivity using mixed oligonucleotides was determined to be 0.3%. The T790M mutation was detected in 21 of the 75 plasma samples (28%). The presence of the T790M mutation was confirmed by subcloning into sequencing vectors and sequencing in 14 of the 21 samples (66.6%). In this cohort of 75 patients, the median progression-free survival (PFS) of the patients with the T790M mutation ($n = 21$) was not statistically different from that of the patients without the mutation ($n = 54$, $P = 0.94$). When patients under 65 years of age who had a partial response were grouped according to their plasma T790M mutation status, the PFS of the T790M-positive patients ($n = 11$) was significantly shorter than that of the T790M-negative patients ($n = 29$, $P = 0.03$). The SABER method is a feasible means of determining the plasma T790M mutation status and could potentially be used to monitor EGFR-TKI therapy. (Cancer Sci, doi: 10.1111/cas.12211, 2013)

Despite responses to epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs), such as gefitinib and erlotinib, in the majority of lung cancer patients carrying an EGFR mutation, most of these patients eventually become resistant to EGFR-TKIs.⁽¹⁾ The most common known mechanisms of acquired resistance are MET amplification, increased levels of hepatocyte growth factor, and secondary mutations in EGFR.^(2–5) The substitution of threonine 790 with methionine (T790M) leads to acquired resistance to reversible EGFR-TKIs. The frequency of this secondary mutation is 30–50% among patients who are resistant to EGFR-TKIs. Understanding the mechanisms of resistance to EGFR-TKIs is important because second and third generations EGFR-TKIs, including irreversible EGFR inhibitors, are presently under clinical development with the goal of conquering resistance mechanisms to EGFR-TKIs.

Here, we report the development of a highly sensitive single allele base extension reaction (SABER) method capable

of detecting low levels of T790M mutation. We used this method to assess the T790M mutation status of plasma samples from non-small cell lung cancer (NSCLC) patients to clarify (i) the frequency of tumor samples carrying the T790M mutation after EGFR-TKI treatment; and (ii) the association between the T790M mutation status and the clinical outcome. The detection of the T790M mutation in plasma samples could enable the clinical response to EGFR-TKIs to be monitored.

Materials and Methods

Patients. A total of 75 NSCLC patients with progressive disease (PD) after undergoing EGFR-TKI treatment (gefitinib or erlotinib) at the Thoracic Oncology Center, Cancer Institute Hospital, Japanese Foundation for Cancer Research between 2006 and 2011 were included in this study. Progressive disease was defined as the appearance of a new lesion or a 20% increase in the size of a primary tumor. The period between the detection of PD and the collection of the plasma sample used to determine the T790M mutation status varied (median 44 days; range, 0–803 days). Epidermal growth factor receptor mutation status in tumor samples obtained before treatment with an EGFR-TKI were identified using direct sequencing. Table 1 shows the clinical characteristics of the patients. Plasma samples obtained after discontinuation of EGFR-TKI were used to examine the T790M mutation status. All the patients provided informed written consent, and the study was approved by the Institutional Review Board at the Cancer Institute Hospital and the Kinki University Faculty of Medicine.

Sample processing. Plasma DNA was purified using a QIAamp Circulating Nucleic Acid Kit (Qiagen, Valencia, CA, USA). The extracted DNA was stored at -80°C until analysis.

Assay design. The assay was designed using MassARRAY Assay Design 4.0 software (Sequenom, San Diego, CA, USA) with a slight modification to enable use with the SABER method; the assay was intentionally designed so that it would only include terminators for the mutated nucleotide, and not the terminator for the wild-type nucleotide. A schematic diagram of the assay is shown in Figure 1. An amplification control assay was incorporated into each reaction using a conserved sequence in the amplified EGFR transcript so that

⁴To whom correspondence should be addressed.
E-mail: knishio@med.kindai.ac.jp

Table 1. Clinical characteristics of patients with progressive disease (PD) after epidermal growth factor-tyrosine kinase inhibitor (EGFR-TKI) treatment (n = 75)

EGFR-TKI-treated Patients	All patients (n = 75) No. (%)	Post-treatment T790M mutation positive patients (n = 21)	Post-treatment T790M mutation negative patients (n = 54)	P†
Age, years(mean, 61.6 ± 8.5 years)				
≤65	47 (62.7)	11	36	0.294
>65	28 (37.3)	10	18	
Sex Male				
Female	21 (28.0)	7	14	0.573
Smoking	54 (72.0)	14	40	
No				
Yes	44 (58.7)	12	32	1.000
Histology	31 (41.3)	9	22	
Ad				
Large/Sq	71 (94.7)	19	52	0.311
EGFR mutation (pre-treatment, tumor)	4 (5.3)	2	2	
Activating mutation				
Wild type/unknown	60 (80.0)	18	42	0.535
Response to EGFR-TKI	15 (20.0)	3	12	
PR				
SD/PD/NE	60 (80.0)	20	40	0.053
	15 (20.0)	1	14	

†Fisher's exact test. Ad, adenocarcinoma; Large, large cell carcinoma; NE, not evaluable; PD, progressive disease; PR, partial response; SD, stable disease; Sq, squamous cell carcinoma.

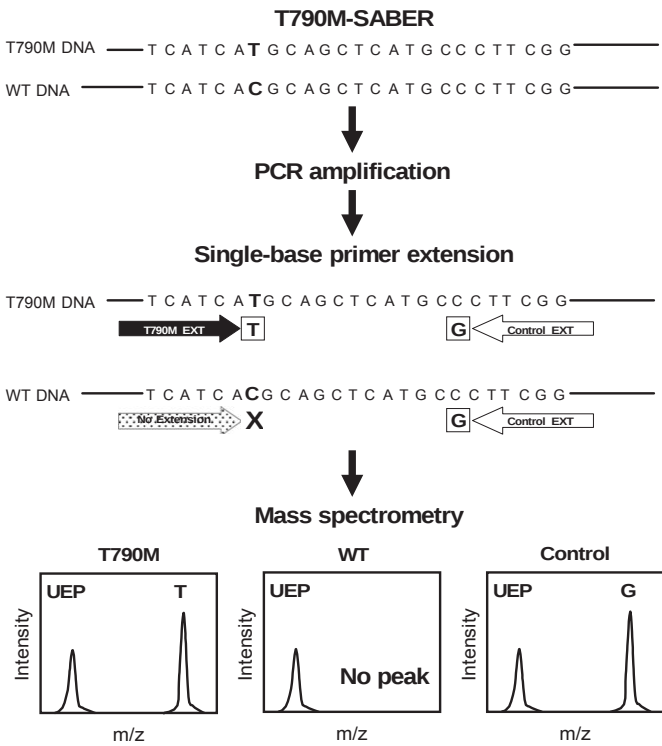


Fig. 1. Schematic diagram of the T790M-SABER method, based on MassARRAY assays. The DNA samples were first amplified using polymerase chain reaction (PCR). The PCR products were then subjected to a single base extension reaction. The T790M-SABER reaction only included terminators for the mutated nucleotide, and not the terminators for the wild-type nucleotide. An internal amplification control was designed to prevent false-negative results caused by PCR inhibitors. The primer extension products were analyzed using matrix-assisted laser desorption ionization-time of flight mass spectroscopy (MALDI-TOF MS). UEP, unextended primer.

Table 2. Primers used in the T790M SABER (single allele base extension reaction) method

Sequences	
PCR	
Forward	5'- ACGTTGGATGATCTGCCTCACCTCCACCGT -3'
Reverse	5'- ACGTTGGATGTGTTCCCGACATAGTCCAG -3'
Extension	
T790M	5'- CACCGTGCAGCTCATCA -3'
Internal control	5'- GTCCAGGAGGCAGCCGAAG -3'

PCR, polymerase chain reaction.

amplification would always occur in the presence of input template DNA. The PCR and extension primer sequences are listed in Table 2.

SABER method. The SABER method, where the primer extension was restricted to the mutant-specific allele, was performed using Sequenom iPLEX Pro biochemistry with resultant products detected with the MassARRAY platform. The PCR reactions were performed in 5-1L volumes containing 1.5 1L of eluted serum DNA, 200 nM of each primer, 50 1M of dNTPs (Sequenom), 0.2 U of PCR Taq DNA polymerase (Sequenom), 2.0 mM of MgCl₂, and 1 9 PCR buffer (Sequenom). The thermal cycling for the PCR was performed as follows: 2 min at 94°C, followed by 45 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min. The program was terminated after a final incubation at 72°C for 5 min. After the completion of the PCR, 2 1L (0.5 U) of shrimp alkaline phosphatase (Sequenom) was added to each reaction and the resulting mixture was incubated for 40 min at 37°C before enzyme inactivation by incubating the sample for 5 min at 85°C. The single-base primer extension reaction (SABER) was then performed in a final volume of 9 1L, containing 1 1mol of each extension primer, a mixture of three iPLEX mass-modified terminators (Sequenom), and 2.56 U of

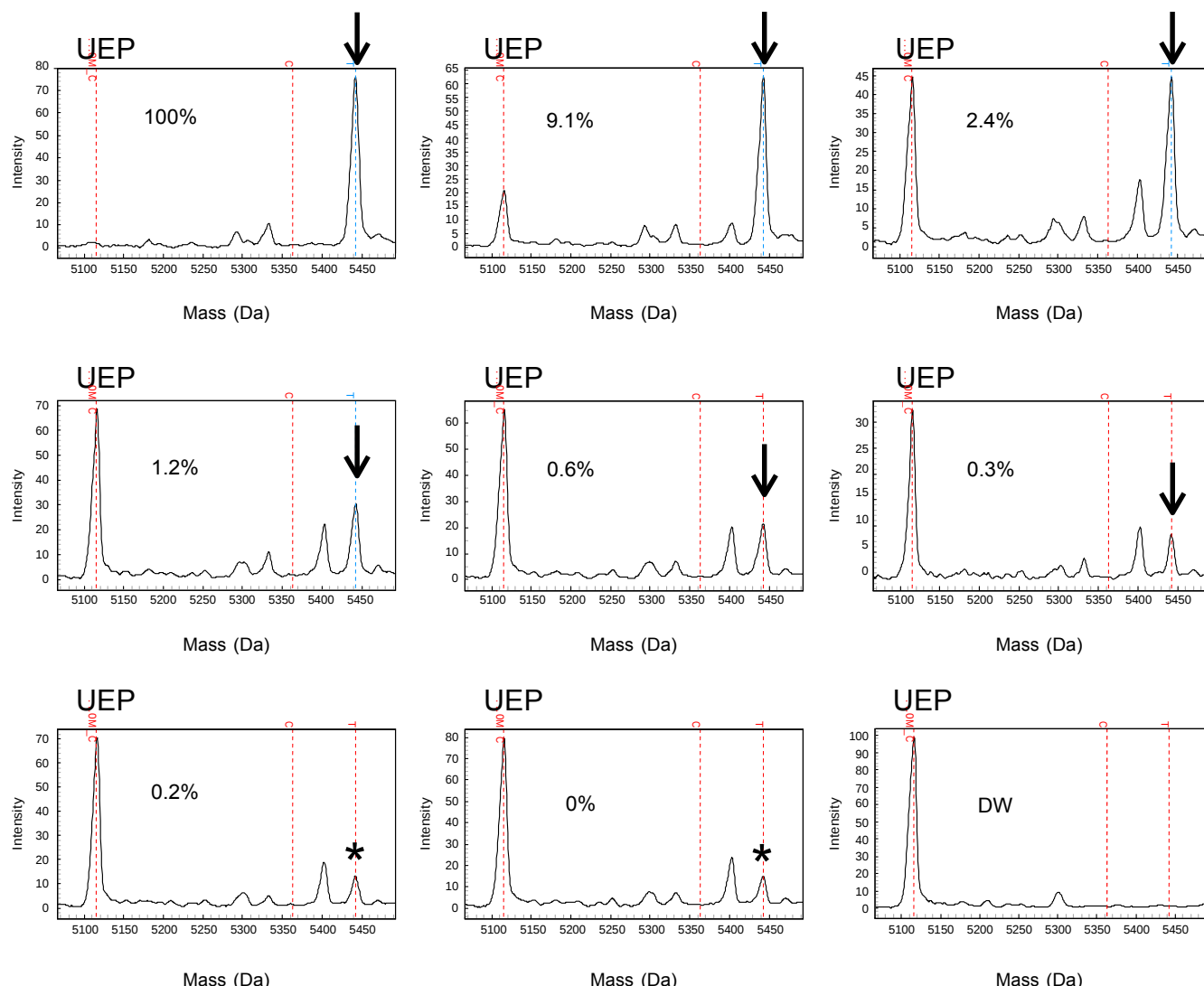


Fig. 2. Sensitivity of T790M detection. The percentages indicate the calculated proportion of T790M DNA in a mixture with wild-type DNA. An arrow at 5442 Da indicates the detection of the T790M mutation. An asterisk indicates a non-specific background peak. Three independent experiments were performed in duplicate, with identical results. DW, distilled water; UEP, unextended primer.

ThermoSequenase (Sequenom). The thermal cycling program for the reaction included an initial denaturation for 30 s at 94°C followed by five cycles of 5 s at 52°C and 5 s at 80°C. Forty additional annealing and extension cycles (5 s at 94°C, 5 s at 52°C, and 5 s at 80°C) were then performed. The final extension was performed at 72°C for 3 min, and the samples were then cooled to 4°C. The reaction products were desalted by dilution with 41 μ L of distilled water, the addition of 15 mg of ion-exchange resin (Sequenom), and subsequent separation of the resin by centrifugation. The products were spotted on a SpectroChip II (Sequenom), processed, and analyzed using a Compact Mass Spectrometer and MassARRAY Workstation (version 3.3) software (Sequenom). The data analysis was performed using MassARRAY Typer software, version 4.0 (Sequenom).

Sequencing analysis. The PCR products were subcloned into a pTA2 vector (Toyobo, Osaka, Japan) and sequenced using an automated sequencer (ABI Prism 3100 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA) and M13 universal primers (Applied Biosystems).

Scorpion ARMS analysis. Scorpion ARMS analysis used the DxS EGFR Mutation Test Kit for research use only (Qiagen)

and was carried out according to the manufacturer's instructions.

Statistical analyses. The Fisher exact test was used to assess the relationship between the T790M mutation status and different clinical characteristics, including patient sex and the primary mutation status. The objective tumor response (partial response [PR], stable disease [SD], or progressive disease [PD]) was evaluated according to the Response Evaluation Criteria in Solid Tumors guidelines. Progression-free survival (PFS) was defined as the period from the start of treatment until the date when disease progression was observed. The survival curves were derived using the Kaplan–Meier method and were compared using the log-rank test. All the statistical analyses were performed using JMP software (version 10; SAS Institute, Cary, NC, USA). A P-value < 0.05 was considered statistically significant.

Results

Assay sensitivity. The assay sensitivity was determined using T790M DNA oligonucleotide (5.0×10^{-16} , 2.5×10^{-16} , 1.25×10^{-16} , 6.25×10^{-17} , 3.125×10^{-17} , 1.5625×10^{-17} , 7.8125×10^{-18} , 3.90625×10^{-18} , 1.953125×10^{-18} , 9.765625×10^{-19} , $4.8828125 \times 10^{-19}$, $2.44140625 \times 10^{-19}$, $1.220703125 \times 10^{-19}$, $6.103515625 \times 10^{-20}$, $3.0517578125 \times 10^{-20}$, $1.52587890625 \times 10^{-20}$, $7.62939453125 \times 10^{-21}$, $3.814697265625 \times 10^{-21}$, $1.9073486328125 \times 10^{-21}$, $9.5367431640625 \times 10^{-22}$, $4.76837158203125 \times 10^{-22}$, $2.384185791015625 \times 10^{-22}$, $1.1920928955078125 \times 10^{-22}$, $5.9604644775390625 \times 10^{-23}$, $2.9802322387695312 \times 10^{-23}$, $1.4901161193847656 \times 10^{-23}$, $7.450580596923828 \times 10^{-24}$, $3.725290298461914 \times 10^{-24}$, $1.862645149230957 \times 10^{-24}$, $9.313225746154785 \times 10^{-25}$, $4.656612873077392 \times 10^{-25}$, $2.328306436538696 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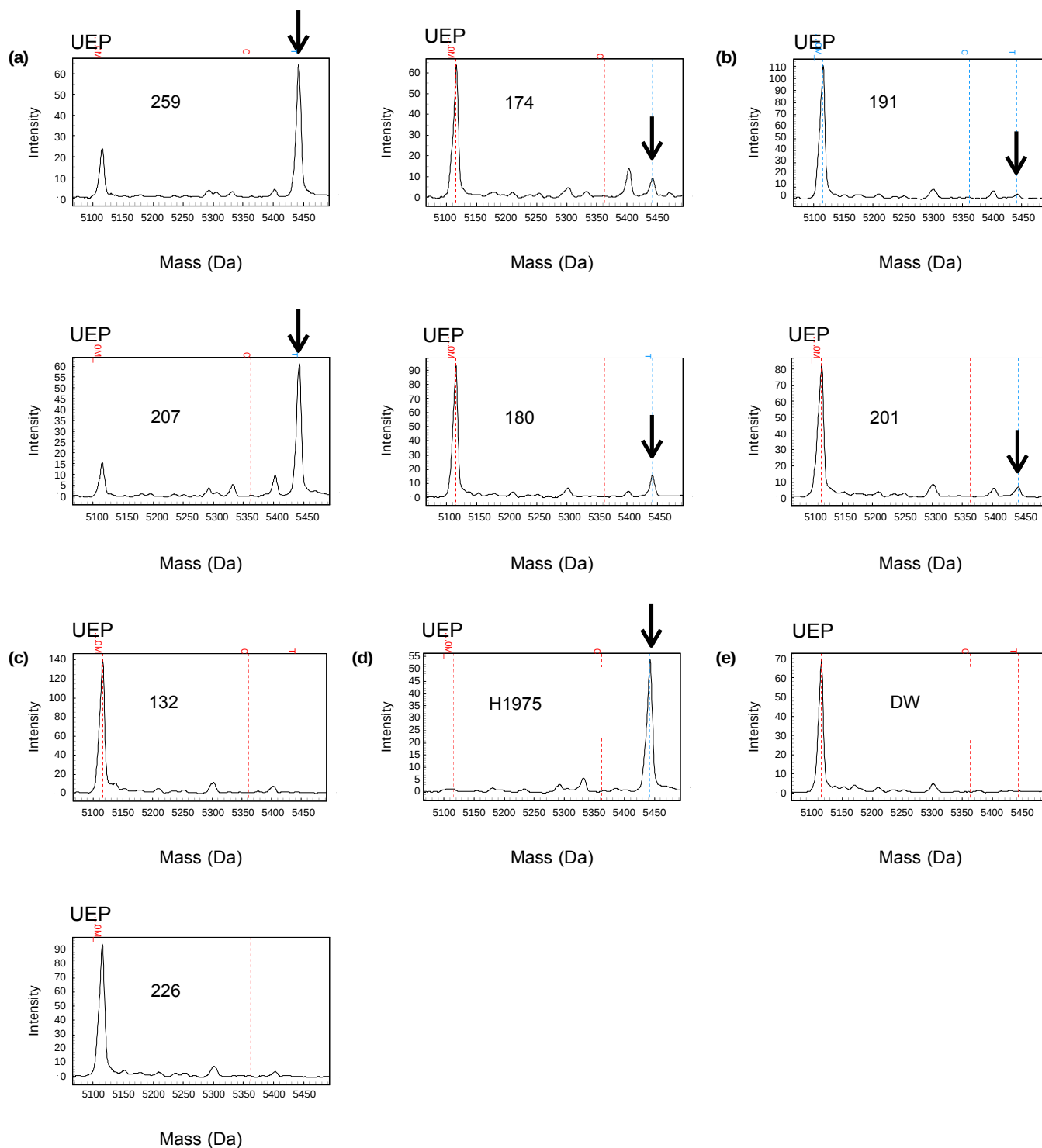


Fig. 3. Mass spectrometry (MS) analysis of the T790M mutation in plasma DNA. An arrow at 5442 Da indicates the detection of the T790M mutation. (a) Example of a T790M-SABER/subcloning double-positive sample. (b) Example of a T790M-positive case that was only detected using the T790M SABER method. (c) Example of a T790M-negative case, as detected using the T790M SABER method. (d) Positive control for the T790M-SABER method using T790M-positive human non-small cell lung cancer (NSCLC) H1975 cells. (e) Negative control for the T790M-SABER method using distilled water. The experiment was repeated twice with identical results. DW, distilled water; UEP, unextended primer.

7.8×10^{-18} mol/reaction (i.e. 4.8%, 2.4%, 1.2%, 0.6%, 0.3%, 0.2%, and 0.1%, respectively) mixed with a fixed amount (1×10^{-14} mol/reaction) of wild-type DNA oligonucleotide. Figure 2 shows the MALDI-TOF MS spectra. Concentrations as low as 3.1×10^{-17} mol of T790M DNA mixed with

1×10^{-14} mol of wild-type DNA could be detected, indicating a detection sensitivity of approximately 0.3%, which is in agreement with previous studies using SABER.⁽⁶⁾

Detection of T790M in plasma DNA. We examined the T790M mutation status in plasma samples obtained after dis-

Table 3. Clinical characteristics of T790M mutation positive patients with acquired resistance to epidermal growth factor-tyrosine kinase inhibitor (EGFR-TKI)

No.	Age	Sex	Histology	Pre-treatment EGFR mutation status (tumor) [†]		Response to EGFR-TKI	From PD to plasma collection (days)	Post-treatment T790M mutation status (plasma) [‡]	
				Direct sequencing	SABER			SABER	Clonal analysis T790M clones/Total clones
147	69	F	Ad	unknown	-	PR	139	+	0/105
162	76	F	Ad	L858R	-	PR	190	+	0/105
174	60	F	Ad	del E746-A751	n.t.	PR	272	+	2/20
180	67	F	Ad	del E746-A751	n.t.	PR	34	+	3/25
185	75	M	Ad	G719X	+	PR	17	+	1/55
191	56	F	Ad	L858R	-	PR	17	+	0/105
192	68	M	Ad	del E746-A751	-	PR	65	+	0/105
199	72	M	Ad	L858R	-	PR	0	+	0/100
201	73	F	Ad	del E746-A751	-	PR	390	+	0/105
207	64	F	Ad	L858R	-	PR	44	+	4/35
214	61	F	Ad	L858R	-	PR	60	+	2/65
217	71	F	Ad	unknown	n.t.	PR	128	+	3/20
235	69	F	Ad	unknown	n.t.	PR	222	+	1/35
243	59	M	Ad	L858R	-	PR	14	+	1/25
246	54	F	Ad	del E746-A751	-	PR	182	+	1/20
248	42	F	Large	del E746-A751	n.t.	PR	23	+	1/30
258	44	M	Ad	del E746-A751	-	PR	299	+	1/100
259	60	F	Ad	L858R	-	PR	237	+	2/25
279	71	F	Ad	L858R	n.t.	SD	379	+	1/30
306	43	M	Ad	del E746-A751	n.t.	PR	34	+	1/57
308	65	M	Sq	del E746-A751	n.t.	PR	42	+	0/105

[†]The EGFR mutation status of each tumor sample was analyzed using direct sequencing and SABER (single allele base extension reaction) method.

[‡]The EGFR mutation status of each plasma sample was analyzed using the SABER method and was confirmed using clonal analysis. +, T790M positive; -, T790M negative; Ad, adenocarcinoma; Large, large cell carcinoma; n.t., not tested; PD, progressive disease; PR, partial response; SD, stable disease; Sq, squamous cell carcinoma.

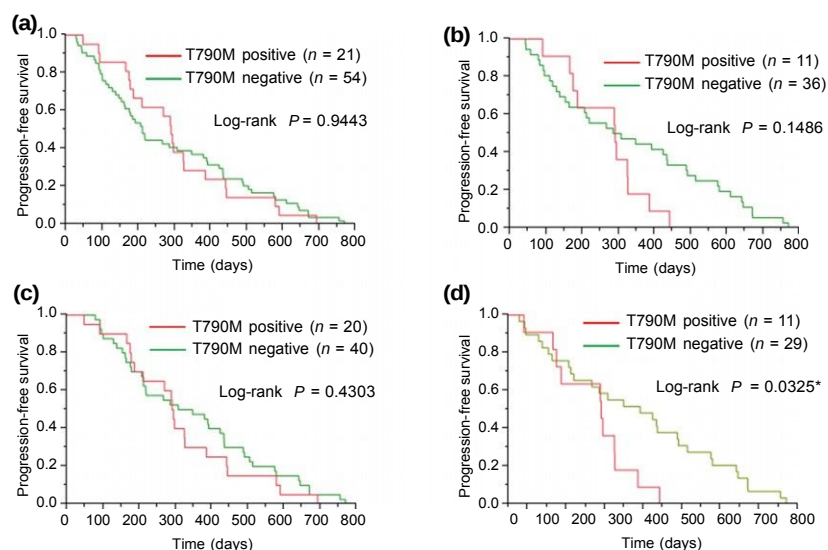


Fig. 4. Progression-free survival according to T790M mutation status as measured in plasma DNA. The Kaplan-Meier progression-free survival curves are shown for all the T790M-positive and T790M-negative patients ($n = 75$) in (a), for patients aged 65 years or younger ($n = 47$) in (b), for patients with a partial response to epidermal growth factor-tyrosine kinase inhibitors (EGFR-TKIs) treatment ($n = 60$) in (c), and for patients aged 65 years or younger who had a partial response to EGFR-TKI treatment ($n = 40$) in (d).

continuation of EGFR-TKI. Using the SABER method, the internal control was successfully amplified and detected in all the samples (data not shown). The T790M mutation was detected in 21 of the 75 samples (28%). The key results of the SABER method are shown in Figure 3. The clinical characteristics of the T790M-positive patients are shown in Table 3.

The presence of the T790M mutation was confirmed by subcloning into sequencing vectors and sequencing. When up to 105 colonies were selected and sequenced (theoretical median limit of detection of 0.95%), the T790M mutation was confirmed in 14 of the 21 (66.6%) PCR products. The T790M mutation in plasma was also identified by the Scorpion ARMS (Table S1).

T790M was detected in 6/75 cases (8%). Of these cases, 5/6 were also positive by the SABER method. Compared to the positive rate (28%, 21/75) when using the SABER method, the rate detected by the Scorpion ARMS was relatively low.

Detection of T790M in pretreatment tumor specimens. We analyzed the T790M mutation status in the tumor samples using the SABER method. All of the tumor samples were obtained before the EGFR-TKI treatment. No tumor sample was obtained by the re-biopsy after EGFR-TKI treatment. T790M was detected in two tumor samples (Table S1). One case (no. 185) was double positive (tissue +, plasma +). Another case (no. 167) was T790M positive in tumor samples only. The positive rate (7%, 2/28) was relatively low as compared with that of plasma samples (46%, 13/28). These data suggest that the T790M mutation detected in the plasma is acquired by EGFR-TKI treatment and that detection of T790M in the plasma is feasible to detect EGFR-TKI refractory cases.

Correlation between plasma T790M mutation status and clinical outcome. There was no significant difference in clinical characteristics between patients with or without T790M mutation (Table 1). T790M positive detection tends to be observed more frequently in PR patients compared with non-PR patients although the correlation is not highly significant ($P = 0.053$; Table 1).

In our cohort of 75 patients, the median PFS of the patients with the T790M mutation ($n = 21$) was not statistically different from that of the patients without the mutation ($n = 54$) ($P = 0.9443$), being 289 days and 210 days, respectively (Fig. 4a). When patients aged 65 years or younger were subdivided into two groups according to their plasma T790M status, the median PFS of the T790M-positive patients ($n = 11$) tended to be shorter than that of the T790M-negative patients ($n = 36$, $P = 0.1486$; Fig. 4b). We also compared the PFS of patients according to their response to the EGFR-TKIs and found no statistical differences between the responders (PR) and the non-responders ($P = 0.4303$; Fig. 4c). When patients aged 65 years or younger who had a PR were grouped according to their plasma T790M mutation status, the PFS of the T790M-positive patients ($n = 11$) was significantly shorter than that of the T790M-negative patients ($n = 29$, $P = 0.0325$; Fig. 4d) being 289 days and 391 days, respectively.

Discussion

Our findings show that the T790M mutation can be detected in plasma samples obtained after discontinuation of EGFR-TKI and that the SABER method is a feasible means of determining the plasma T790M mutation status. We detected the T790M mutation in 21 out of 75 plasma samples that were obtained from patients after discontinuation of EGFR-TKI (28%). This frequency seems to be lower than the positive rate (~50%) in tumor tissue samples reportedly.⁽⁷⁾ However, the positive rate when using SABER is relatively higher than when using Scorpion-Arms (Table S1) for the same samples and our previous report.⁽⁸⁾ We can speculate that the sensitivity of the SABER assay for circulating samples (plasma or serum) is much improved. In this study, plasma samples were collected following therapy with the EGFR-TKI; therefore, the time between the detection of PD and that of sample collection was varied. To conclude whether this sensitivity is enough or

not, it will be necessary to conduct a prospective comparison study using the paired samples of plasma and re-biopsy samples as the next step. Previously, we attempted to determine the EGFR mutation status in serum samples using the highly sensitive Scorpion-Arms method.^(8,9) In that cohort, a high false-negative rate was observed, and more sensitive methods of detecting EGFR mutations in serum samples are desirable. Another group reported the results of a serum EGFR mutation analysis using the MBP-QP method,⁽¹⁰⁾ which yielded a detection sensitivity equivalent to that of the SABER method used in the present report. Automated and high throughput analysis is an advantage of SABER method.

Direct sequencing of the subcloned PCR products confirmed the presence of the T790M mutation, suggesting that this SABER method is highly specific. However, the clonal analysis did not detect the T790M mutation in seven of the 21 (33.3%) samples that were found to be T790M-positive using SABER. In this cohort, we selected and sequenced up to 105 colonies (theoretical median limit of detection of 0.95%). Therefore, the number of tested colonies might not have been large enough to detect the low frequency of mutant clones with normal sampling error distribution.

In our study, the median PFS of the T790M-positive patients was significantly shorter than that of the T790M-negative patients with a clinical PR among younger (≤ 65 years) patients. It is likely that the prognosis of patients with an activating mutation who acquire a resistance mutation is less favorable. However, Oxnard et al.⁽¹¹⁾ showed that EGFR-TKI resistant patients with T790M identified in re-biopsy specimens had a relatively favorable prognosis compared with patients without the T790M mutation. Their data seems to be inconsistent with our result. Different materials and detection methods may be the cause of this discrepancy between two studies.

The early detection of T790M mutation may be beneficial to such patients.

The intrinsic existence of T790M clones in pretreatment tumors has been previously suggested. Indeed, the T790M mutation has been detected in a few pretreatment tumor samples.^(12,13) The T790M-SABER method is highly sensitive and is capable of detecting this mutation in plasma samples; consequently, the detection of the T790M mutation before or during EGFR-TKI treatment may be possible. Consequently, the T790M-SABER method is a promising tool for the detection of T790M mutation in a diagnostic setting.

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Disclosure Statement

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Supporting Information

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

Table S1. A comparison of T790M detection by SABER and Scorpion-ARMS methods.



次世代シーケンス技術を応用したがん薬物療法最適化への試み

土原 一哉*

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Clinical Application of Next-Generation Sequencing Technologies to Achieve Cancer Precision Medicine: Katsuya Tsuchihara (Division of Translational Research and Clinical Trial Center, National Cancer Center)

Summary

Clinical applications of genomic biomarkers have been rapidly expanding, as has the development of molecular targeted therapies for various cancers. Ras activation has been regarded as a negative predictive marker for anti epidermal growth factor receptor (EGFR) antibody therapy for colorectal cancer. Detection of mutations in KRAS codons 12 and 13 is widely

used in clinical settings. Recent studies revealed that minor activating mutations such as those in KRAS codons 61 and 146 as well as NRAS mutations, contribute to resistance. Multiplex mutation testing that includes analysis of these mutations will be clinically available in the near future. We have conducted translational research in which novel biomarker candidates were evaluated using whole-exome sequencing based mutation profiles of anti-EGFR antibody-treated samples from multiple centers in Japan. Development of the necessary infrastructure to ensure that genetic testing data are properly handled and

要旨 分子標的療法の進展に伴い、治療効果予測に用いられるゲノムバイオマーカーの数は増加している。大腸がん抗 EGFR 抗体療法の効果を予測する RAS 遺伝子検査は、KRAS コドン 12・13 について行われてきたが最近の研究結果より、これまでマイナーな変異とされてきた KRAS の他のコドンや NRAS 変異に拡大することで臨床的有用性が向上することが示された。さらに新規のバイオマーカーを探索するために、詳細な臨床情報を附随する組織標本を全国から集積し次世代シーケンサーによる大規模ゲノム解析を行う臨床研究も進行している。一方で、臓器の枠を越えてゲノムバイオマーカーを有効に利用するために実地臨床での次世代シーケンサーを利用した変異検索の可能性を評価することも重要である。国立がん研究センターにおけるクリニカルシーケンスの現状を紹介し、今後の課題について考察する。

1. 大腸がん治療効果予測バイオマーカーの拡大

いわゆる次世代シーケンサーの実用機の登場から 5 年以上が経過し、がん研究の領域でもゲノムワイドの高精度変異解析が一般的になった。国際がんゲノムコンソーシアムを中心とした各種がんゲノムのカatalog作りも順調に進捗し、新たながん関連遺伝子の同定や、特徴的なゲノム DNA 塩基置換のシグネチャーからがんの発生要因との関連を探索する取り組みなど、基礎研究領域における寄与は多大なものがある¹⁾。一方、高速シーケンス

技術の臨床応用も着実に進んでいる。その背景には、分子標的治療の効果を予測するバイオマーカーの重要性が広く認識され、その種類も増え続けていることがある。代表的なものは、非小細胞肺癌において EGFR 阻害剤の奏効と関連する EGFR の活性型変異であり、また大腸がんの抗 EGFR 抗体薬の不応に関連する KRAS の活性型変異である。EGFR・KRAS 遺伝子の変異 (挿入欠失変異および塩基置換変異) 検査は国内でも汎用されており、品質が保証された対外診断薬キットが承認されている。これらのキットは単一遺伝子の数か所のホットス

* 国立がん研究センター 早期・探索臨床研究センター・トランスレーショナルリサーチ分野

ポット変異を同時に検出するように設計されており、変異箇所の認識には、変異アレル特異的プライマーを用いた PCR (Scorpion-ARMS 法) や、ラベルされた特異的プローブと変異アレルとのハイブリダイゼーション (xMAP 法) が利用されている^{2,3)}。いずれもサンガー法によるダイレクトシーケンスに比べ感度・特異度に優れていること・特殊な操作・技術を必要とせず・検査に必要な機器も普及していること・検査結果を数量化でき客観的な判定基準を規定しやすいこと・検査に掛かるコストが比較的安価であることなど、臨床検査として成立するための要件を満たしている。

KRAS コドン 12・13 に変異をもつ大腸がん症例に抗 EGFR 抗体薬であるセツキシマブの治療効果が期待できないことは、複数の第Ⅱ相・第Ⅲ相臨床試験のサブセット解析から明らかにされた⁴⁻⁷⁾。これらを根拠として治療前 KRAS 遺伝子検査が開始されたのは前述のとおりであるが、Ras 蛋白質の立体構造上・GTP 結合部位を構成するコドン 61 やコドン 146 にミスセンス変異が生じても GTPase 活性が低下し・細胞増殖のシグナルを「オン」にすることは生物学的にはよく知られている。コドン 12・13 と同じくこれらの変異も治療抵抗性の予測マーカーとなる可能性が想定されたが、変異の発生頻度の低さがネックとなり臨床的な検証は難しいとされてきた。英国 Sanger 研究所が各種のがん組織における体細胞変異の文献情報を収集しているデータベース・COSMIC の最新版 (v67) には、大腸 (large intestine) の腺がん (adenocarcinoma) 44,982 例において KRAS 変異例は 16,276 例 (36%) が登録されている。このうち最も頻度が高い G12D 変異 (5,628 例) を筆頭に、コドン 12 変異 12,725 例 (変異症例の 78%)・コドン 13 変異が 3,360 例 (変異症例の 20%) を占めるのに対し、コドン 61 変異 214 例・コドン 146 変異は 97 例にとどまっている。これらのデータから、変異検索をコドン 12 および 13 にかぎっても KRAS 変異例のほとんどがカバーでき、臨床に十分な性能が期待できると考えられてきた。

国立がん研究センター東病院の吉野孝之医長と筆者は大腸がんゲノムバイオマーカーの適正化をめざし、消化器がんの治療開発の経験が豊富な施設によって構成された研究グループを主宰しており、KRAS 変異検査の性能が実地臨床においても満足できるものかを検証することにした。北米・欧州と異なり日本におけるセツキシマブの承認 (2008 年) と KRAS 検査の承認・普及 (2010 年) には時間差があった。残念ながらこれは日本における明白なデバイスラグを示すものであるが、翻って市販後 KRAS コドン 12・13 変異例に対する治療効果の検討は日本でしか行えないとも考えられた。後方視的解析では

あるが上記のラグの期間に国立がん研究センター東病院・北海道大学・がん研有明病院・四国がんセンター・埼玉県立がんセンター・静岡がんセンター・富山大学においてセツキシマブ・イリノテカン併用療法が施行された 376 例から、規定に従った治療前後の画像診断の有無など十分に評価に耐え得る 109 例を抽出した。Scorpion-ARMS 法・xMAP 法によるコドン 12 変異検出例 26 例・コドン 13 変異例は 7 例であった。一部の施設で先進医療として KRAS 変異検査の結果を診療に利用していたことなどを考慮しても、そのプロファイルは COSMIC 登録例と大きく変わらないことが確認された。コドン 12・13 野生型 76 例において 23 例 (30%) の奏効が得られたのに対し、コドン 13 変異例の 1 例を除き KRAS 変異例に奏効はなく、無増悪生存期間・全生存期間においても既報とほぼ一致した結果となっていた。コドン 13 変異例において統計学的有意差には至らないものの、無増悪生存期間・全生存期間の延長の傾向が認められたのも、過去の臨床試験のサブセット解析の結果と矛盾しないものと考えられた。これらの結果はコドン 12・13 変異検査の negative predictive marker としての有用性を担保するものであった⁸⁾。

ただし、依然低い KRAS 野生型の奏効率が次の課題になった。薬理学的その他の原因も考えられたが、まず KRAS コドン 12・13 以外の変異・KRAS 以外の EGFR 下流分子の変異の寄与を検討することにした。改めて組織検体が入手可能な 82 例を抽出し、既承認の KRAS コドン

12・13 検査 (MEBGENTM KRAS 遺伝子変異検出キット・株式会社医学生物学研究所) と同じ原理 (xMAP 法) により、ほぼ同等の性能で BRAF・KRAS・NRAS・PIK3CA の一塩基置換変異を検出するキット (GENOSERCHTM Mu-PACKTM・株式会社医学生物学研究所) を開発し、これを用いて変異検索を行った^{9,10)}。82 例中計 21 例の KRAS コドン 12・13 変異例があったのに対し、KRAS コドン 61 変異 3 例・コドン 146 変異 2 例・NRAS 変異 2 例などが検出され、母集団の小ささを考慮しても大腸がんにおける Ras ファミリーのマイナー変異の割合が無視できないことが示唆された (図 1)。改めて上述の COSMIC データベースにおける変異頻度の解釈には収集された各研究における変異検出範囲・方法のバイアスを考慮する必要があると考えられる。実際、米国のがんゲノム解析コンソーシアム・TCGA が行った系統的な網羅的全エクソン解析の対象例にかぎると、KRAS 変異陽性 118 例中コドン 12 変異 51 例・コドン 13 変異 12 例に対し、コドン 61 変異 8 例・コドン 117 変異 4 例・コドン 146 変異 11 例が同定されており、筆者らの実感とも一致

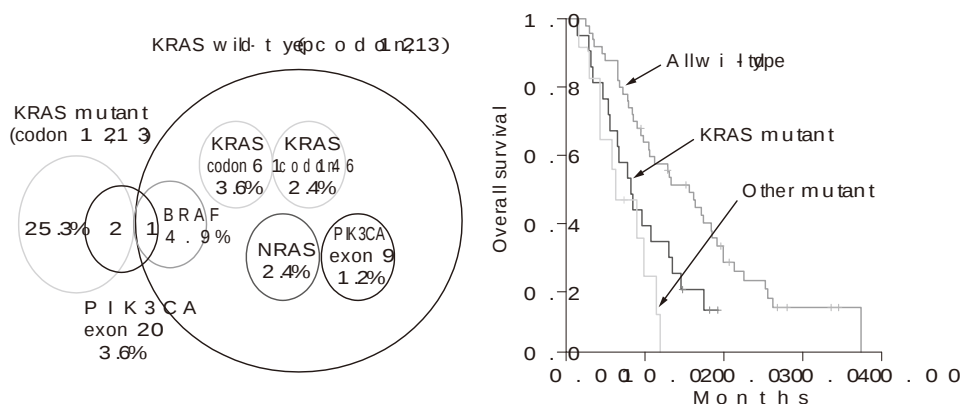


図 1 マルチプレックスゲノムバイオマーカーによる抗 EGFR 抗体療法の最適化 (文献⁹⁾を改変)

イナー変異に BRAF・PIK3CA 変異を加えた 12 例に奏効例はなく・無増悪生存期間 1.6 か月・全生存期間 6.3 か月も KRAS コドン 12・13 変異 21 例における無増悪生存期間 2.7 か月・全生存期間 8.2 か月と有意差を認めなかった (図 1)。⁹⁾ KRAS コドン 12・13 変異のみを除外した際の奏効率 31.1% に対し・それ以外の変異も除外すると奏効率は 38.8% に向上することも示され・大腸がん抗 EGFR 抗体薬の治療効果予測における KRAS コドン 12・13 以外の変異検出の有用性が示唆された。最近・もう一つの抗 EGFR 抗体薬であるパニツムマブの FOLFOX4 療法における上乗せ効果を示した PRIME 試験の後解析が発表された。同試験における KRAS コドン 12・13 野生型 512 例のうち 108 例に KRAS コドン 61・117・146 変異もしくは NRAS 変異が検出され・これらの症例では KRAS コドン 12・13 変異例と同様にパニツムマブの効果がみられなかった。なお・この論文では BRAF 変異については・従前の報告と同様予後予測因子としての側面が強いことが報告されている¹¹⁾。

これらの結果は・生物学的な基礎研究の結果から推測された抗 EGFR 抗体薬の治療効果を修飾する因子が・実際の臨床例においても重要な役割を果たしていることを示す合理的なものであり・拡大した RAS 遺伝子変異をバイオマーカーとして臨床応用することの必要性を示している。そのためには・性能・価格など遺伝子診断の基準に適合した検査システムの開発が必要である。検索しなければならぬ変異の増加により・ダイレクトシーケンス法や Scorpion-ARMS 法では検査手順が煩雑になり・また検査に必要な検体の量も増大する。筆者らが用いたマルチプレックス PCR と Luminex 技術を組み合わせた方法であれば 100~200 か所程度の変異検索を従来の KRAS 検査法とほぼ同じ手順で解析可能である。現在この方法を応用し・さらに感度を向上させた診断試薬について早期の保険承認をめざした臨床性能試験 (UMIN000011784・Luminex 技術を用いた新規 RAS 遺

伝子変異検査試薬の臨床性能試験・責任研究者 吉野孝之・実施責任組織 株式会社医学生物学研究所) が進行中である。

・全エクソン解析を応用した新規バイオマーカー探索の試み

筆者らの研究グループでは・抗 EGFR 抗体薬の効果を修飾する因子をより広範に探索することで・治療効果が期待できる患者集団をさらに絞り込める可能性を追求している。愛知県がんセンターを加えた上述の臨床研究グループに東京大学・国立国際医療研究センターのゲノム解析グループ・診断薬開発企業 (G & G サイエンス株式会社) が参加したコンソーシアムを形成し・科学技術振興機構の研究成果最適展開支援プログラム (A-STEP) の補助を受け研究を開始した。研究成果によってがんの最適化治療実現のために風穴を開けることをめざして・BREAC study と呼称している「網羅的遺伝子解析技術を用いた抗 EGFR 抗体薬治療効果予測バイオマーカーの探索研究に関する多施設共同研究」では・原則として KRAS コドン 12・13 に変異をもたない大腸がん抗 EGFR 抗体薬投与例の組織を集積し全エクソン解析を行い・詳細な臨床情報と併せて治療効果と関連する遺伝子変異・多型を探索する (図 2)。

この研究には二つの技術的な特徴がある。まず・対象症例を抗 EGFR 抗体薬投与時期の違いによって「探索用コホート」・「統計推測用コホート」に分けた。効果に関連する特徴的な変異を濃縮するため探索用コホートでは「著効例」・「無効例」のみを各施設から抽出して登録している。著効・無効の判定は各参加施設の腫瘍内科医に加え・画像診断・病理診断の専門医・生物統計学の専門家が加わったボード全員の合議で行うことにより・その信頼性を高めている。探索用コホートの解析で得られる候補遺伝子変異の妥当性は・各施設で当該期間に治療された連続的な症例を集積する統計推測コホートを用い検証

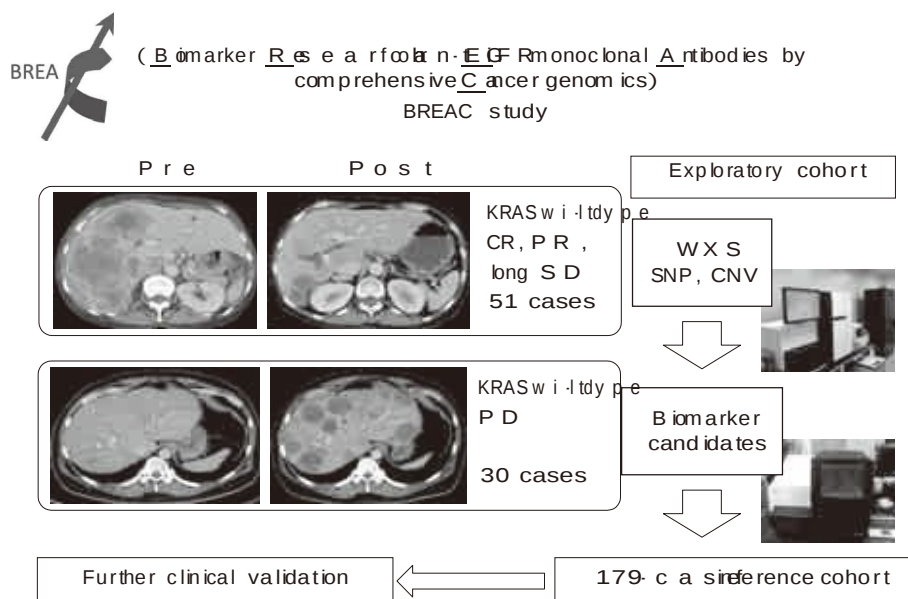


図 2 新規抗 EGFR 抗体療法バイオマーカーを探索するための BREAC study の概要
WXS: 全エクソンシーケンス・CNV: コピー数異常

する。いずれのコホートにおいても、各施設から送られてくる症例報告書は国立がん研究センター早期・探索臨床研究センター先端医療開発支援室、および臨床試験支援室で入念なチェックが行われ逸脱を防いでいる。ゲノム試料を用いた大規模な研究であり、個人情報管理についても各参加施設の倫理審査委員会の承認の下、連結可能匿名化を国立がん研究センター東病院個人情報管理室で一元的に行っている。バイオマーカー研究において解析に用いる臨床情報の質と検体提供者の保護は最重要項目であり、筆者らの取り組みは多施設共同研究における今後のモデルケースになると考えている。

一方、ゲノム解析では探索用コホート全例についてゲノム中の蛋白質をコードする全領域とマイクロ RNA の標的となる UTR 領域計約 50 Mb を標的としたターゲットシーケンスを行い、著効・無効群に有意に集積する変異遺伝子を同定した後、統計推測コホート試料について候補遺伝子変異をカバーしたカスタム化キャプチャーパネルを用いたターゲットシーケンスを行う。多施設において日常診療で得られる組織検体を解析する本研究では、ゲノム DNA のソースをホルマリン固定パラフィン包埋組織に求める必要があった。従来、国際コンソーシアムなどのがんゲノム研究では質の高い DNA 検体を確保するために凍結組織の提出を必須にするものが多い。しかし、ホルマリン固定後の DNA を利用することで、より臨床の実地に近いサンプル、希少症例のサンプルが得やすくなり、研究のスピードも向上することが期待される。筆者らはホルマリン固定後の DNA の抽出、シーケンスライブラリー作製について手法の改良と適切な品質評価法を工夫し、これまでに数百例の全エクソン解析

を成功させている(文献¹²⁾・投稿中)。この経験を生かし BREAC study においても著効 51 例・無効 30 例のシーケンスを終了し、抗 EGFR 抗体薬治療効果と相関するいくつかの変異遺伝子候補を見いだしている。

臓器横断型ゲノムバイオマーカー診断の実現可能性

大腸がんの EGFR 標的療法にかぎらず、今後の分子標的治療においてゲノムバイオマーカーの増加は避けられない。また、他臓器のがんで治療開発が進んでいる標的分子と同様の活性変化が示唆される遺伝子変異が低頻度で見つかるケースもあり、こうした症例を対象にした臨床試験の実施や、適応外使用のチャンスが広がることも予想される。こうした状況に対応するためにも、臓器の枠を越えてゲノムバイオマーカー診断が可能な体制を整備する必要がある。また、これらの検査は進行・再発症例を対象にすることが多く、内視鏡生検、針生検やいわゆる liquid biopsy によって得られる微量の組織サンプルを利用できるようにしなければならない。国立がん研究センター早期・探索臨床研究センター・東病院では近未来に予想される biomarker-based drug selection の実施可能性を検証するための ABC study(切除不能・進行・再発固形がんに対するがん関連遺伝子変異のプロファイリングと分子標的薬耐性機構の解明のための網羅的体細胞変異検索)を各科横断的に実施している(図 3)。ABC study は二段階の構成になっており、第一段階を target sequencing stage としている。文書による同意を得られた進行・再発固形がん患者の治療前生検の余剰検体からゲノム DNA を抽出し、市販のシステム(IonAmpliSeq™

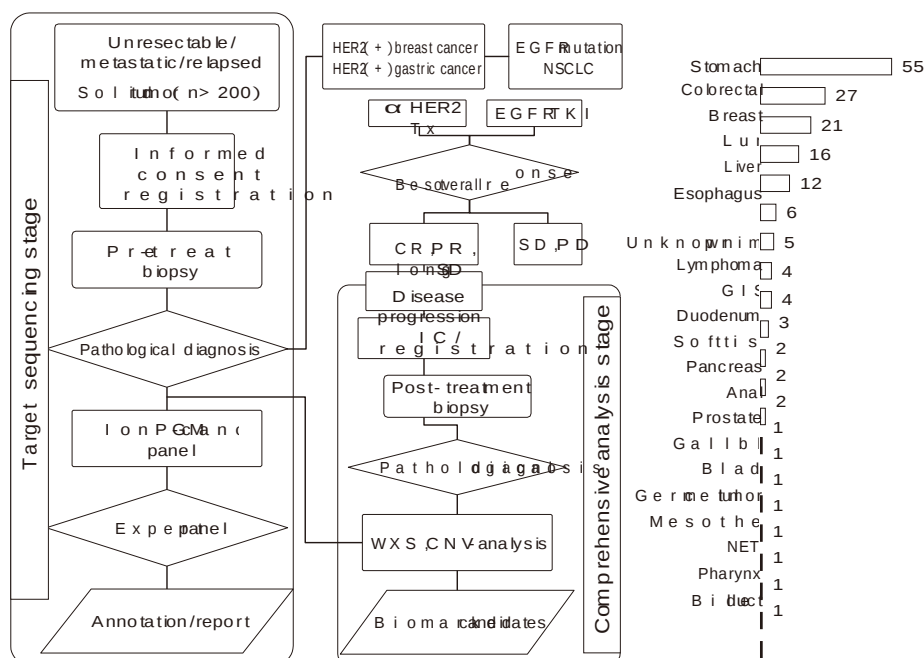


図 3 国立がん研究センター柏キャンパスで実施されている ABC study の概要と 2013 年 8 月現在での臓器別登録状況

Cancer Hotspot Panel v1 および v2)を用いマルチプレックス PCR で増幅した約 50 遺伝子のホットスポット変異を、半導体型次世代シーケンサーでシーケンス、変異検出する。その後、生物学的特徴、変異頻度など臨床疫学的な特徴、既承認および開発中の治療法に関する薬理学的情報を研究事務局で集積し、センター内の病理学、臨床腫瘍学、臨床遺伝学、分子生物学、生物統計学の専門家からなるエキスパートパネルで内容を討議した後に臨床担当医にレポートする。第二段階として target sequencing stage に登録された症例のうち、標準治療として分子標的治療が施行されたものを追跡し、いったん奏効した後、病勢が制御できなくなった際に再同意を得て生検を行い、治療前後の試料について全エクソン解析を含む網羅的ゲノム解析を行い、治療抵抗性の原因を探索するステップを comprehensive analysis stage としている。target sequencing stage には 2012 年 7 月の研究開始以降、約 1 年で想定を上回る 160 例以上の症例が集積された。国立がん研究センター東病院における診療実績をほぼ反映して多臓器の症例が登録されている。比較的早期の治療ラインで target sequencing stage に登録されていることもあり、comprehensive analysis stage は現状では 5 例にとどまっているが、今後登録が進むと予想している。target sequencing stage では 99%以上の同意取得が得られ、組織学的にがん細胞が確認された症例の 99%でアンプリコンシーケンスに成功している。46 遺伝子の 739 変異をカバーする初期型の Cancer Panel を用いた 93 例の解析では 1 検体当たり平均 1.6 個の変

異が検出され、PIK3CA や BRAF・EGFR など既存の分子標的療法の治療効果との関連が想定される何らかの変異が約 40%の症例で見いだされている。ABC study は腫瘍内科医、分子生物学者だけでなく、生検を担当する医師、看護師、検体を処理する検査技師に加え、データマネージャーなどセンター内の各職種の協力なしには成り立たない。また、今後 BRCA1/2 など遺伝性腫瘍にかかわる変異の診断にも対応できるよう家族性腫瘍外来の整備を進めたことも、この研究の成果であった。現在 DNA 抽出からアンプリコンシーケンスのステップは一連の検査工程のバリデーションを定期的実施している外部の臨床検査機関（株式会社エスアールエル）に委託している。今後、次世代シーケンサーを利用したがん遺伝子検査を実施していくためには国内では未整備の検査機関、手法の標準化も避けて通れない課題である。

おわりに

新たなドライバー変異の発見とそれに基づく治療法の開発など、基礎研究と臨床の距離は極めて近くなっている。新たな知見の有効性、妥当性をいち早く検証する先進的で臨床的な研究体制の構築に合わせ、こうした「ショールーム」で得られた結果を全国に展開するシステムの構築にも今後力を注ぎたいと考えている。

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