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Original

Frequency of *cis* and *trans* EGFR T790M and activation mutations in tumors treated with EGFR inhibitors

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Abstract

The exon 20 T790M mutation confers resistance to 1st- and 2nd-generation EGFR-TKIs while the C797S mutation confers resistance to the 3rd-generation EGFR-TKI, osimertinib. The presence of C797S in *trans* with T790M restores sensitivity to 1st/2nd-generation EGFR-TKIs. In the current study, we analyzed whether the T790M mutation was present in *cis* or *trans* with activating mutations in tumors treated with osimertinib.

We analyzed tissue specimens harboring both T790M and activating mutations in patients with non-small cell lung cancer obtained from May 2016 to June 2018. After the presence of EGFR mutation was confirmed using the PNA-LNA PCR clamp method, we determined whether activating mutations were located in *cis* or *trans* with the T790M mutation using RT-PCR. Results were validated using colony PCR followed by Sanger sequencing, which allowed us to calculate the allele frequency of each. Both EGFR mutations were detected in 11 out of 23 cases. All 11 cases had *cis* configuration. The low allele frequency of T790M might be related to the short duration of osimertinib treatment.

The *cis* configuration of T790M and activating mutations in all specimens was confirmed, and this result was in contrast to the existence of *trans* configuration of C797S and T790M.

Key words : *EGFR gene mutation, cis configuration, trans configuration, allele frequency, osimertinib*

I. Introduction

Of the 9.6 million cancer deaths worldwide in 2016, more than half (54%) were due to the top 10 tumor types. Among these, lung cancer claimed 1.7 million deaths in 2016 [1]. Epidermal growth factor receptor (EGFR) mutations exist in about 30–50% of non-small cell lung cancers, and are associated with nonsmokers, females, adenocarcinomas, and Asians [2]. Mutant EGFR-driven signaling promotes carcinogenesis, cancer cell proliferation,

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invasion, and metastasis. The high sensitivity of EGFR mutated non-small cell lung cancer (NSCLC) to EGFR tyrosine kinase inhibitor (EGFR-TKI) was discovered in 2004 [3,4]. The major EGFR mutations are exon 19 deletion (Ex 19 del) and exon 21 point mutation (L858R), which are found in 44% and 41% of all NSCLC cases, respectively [5]. The mutated EGFR continuously stimulates signal transductions to the downstream growth signal pathway without functional stimulation of ligands such as EGF and Amphiregulin. This is called an activating mutations.

The efficacy of EGFR TKI treatment has been demonstrated in several Phase III clinical trials in comparison with platinum combined chemotherapy for EGFR mutation-positive cases. For example, the benefits of gefitinib, erlotinib and afatinib have been demonstrated in the NEJ 002 study [6] and the WJTOG 3405 study [7], in the OPTIMAL study [8] and EUROTAC study [9], and in the LUX-LUNG 3/6 study [10,11], respectively.

Though EGFR TKIs are remarkably effective against EGFR mutant tumors, half of these malignancies develop resistance to the drugs within a year. A frequent TKI resistance mechanism is the selection for the exon 20 point mutation, T790M, a mutation with accounts for 40-60% of resistant cases [12]. The 3rd-generation EGFR TKI, osimertinib, was developed in order to counteract T790M-driven resistance. The combined analyses from both a global Phase I / II study (AURA) and a Phase II study (AURA 2 study) using osimertinib showed a progression-free survival of 9.7 months and response rate of 66.1 % [13-15]. Osimertinib can overcome resistance to 1st- and 2nd-generation TKIs if it is driven by T790M. However, 10 months after initiation of osimertinib treatment, half of the tumors develop resistance to osimertinib; in some cases, this is due to the emergence of the C797S mutation. The mechanism of resistance engendered by the C797S mutation was reported in 2015 [16]. This report demonstrated that the C797S mutation occurred in 6 of 15 cases that were resistant to osimertinib. In addition, studies of the mechanisms of resistance to the 3rd-generation EGFR inhibitor, osimertinib, showed that the T790M and C797S mutations can occur on both the same allele (*cis* configuration), or on separate alleles (*trans* configuration). The trans configuration of T790M and C797S restores sensitivity to 1st- and 2nd-generation EGFR-TKIs [16] (Fig. 1). Although the *trans* configuration of T790M and C797S was reported in several reports, there have been few reports regarding the *cis* or *trans* configurations between activating mutations and the T790M mutation. In this report, we designed the current study to address this important gap in our knowledge.

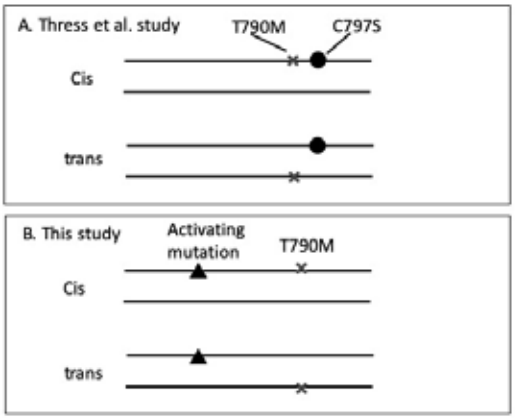

Fig 1. A. Cis and trans configuration between C797S and T790M.
B. Cis and trans configuration between activating mutation and T790M.

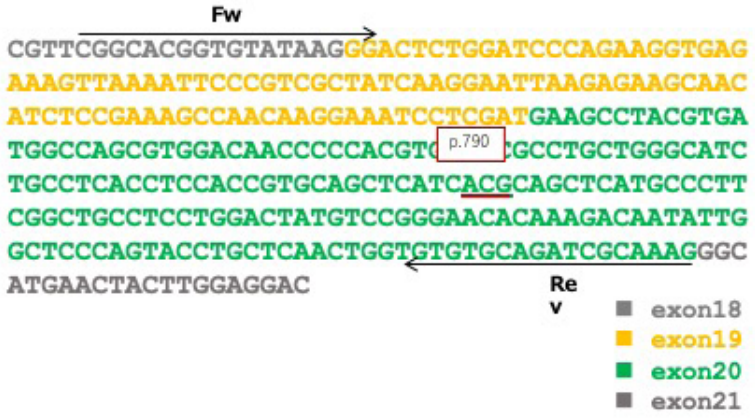

Fig 2. cDNA sequence of exons 19-20 and primers used.
Sequence of cDNA produced by mRNA transcribed from exon 19-20 DNA of EGFR are shown. Fw and Rev depict the locations of forward and reverse primers, respectively.

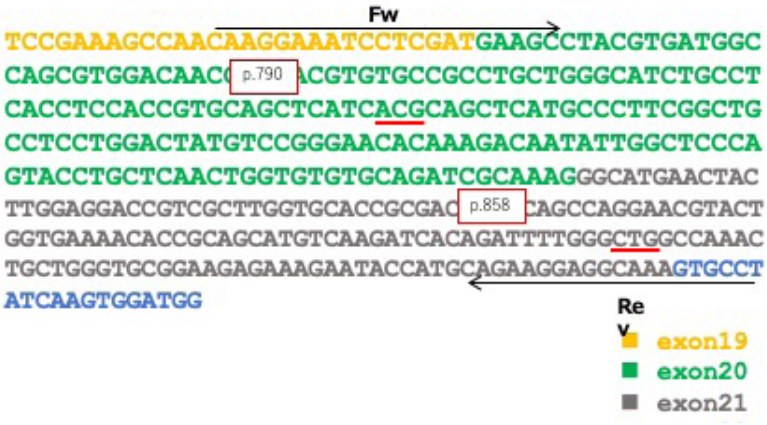

Fig 3. cDNA sequence of exon 20-21 and primers used.
The sequences of cDNA produced by mRNA transcribed from exon 20-21 DNA of EGFR are shown. Fw and Rev depict the locations of forward and reverse primers, respectively. Fw, forward primer; Rev, reverse primer.

II. Methods

1. Patients and specimen collection

Patients with non-small cell lung cancer (NSCLC) with both active type mutations [Ex 19 del (2235–2249 del GGAATTAAGAGAAGC) or L858R (CTG; CGG)] and the T790M (ACG; ATG) mutation, and who had undergone biopsy or operation between May 2016 and Sep 2018, were selected. Eligible cases were those who had received osimertinib treatment. The main criteria for eligibility were 1) patients with metastatic or postoperative recurrent NSCLC, 2) previous treatment with 1st- or 2nd-generation EGFR-TKIs, 3) presence of activating mutations (Ex 19 del, L858R mutation) at diagnosis, and 4) confirmed activa ting EGFR gene mutations and T790M mutations in tumor tissues or cell blocks obtained by re-biopsy before osimertinib treatment. Cases with no EGFR gene mutation were excluded. The specimens analyzed i n this study were taken after the disease progressed during 1st- or 2nd-generation EGFR-TKI treatment, or after treatment cessation due to toxicity.

Table 1. Primer sequences for the present study

Target	Primer	Sequence
Primers for RT-PCR	Fw	5'-GGCACGGTGTATAAGGGACTC-3'
Ex19del + T790M	Rev	5'-TTCATGCCCTTTGCGATCTG-3'
	Fw	5'-GCCAACAAGGAAATCCTCGATGAA-3'
L858R + T790M	Rev	5'-ATAGGCACTTTGCCTCCTTCTGC-3'

Table 2. Patient characteristics

	All	A(+/−)T(−)*	A(+)T(+)
	N = 23	N = 12	N = 11
Age			
Median(range)	68 (47-81)	67 (47-81)	71 (56-80)
≧ 75	9	5	4
< 75	14	7	7
Male sex	7	0	7
ECOG PS †			
0	16	8	6
1	6	3	3
2	1	1	0
Smoking status			
Current or former	11	7	6
Never	12	5	5
Histologic features			
Adeno	23	12	11
EGFR ‡ mutation			
Exon19	16	7	9
L858R	7	5	2
Staging			
Post-operativerecurrence	13	5	8
Ⅲ A	1	1	0
Ⅳ B	9	6	3
Metastases			
Brain	9	5	4
Pleural	5	2	3
Methods to collect specimen			
Bronchoscopy	12	5	7
Pleurocentesis	1	0	1
Operation	2	2	0
Percutaneous needle biopsy	8	5	3
Site of collection			
Lung	20	11	9
Lymph node	1	1	0
Pleural effusion	1	0	1
Liver	1	0	1
Kinds of specimens			
Tissue	22	12	10
Cell block	1	0	1

*A, activating mutation; T, T790M mutation.
† ECOG PS, Eastern Cooperative Oncology Group performance status.
‡ EGFR, epidermal growth factor receptor.

This study was conducted according to the provisions of the Helsinki Declaration and approved by the Ethics Committee of Iwate Medical University School of Medicine (MH2018-026) and Miyagi Cancer Center.

2. Preparation of DNA for mutation analysis

Total RNA was extracted from the tumor regions of formalin fixed paraffin embedded (FFPE) specimens using the RNeasy FFPE Kit (QIAGEN, Hilden, German). cDNA was synthesized with the use of SuperScript II Reverse Transcriptase (Invitrogen, California, USA) according to the manufacturer's manuals. The synthesized cDNA was subjected to a set of PCR reactions to amplify two overlapping regions of EGFR encompassing exons 19–22, which includes both sites of activating mutations and T790M, using two sets of primers, Ex 19del+T790M and L858R+T790M (Fig. 2,3). Primer sequences are shown in Table 1.

3. Sequence analysis

The PCR products were purified and cloned into the TA-cloning vector pGEM-T Easy (Promega, Wisconsin, USA) to transform *E. coli* JM109 competent cells (Takara Bio, Kusatsu, Japan), and subjected to colony PCR using M13 universal primers [17]. After purification, the amplicons were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Massachusetts, USA) with T7 and SP6 primers on an ABI 3130 sequencer (Thermo Fisher Scientific, Massachusetts, USA). Based on the resulting sequences, clones were classified according to whether they carried mutations Ex 19del, L858R or T790M in order to then evaluate whether two EGFR mutations (T790M and either of the activating mutations) were present in *cis* or in t*rans*.

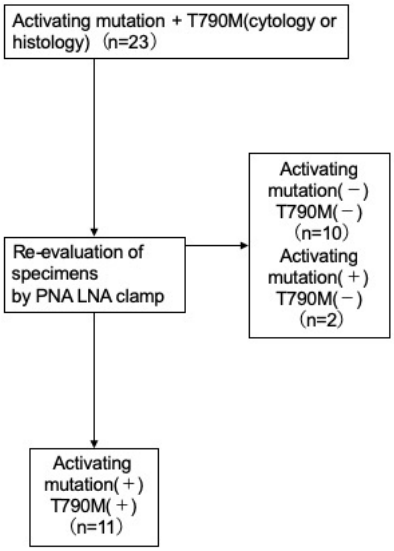

Fig 4. Consort. Tumors harboring an activating mutation (A) and T790M (T) were re-evaluated using the PNA-LNA clamp PCR method. Seven cases were eligible in this study.

To confirm configuration of *cis* or *trans*, colony PCR was performed. Plasmids including the prepared cDNAs were produced and transfected into *E. coli.* Transformants were picked up and the existence of the activating gene mutation and the T790M mutation was confirmed by PCR in each clone.

4. Statistical analysis

The ratio of *cis* or *trans* configurations and the relationship between allelic frequency and osimertinib efficacy were statistically evaluated using the chi-square test. Practically, the success with which targets could be amplified is dependent on two variables: the amount of mRNA available for cDNA synthesis and the length of time the sample is stored prior to analysis. The impact of these variables on successful target amplification was determined using the Mann-Whitney

test. Statistical analysis was performed using GraphPad Prism for Windows (GraphPad Software, California, USA).

III. Results

1. Characteristics

A total of 23 eligible cases with tumors with both an activating mutation and a T790M mutation were collected from Miyagi Cancer Center and Iwate Medical University (n =18 and n = 5, respectively). The median age was 68 (range, 47–81), 9 cases were over 75 and 10 cases were male. ECOG Performance Status (PS) was PS 0 in 17 cases, PS 1 in 5 cases, and PS 2 in 1 case. Tissue type was adenocarcinoma in all cases. In terms of EGFR gene mutation, 16 cases had Ex 19 del and 7 cases had L858R. Recurrence after operation was predominant. Eight cases had brain metastasis and 5 cases had pleural metastasis. The biopsy site was the lung in 15 cases, lymph node in 6 cases, and 1 case each for pleural effusion and the liver. The specimens were 22 tissue samples and 1 cell block. There was no difference between 12 untestable and 11 testable cases, with the exception of male predominance in the untestable cases (Table 2).

2. *Cis* or *trans* configuration of EGFR mutation

RT-PCR identified 11 out of 23 cases in which activating mutations and the T790M mutation were present. The consort of this study is shown in Fig. 4.

Comparing the testable 11 cases to 12 untestable cases, the amount of RNA available for RT-PCR in the testable group was higher than in the untestable group (testable group, 277 ng; untestable group, 134 ng), although the difference was not statistically significant (Fig. 5).

Table 3. Individual data of

ID	Age	Sex	ECOG PS [‡]	TNM [§]
G-04-T	80	male	0	pT2aN0M0
G-05-T	75	male	0	pT2aN2M0
G-06-T	72	male	0	pT2aN0M0
G-09-T	60	male	0	pT1bN2M0
G-14-T	63	female	1	pT1aN1M0
G-16-T	58	female	1	T2aN2M1c
G-17-T	75	male	0	T2N1M1c
G-20-T	56	female	1	T1aN0M0
G-21-T	68	male	0	T4N2M1b
G-22-T	71	male	0	T1aNxM0
G-23-T	76	male	0	T2aN2M0

[‡]ECOG PS, Eastern Cooperative Oncology Group performance status.
[§]TNM: T, primary tumor; N, regional lymph nodes; M, distant metastasis.

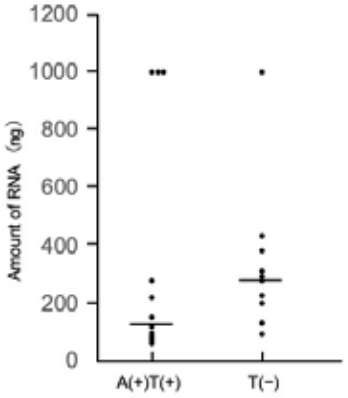

Fig 5. Relationship between the RNA yield and PCR amplification. The RNA yield was compared between group [A (+) T (+)] in which T790M was detected using the PNA LNA Clamp method and group [A (+/−) T (−)] in which T790M was not detected. A, Activating mutation; T, T790M mutation.

We next tested whether the RNA yield was influenced by duration of specimen storage. Storage duration tended to be longer in the untestable group than the testable group (median of 848 days and 617 days, respectively

patients with tumor harboring confirmed double mutation

Staging	Histologic features	EGFR †	Best response	Allele frequency (%)				Cis/Trans
				A(+) T(+)*	A(+) T (−)	A (−) T(+)	A (−) T (−)	
Ⅰ B Post-ope	Adenocarcinoma	exon19	SD#	29.2	54.10	0	16.70	Cis
Ⅲ A Post-ope	Adenocarcinoma	exon19	SD	100	0	0	0	Cis
Ⅰ B Post-ope	Adenocarcinoma	L858R	PR¶	100	50	0	0	Cis
Ⅲ A Post-ope	Adenocarcinoma	exon19	PR	28.6	10.5	0	21.40	Cis
Ⅱ B Post-ope	Adenocarcinoma	exon19	PR	89.5	63.9	0	0	Cis
Ⅳ B	Adenocarcinoma	exon19	PR	30.5	21.7	0	5.60	Cis
Ⅳ B	Adenocarcinoma	L858R	PR	17.4	83.3	0	60.90	Cis
Ⅰ A	Adenocarcinoma	exon19	PR	16.7	62.5	0	0	Cis
Ⅳ B	Adenocarcinoma	exon19	PR	25	54.2	0	12.50	Cis
Ⅰ A	Adenocarcinoma	exon19	SD	25	37.5	0	20.80	Cis
Ⅲ A	Adenocarcinoma	exon19	SD	16.7		0	41.60	Cis

†EGFR, Epidermal growth factor receptor.
※ A, activating mutation; T, T790M mutation.
SD, stable disease.
¶ PR, partial response.

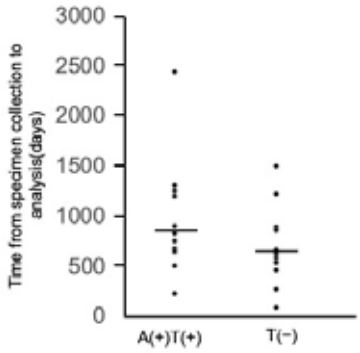

Fig 6. Relationship between PCR amplification and time elapsed from specimen collection to analysis. The period from specimen collection to analysis was compared between group [A (+) T (+)] in which T790M (T) was detected using PNA LNA Clamp method and group [A (+/−) T (−)] in which it was not detected. A, activating mutation; T, T790M mutation.

(Fig. 6). Indeed, the quality and quantity of RNA is important for successful analyses, as has been reported previously [18].

Next, cDNA was synthesized using reverse transcriptase according to the amount of RNA extracted from specimens. Complementary probes to the activating mutation and T790M mutation of the cDNA were prepared for PCR tests. Simultaneous detection of both an activating mutation and the T790M mutation by RT-PCR indicates that the two mutations are located in the *cis* configuration. In our study, the cis configuration was found in all 11 cases in which each mutation was detected by PCR. However, even in these cases there may be a minor cellular subpopulation in which the mutations are present in the trans position, and which is not detected by PCR due to the low abundance of target mRNA. To confirm whether the *trans* and *cis* configurations coexisted, we performed colony PCR. The results showed that both the activating mutation and the T790M mutation were found in all clones derived from two patients, whereas clones from all other patients had a configuration of the activating mutation and T790M, or the activating mutation alone (Table 3). No *trans* mutations were detected in any of the 11 cases, since there were no clones that

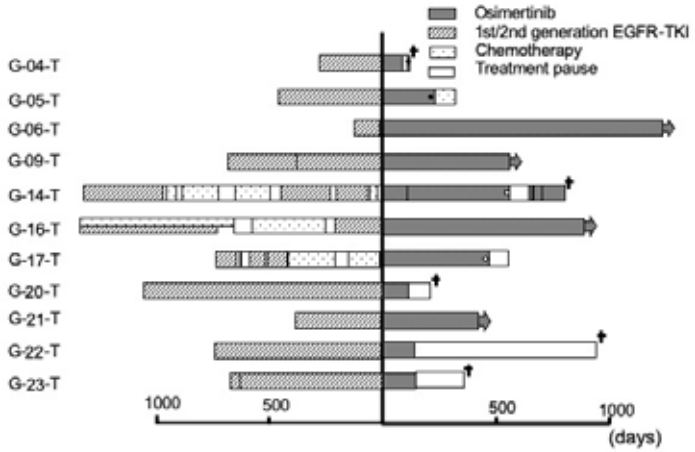

Fig 7. Duration of 1st/2nd generation TKI treatment and osimertinib treatment. In seven cases in which both EGFR activating mutations and T790M were detected in specimens. Duration of chemotherapy ( ) and the 1st/2nd generation EGFR-TKIs ( ) before initiation of osimertinib treatment and the duration of osimertinib treatment ( ) are shown. Blank( ) represents the observation period without treatment.
Diamonds indicate progressive disease (PD), circles indicate discontinuation due to toxicities, and crosses indicate death. Vertical lines show timing of rebiopsy and initiation of osimertinib treatment, respectively.

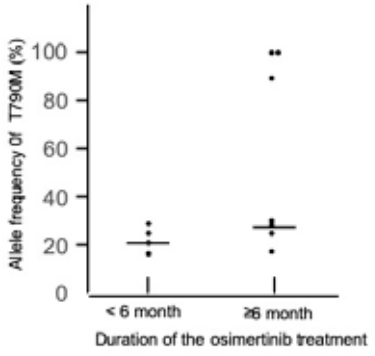

Fig 8. Allele frequency of T790M mutation according to duration of osimertinib treatment. Vertical axis shows percent of allele frequency of T790M. Allele frequencies are indicated each less than 6 months or more than 6 months duration of osimertinib treatment.

carried only the T790M mutation. In addition, the allele frequency of T790M was different between tumor samples taken from different patients.

3. Osimertinib treatment effect and allele frequency

Fig. 7 shows the pretreatment history before osimertinib and the treatment duration of osimertinib. Treatment with osimertinib achieved an 81% (9/11) response rate and 5 patients experienced failure of osimertinib treatment within 1 year, whereas 6 others were continued on osimertinib for the long term. Though there was no association between the T790M allele frequency and duration of the osimertinib treatment in general, patients receiving less than 6 months duration of osimertinib treatment had low allele frequency of T790M in tumors (Fig. 8). The lack of osimertinib efficacy we observed in two patients may have been due to the presence of pleural lesions. There was no relationship between duration of $1^{st}/2^{nd}$-generation TKI treatment before osimertinib treatment and the allele frequency of T790M.

IV. Discussion

When the distance between two target mutations on a stretch of DNA is short, such as the distance between T790M and C797S, the evaluation of whether these mutations are in *cis* or *trans* with respect to their alleles can be easily analyzed by Next Generation Sequencing (NGS). However, evaluating *cis* and *trans* configuration over longer distances (such as that between EGFR activating mutations and the T790M mutation) is better done by preparing cDNA from RNA and then performing colony PCR.

Our current results show that, when present, co-occurring mutations existed in *cis* and never in *trans*. This result is consistent with a recently published article, which showed resistance mutations definitely occurring in the activating mutant allele [19]. Leone discussed the mechanism by which activating and resistance mutations occur in the same allele [20]. In cases where the mutations exist in *trans*, there is a possibility that clones become sensitive to 1st/2nd-generation EGFR-TKI. Actually, it was reported that a case had tumors with the localization of C797S and T790M in *trans*, which is clinically important because such tumors respond to combination therapy of 1st- or 2nd-generation EGFR-TKIs and osimertinib [21]. Our current study showed that the T790M mutation was never located in *trans* with an activating mutation. Thus, at present, we infer that *cis* or *trans* configuration of EGFR does not have a clinical impact, with the clear exception of C797S and T790M in *trans*.

It was reported that when the EGFR resistance mutation is present in *trans* (approximately 20% of cases), a combination of 1st- and 2nd-generation TKI becomes effective [16]. On the other hand, multiple resistance mutations in the same allele of the anaplastic lymphoma kinase (ALK) fusion gene restore sensitivity to a 1st-generation ALK-TKI through induction of a conformational change [22]. Although oncogenic ALK gene fusions are clearly different to oncogenic EGFR gene mutations, the mechanisms of resistance and restoration of sensitivity conferred by mutational allelic configurations in these two genes may be similar.

On the other hand, it was found that the T790M mutation developed after the existence of activating mutation according to this analysis of allele frequencies. This phenomenon is attributed to the sequence of EGFR-TKI treatment. This study showed that the allele mutation frequency varies in each case. When the relationship between the allele frequency and the effect of the 3rd-generation TKI osimertinib was evaluated, no significant relationship was observed due to the small sample size. However, all tumors low response to osimertinib, less than 6 months PFS, showed low allele frequency of the T790M mutation. The efficacy of osimertinib is influenced by various factors. Regarding tumors these include heterogeneity, gene amplification, gene copy number gain, and so on, and regarding patients there are various characteristics of patients. For example, the presence of pleural effusion might explain why osimertinib was only effective over the short term.

Finally, our results confirm that colony PCR is a reliable method for determining the presence of *cis* or *trans* mutations and their allele frequency. The method can even

be used with short fragmented RNA from paraffin embedded tissue. However, evaluation of concordance between the present method and NGS is required.

Conflicts of interest: Makoto Maemondo received a lecture fee from AstraZeneca, Chugai, and Boehringer, and received funding support from Boehringer. Other authors have no competing interest.

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EGFR 阻害薬で治療された腫瘍における T790M 耐性変異および活性型変異のシスおよびトランス頻度

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要旨

Exon20 T790M 変異は，第 1，2 世代 EGFR-TKI 耐性の原因となり，C797S 変異は，第 3 世代のオシメルチニブ耐性に関連する．T790M と C797S トランスの関係にあると第 1，2 世代 EGFR-TKI への感受性が回復する．本研究では 2016 年 5 月～ 2018 年 6 月に得られた T790M および活性型変異の両方がある非小細胞肺癌の組織標本を T790M 変異がシスまたはトランスに存在するかを RT-PCR で分析した．また，コロニー PCR に続いてサンガーシーケンス法で検証し，アレル頻度を得た．両方の EGFR 変異が 23 例中 11 例で検出され，すべてシスであった．T790M 遺伝子のアレル頻度が低い場合は，オシメルチニブ治療が短期間になる傾向が認められた． T790M と活性型変異のシスの関係がすべての標本で確認され，C797S と T790M のトランスの存在と対照的であった．