JOURNAL OF CLINICAL ONCOLOGY ORIGINAL REPORT

Pretreatment Epidermal Growth Factor Receptor (*EGFR*) T790M Mutation Predicts Shorter EGFR Tyrosine Kinase Inhibitor Response Duration in Patients With Non–Small-Cell Lung Cancer

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Submitted July 15, 2011; accepted October 7, 2011; published online ahead of print at www.jco.org on January 3, 2012.

Supported by the National Science Council and Department of Health of the Republic of China (Grants No. NSC97-3112-B-002-033, NSC97-3112-B-002-049, NSC98-3112-B-002-031, NSC 99-2314-B-001-003-MY3, NSC100-2325-B-002-062, DOH100-TD-B-111-001, and NSC100-2627-B-002-010). K.-C.L. was supported in part by Mathematics in Biology, Institute of Statistical Science, Academia Sinica, and the National Science Council (Grant No. NSC98-2314-B-001-001-MY3).

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Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

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0732-183X/11/3099-1/$20.00

DOI: 10.1200/JCO.2011.38.3224

ABSTRACT

Purpose
Patients with non–small-cell lung cancer (NSCLC) with epidermal growth factor receptor (*EGFR*)–activating mutations have excellent response to EGFR tyrosine kinase inhibitors (TKIs), but T790M mutation accounts for most TKI drug resistance. This study used highly sensitive methods to detect T790M before and after TKI therapy and investigated the association of T790M and its mutation frequencies with clinical outcome.

Patients and Methods
Direct sequencing, matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) and next-generation sequencing (NGS) were used to assess T790M in the following two cohorts of patients with NSCLC: TKI-naive patients (n = 107) and TKI-treated patients (n = 85). Results were correlated with TKI treatment response and survival.

Results
MALDI-TOF MS was highly sensitive in detecting and quantifying the frequency of *EGFR*-activating mutations and T790M (detection limits, 0.4% to 2.2%). MALDI-TOF MS identified more T790M than direct sequencing in TKI-naive patients with NSCLC (27 of 107 patients, 25.2% *v* three of 107 patients, 2.8%, respectively; $P < .001$) and in TKI-treated patients (before TKI: 23 of 73 patients, 31.5% *v* two of 73 patients, 2.7%, respectively; $P < .001$; and after TKI: 10 of 12 patients, 83.3% *v* four of 12 patients, 33.3%, respectively; $P = .0143$). The *EGFR* mutations and their frequencies were confirmed by NGS. T790M was an independent predictor of decreased progression-free survival (PFS) in patients with NSCLC who received TKI treatment ($P < .05$, multivariate Cox regression).

Conclusion
T790M may not be a rare event before or after TKI therapy in patients with NSCLC with *EGFR*-activating mutations. The pretreatment T790M mutation was associated with shorter PFS with EGFR TKI therapy in patients with NSCLC.

J Clin Oncol 30. © 2012 by American Society of Clinical Oncology

INTRODUCTION

Lung cancer is the leading cause of cancer-related death worldwide.[1] Platinum-based chemotherapy is the standard treatment for patients with advanced-stage non–small-cell lung cancer, and modest improvements in survival and quality of life have been achieved.[2] A major advance in NSCLC management is the understanding of molecular biology, development of molecule-targeting agents, and identification of biomarkers for patient selection for targeted treatment.

The activation of epidermal growth factor receptor (EGFR) pathways results in the initiation of cancer proliferation, increased metastasis potential, and neoangiogenesis. Inhibition of EGFR kinase activities by EGFR tyrosine kinase inhibitors (TKIs) such as gefitinib and erlotinib results in effective treatment for patients with NSCLC.[3-5] *EGFR*-activating mutations in exon 19 (Del19) and exon 21 (L858R) that correlated with a 70% TKI treatment response rate and prolonged progression-free survival (PFS) have been identified.[6-11] Such mutations are present in up to 17% of white and 50% of East Asian patients with lung adenocarcinoma.[6-9,12,13] The Iressa Pan-Asia Study (IPASS) clinical trial in Asia[14] and randomized controlled studies

on advanced NSCLC[11,15] have confirmed EGFR-activating mutations as the main predictor of clinical outcome with TKI therapy for NSCLC, and this has led to a paradigm shift of using TKI as standard first-line treatment for patients with NSCLC with *EGFR*-activating mutations.

However, acquired TKI resistance ultimately has developed in the vast majority of patients with NSCLC.[16-17,18] Although multiple mechanisms are involved, EGFR mutation at T790M accounts for more than 50% of acquired TKI resistance,[16,18,19] through steric hindrance to EGFR TKIs in crystal structure analysis[17,20] or by increased affinity for adenosine triphosphate.[21] Nonetheless, how tumor cells acquire the T790M mutation remains unclear. Some evidence suggests that T790M may exist at a low frequency within the tumor cells before TKI treatment and becomes the dominant clone after TKI drug selection pressure.[18,22-24] Whether TKI resistance is de novo rather than acquired is unclear because of the limited sensitivity of detection methods like direct sequencing.

Several challenges exist in the detection and assessment of T790M clinically, including how to detect a small frequency of T790M mutation with the allelic dilution of T790M among a large *EGFR* wild-type background,[18,25] the relatively low detection sensitivity of traditional sequencing methods,[26] the availability of tumor specimens, and other resistance mechanisms. It is also unclear whether using high-sensitivity detection techniques to detect low copy number of T790M present in patients with NSCLC before TKI treatment will have any correlation with clinical outcome.

This study used direct sequencing, highly sensitivity matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), and next-generation sequencing (NGS) methods to investigate *EGFR* T790M in patients with NSCLC before and after TKI treatment and correlates the results with clinical outcome.

PATIENTS AND METHODS

Study Population

Two independent cohorts were recruited for this study. The first cohort, which contained 107 surgical tumor samples from EGFR TKI–naive patients collected from March 2000 to January 2008 at Taichung Veterans General Hospital, was used to pilot the assay. The second cohort contained 88 tumor biopsy samples from EGFR TKI–treated patients with pathologically confirmed stage IIIB or IV NSCLC from National Taiwan University Hospital, of whom 76 had samples collected before EGFR TKI treatment (pre-TKI) to assess *EGFR* mutations and T790M detection; 73 samples were analyzed for correlation with TKI treatment response. Twelve patients had tumor samples (cell blocks, n = 8; echo-guided biopsy, n = 1; soft tissue excisional biopsy, n = 3) obtained after EGFR TKI therapy (post-TKI). Among the 76 pre-TKI patients, three had uncommon mutations other than typical L858R, Del19, or T790M.

Responders to EGFR TKI treatment were defined as patients with partial response, whereas patients with stable disease or progressive disease were considered nonresponders. Clinical data of patients, including demographic information, cell type of cancer, smoking status, and image studies, were reviewed. Never-smokers were defined as patients who had smoked less than 100 cigarettes in their lifetime.

The Institutional Review Boards of Taichung Veterans General Hospital and National Taiwan University Hospital approved the study. All patients provided written informed consent in the procurement of tumor specimens.

Detection of EGFR-Activating Mutations and T790M by Direct Sequencing, MALDI-TOF MS, and NGS

Detection of *EGFR* mutations by direct sequencing was as previously described (Data Supplement).[27] The detection of *EGFR* T790M, L858R, and Del19 mutations was performed by MALDI-TOF MS according to the user's manual of the MassARRAY system (Sequenom, San Diego, CA). The customized primers and probes for detecting L858R, Del19, and T790M and for analysis of the mutations were described in a previous report.[28] To validate mutations by NGS, tumor samples with adequate amount and quality of DNA were subjected to NGS analysis (Data Supplement).

Quantification of T790M Mutation Frequency and Determination of Cutoff Values

For MALDI-TOF MS, quantification and cutoff value determination are described in detail in the Data Supplement. The mutation frequency of NGS was calculated as the ratio of mutant reads among total reads. The detection of mutations by NGS was assessed by receiver operating characteristic curve. The optimal cutoff value for T790M was selected to maximize the sum of sensitivity and specificity.

Statistical Analysis

Simple linear regression analysis was used to test the association of diluted mutation percentage and mutation frequency. For categorical data, Fisher's exact test was carried out to compare differences between groups, and the McNemar test was used to test the differences between paired groups. The κ statistic was used to evaluate the agreement between different methods. Kaplan-Meier method was used to estimate survival curves for PFS and overall survival. Log-rank tests were used to compare the survival curves between different *EGFR* patient subgroups, and multivariate Cox proportional hazards regression analysis was used to evaluate independent prognostic factors associated with PFS. The *EGFR* mutation status, age, sex, smoking status, and tumor stage were used as covariates. A two-sided $P < .05$ was considered statistically significant.

RESULTS

Patients Characteristics

The 107 EGFR TKI–naive patients consisted of 66 men and 41 women with stage I to IV NSCLC (Data Supplement). Of these patients, 25 (23.4%) were smokers, whereas the other patients were never-smokers. Another cohort containing 88 EGFR TKI–treated patients consisted of 37 men and 51 women (Data Supplement). Of these patients, 27 (30.7%) were smokers, whereas the other patients were never-smokers, and 83 patients (94.3%) had adenocarcinoma, whereas the other patients had squamous cell carcinoma. Three patients with *EGFR* mutations other than L858R, Del19, and T790M not designed for MALDI-TOF MS in this study were excluded from further analysis.

MALDI-TOF MS Was Highly Sensitive for Detecting T790M and Quantifying T790M Mutation Frequency

To optimize the T790M detection platform, a pilot test was performed by using *EGFR* mutant-type plasmids and genomic DNA from normal human peripheral-blood mononuclear cells (PBMCs) and PC-9 and H1975 cell lines for feasibility and specificity testing (Fig 1). A single allele T790M of plasmids (Fig 1A) can be identified by mass shift as a result of the incorporated T nucleotide (arrows in Fig 1). Normal genomic DNA showed homozygous wild-type signal (Fig 1B), and PC-9 cells (Del19) exhibited no T790M signal (Fig 1C). However, H1975 cells showed both wild-type and T790M heterozygous signals (Fig 1D). These results indicated that the detection probes could specifically identify T790M.

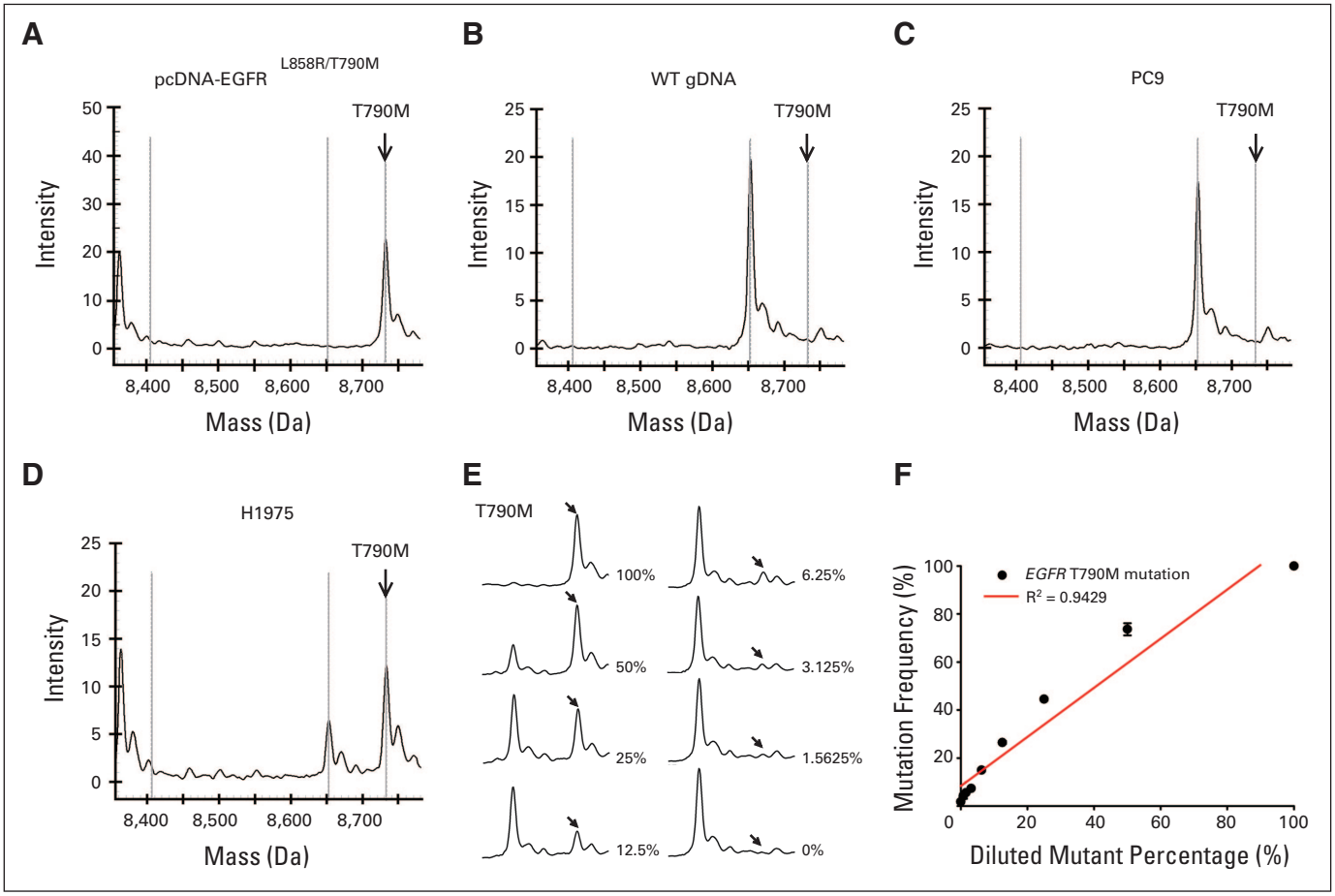


Fig 1. Specificity and sensitivity of matrix-assisted laser desorption ionization–time of flight mass spectrometry for detecting T790M. Mass spectrum of (A) T790M signal in a plasmid with 2,369th nucleotide C to T alteration (T790M), (B) wild-type (WT) genomic DNA from peripheral-blood mononuclear cells in T790M detection, (C) PC-9 lung adenocarcinoma cell line with deletion mutation in exon 19 in T790M detection, and (D) H1975 lung adenocarcinoma cell line with L858R and T790M mutations. (E) Mass spectrum of sensitivity test by plasmids with T790M two-fold serially mixed with wild-type plasmids. The percentage indicates the expected content of mutant DNA in the total input. (F) Linear correlation of mutant DNA expected percentage by serial dilution and mutant DNA frequency calculated by type 4 software. Arrows indicate the signal of T790M (T in 2,369th nucleotide), and dotted lines indicate unextended probes and wild-type signal (C in 2,369th nucleotide). EGFR, epidermal growth factor receptor.

The detection sensitivity was determined by mutant-type plasmid DNAs serially mixed with wild-type ones (Fig 1E). The height of the mutant signal was proportionally decreased with the serial dilution fold in a total of 1,000 copies. The minimal expected diluted percentage with mutant signals detectable by type 4 software was 1.5% for T790M (Fig 1E). The calculated frequency from signal height was 2.25%. Hereafter, these were used as the cutoff values to define the presence of T790M.

Moreover, the mutation frequency and dilution fold showed good linear correlation ($R^2 = 0.9429$; Fig 1F). Wild-type genomic DNA from PBMCs had undetectable mutation frequency (data not shown), which indicated that MALDI-TOF MS was a sensitive, specific, and quantifiable method to determine T790M.

T790M Was Not Rare in Patients With NSCLC With EGFR*-Activating Mutations Even Before TKI Treatment*

T790M was analyzed by direct sequencing, MALDI-TOF MS, and NGS methods in TKI-naive and TKI-treated patient cohorts with NSCLC. In the TKI-naive cohort, direct sequencing could only identify 2.8% of patients (three of 107 patients) with T790M, whereas MALDI-TOF MS detected 25.2% of patients (27 of 107 patients) with T790M ($P < .001$; Table 1). MALDI-TOF MS identified an additional 24 patients with T790M compared with direct sequencing. In addition, smoking is not a factor associated with the occurrence of T790M.

In the 73 TKI-treated patients, MALDI-TOF MS detected 31.5% of patients (23 of 73 patients) with T790M in pre-TKI samples, whereas only 2.7% of patients (two of 73 patients) had T790M by direct sequencing ($P < .001$; Table 1). In the other 12 patients with post-TKI samples available, T790M detection rates significantly increased (83.3% [10 of 12 patients] by MALDI-TOF MS *v* 33.3% [four of 12 patients] by direct sequencing; $P = .0143$). All T790M mutations detected by direct sequencing were also positive by MALDI-TOF MS in both cohorts.

T790M Frequency Detected by MALDI-TOF MS Correlated With Results of NGS

The MALDI-TOF MS detected T790M by NGS. A total of 54 original tumor samples with adequate DNA amount and quality from the two cohorts were examined by NGS with SOLiD3 Plus System (Applied Biosystems, Foster City, CA; Table 2). On the basis of the optimal cutoff value derived from the receiver operating characteristic

Table 1. *EGFR* Mutations Detected by Direct Sequencing, MALDI-TOF MS, and NGS in TKI-Naive and TKI-Treated Patients With NSCLC

Patient Population	Direct Sequencing		MALDI-TOF MS			NGS Validation†			
						MALDI-TOF MS		NGS	
	No.	%	No.	%	*P**	No.	%	No.	%
TKI-naive patients	107	100	107	100		38	100	38	100
EGFR wild type‡	67	62.6	59	55.1		19	50.0	19	50.0
EGFR-activating mutations§	40	37.4	48	44.9	.0196	19	50.0	19	50.0
EGFR-T790M	3	2.8	27‖	25.2	< .001	10	26.3	13	34.2
TKI-treated patients	88		88			16		16	
Pre-TKI	73¶	100	73¶	100		14	100	14	100
EGFR wild type‡	33	45.2	17	23.3		5	35.7	4	28.6
EGFR-activating mutations§	40	54.8	56	76.7	< .001	9	64.3	10	71.4
EGFR-T790M	2	2.7	23	31.5	< .001	1	7.1	2	14.3
Post-TKI	12	100	12	100		2	100	2	100
EGFR wild type‡	3	25.0	0	0.0		0	0.0	0	0.0
EGFR-activating mutations§	9	75.0	12	100		2	100	2	100
EGFR-T790M	4	33.3	10	83.3	.0143	2	100	2	100

Abbreviations: *EGFR*, epidermal growth factor receptor; MALDI-TOF MS, matrix-assisted laser desorption ionization–time of flight mass spectrometry; NGS, next-generation sequencing; NSCLC, non–small-cell lung cancer; TKI, tyrosine-kinase-inhibitor.
*McNemar test.
†Fifty-four DNA samples (38 for TKI-naive patients and 16 for TKI-treated patients) were available and qualified for NGS validation.
‡Patients without *EGFR* L858R or Del19 mutations.
§Patients with *EGFR* L858R or Del19 mutations.
‖Twelve T790M patients without *EGFR* L858R or Del19 mutations in MALDI-TOF MS analysis.
¶Three patients with *EGFR* mutations except L858R and Del19 were excluded from the analysis.

curve, the detection limit for NGS was 3.71% (Data Supplement). The detection sensitivities of EGFR-activating and T790M mutations by NGS were comparable with those of MALDI-TOF MS (50% *v* 50% for EGFR-activating mutations and 34.2% *v* 26.3% for T790M in TKI-naive cohort, respectively; 71.4% *v* 64.3% for *EGFR*-activating mutations and 14.3% *v* 7.1% for T790M in pre-TKI cohort, respectively; Table 1). To compare MALDI-TOF MS results with direct sequencing or NGS in parallel, κ value estimation was used for concordance analysis in these 54 samples. The concordance of T790M detection between MALDI-TOF MS and NGS was statistically significant but not between MALDI-TOF MS and direct sequencing ($\kappa = 0.663$, $P < .001$ *v* $\kappa = 0.176$, $P = .1405$, respectively; Table 2).

In quantitative testing, the strategy of T790M-mutant *EGFR* plasmid serial dilution used in the MALDI-TOF MS method was also applied to the NGS method. The mutation frequency by NGS and dilution fold showed good linear correlation ($R^2 = 0.9998$; Data Supplement). The mutation status of T790M identified by MALDI-TOF MS but not by direct sequencing could be validated by NGS, particularly in patients without T790M (Data Supplement; Table 3). Moreover, the mutation frequency of MALDI-TOF MS and NGS showed high correlation in T790M by linear regression analysis (Data Supplement). Taken together, MALDI-TOF MS could identify the presence small amounts of EGFR mutations among wild-type *EGFR* tumor samples.

Table 2. Concordance of T790M Detection Between MALDI-TOF MS, NGS, and Direct Sequencing

Detection Method	MALDI-TOF MS: T790M Positive		MALDI-TOF MS: T790M Negative		κ	*P*
	No.	%	No.	%		
NGS						< .001
T790M positive	11	84.6	6	14.6	0.663	
T790M negative	2	15.4	35	85.4		
Direct sequencing						.1405
T790M positive	2	15.4	1	2.4	0.176	
T790M negative	11	84.6	40	97.6		

Abbreviations: MALDI-TOF MS, matrix-assisted laser desorption ionization–time of flight mass spectrometry; NGS, next-generation sequencing.

Pretreatment T790M Predicted TKI Treatment Response in Patients With NSCLC With EGFR*-Activating Mutations*

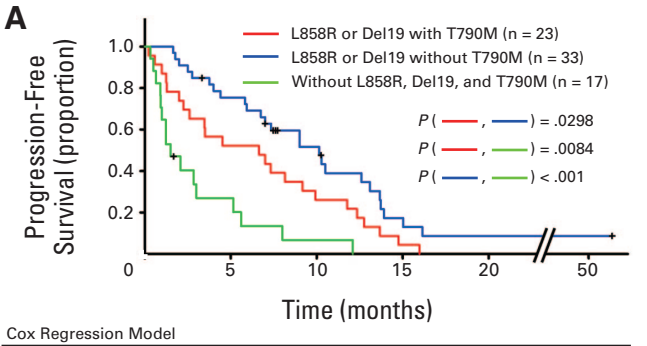
Because MALDI-TOF MS identified T790M mutations in 31.5% of 73 patients in the pre-TKI cohort, the clinical significance of pretreatment T790M mutations in patients with NSCLC treated with EGFR TKIs was further analyzed. All T790M mutations coexisted with EGFR-activating mutations. Of 56 patients with EGFR-activating mutations in the pre-TKI cohort, 23 patients with de novo T790M showed significantly shorter PFS compared with 33 patients without T790M (median PFS, 6.7 *v* 10.2 months, respectively; adjusted hazard ratio, 1.86; 95% CI, 1.044 to 3.292; $P < .05$; Fig 2A and Table 4). However, these patients still had better PFS with TKI compared with 17 patients without EGFR mutations (Fig 2A). The T790M status did not have a significant effect on the overall survival of patients with NSCLC treated with TKIs (hazard ratio, 0.86; 95% CI, 0.416 to 1.797; $P = .697$; Fig 2B).

In the post-TKI patients, T790M was identified in 83.3% (10 of 12 patients) by MALDI-TOF MS but in only 33.3% (four of 12 patients) by direct sequencing (Table 1). These results suggested that de novo T790M could be commonly identified by highly sensitive methods like MALDI-TOF MS and NGS. The presence of

Table 3. Comparison of *EGFR* Mutations Detected by Direct Sequencing, MALDI-TOF MS, and NGS

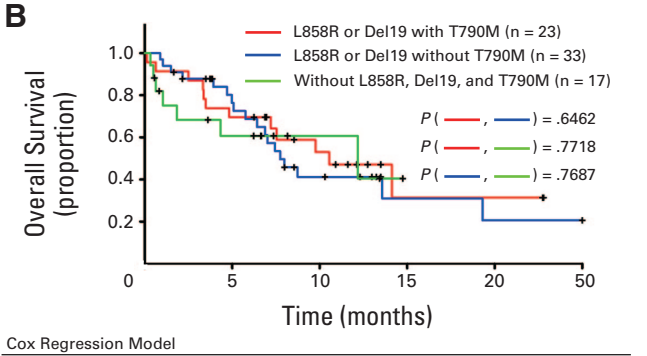
	Direct Sequencing			MALDI-TOF MS					NGS		
Patient No.	L858R	T790M	Del19	L858R	L858R (%)	T790M	T790M (%)	Del19	L858R (%)	T790 M (%)	Del19 (%)
TKI naive											
TCVGH001	No	No	No	No	0.0	Yes	3.3	No	4.500	6.760	0.000
TCVGH008	No	No	No	No	0.0	No	2.0	No	0.080	0.310	0.000
TCVGH009	No	No	No	No	0.0	No	0.1	No	0.360	2.940	0.000
TCVGH010	No	No	No	No	0.0	No	0.4	No	2.060	0.260	0.000
TCVGH011	No	No	No	No	0.0	No	0.0	No	0.326	0.805	0.002
TCVGH013	Yes	No	No	Yes	33.2	No	0.2	No	7.550	3.090	0.000
TCVGH014	No	No	Yes	No	0.0	No	0.3	Yes	0.278	1.046	28.740
TCVGH015	Yes	No	No	Yes	32.5	Yes	6.8	No	20.100	7.740	0.000
TCVGH016	Yes	No	No	Yes	10.9	No	0.0	No	6.420	3.710	0.000
TCVGH017	No	No	Yes	No	0.0	Yes	3.5	Yes	1.500	7.690	66.550
TCVGH018	No	No	No	No	0.0	No	0.0	No	0.090	0.870	0.000
TCVGH021	Yes	No	No	Yes	13.7	No	0.6	No	5.856	5.255	0.000
TCVGH027	No	No	No	No	0.0	No	0.4	No	0.530	1.790	0.000
TCVGH029	No	No	No	No	0.0	No	1.3	No	5.500	6.800	0.000
TCVGH032	No	No	No	No	0.0	No	1.1	No	3.580	1.250	0.100
TCVGH033	No	No	No	No	0.0	No	0.7	No	0.220	1.170	0.100
TCVGH034	Yes	No	No	Yes	52.6	No	0.8	No	52.160	2.920	0.100
TCVGH035	No	No	No	No	0.0	No	0.1	No	0.370	0.752	0.001
TCVGH036	No	No	No	No	0.0	Yes	4.3	No	0.280	3.870	0.000
TCVGH037	No	No	No	No	0.0	No	0.4	No	0.346	1.212	0.019
TCVGH038	No	No	No	No	0.0	No	0.1	No	0.850	0.120	0.000
TCVGH039	No	No	No	No	0.0	No	2.2	No	0.100	5.260	0.000
TCVGH041	No	No	No	Yes	2.1	No	0.7	No	0.655	6.028	0.010
TCVGH043	Yes	No	No	Yes	41.4	No	0.0	No	43.950	2.086	0.014
TCVGH044	No	No	No	No	0.0	Yes	31.1	No	1.822	28.535	0.040
TCVGH045	No	No	No	No	0.0	No	0.1	No	0.384	0.738	0.000
TCVGH046	No	No	No	No	0.0	No	0.8	No	0.389	1.379	0.006
TCVGH047	Yes	No	No	Yes	48.8	Yes	16.8	No	31.640	11.350	0.400
TCVGH056	Yes	No	No	Yes	28.1	Yes	8.9	No	25.320	3.200	0.000
TCVGH059	Yes	No	No	Yes	51.4	Yes	2.9	No	50.600	2.330	0.000
TCVGH062	No	No	No	No	0.0	Yes	3.4	No	0.494	7.423	0.000
TCVGH063	No	No	Yes	No	0.0	No	0.6	Yes	1.180	0.760	29.560
TCVGH064	Yes	No	No	Yes	15.5	No	0.9	No	5.240	0.430	0.000
TCVGH067	Yes	No	No	Yes	22.3	No	0.5	No	16.220	1.080	0.000
TCVGH081	No	No	Yes	No	0.0	No	0.0	Yes	0.400	1.390	31.430
TCVGH084	No	Yes	No	Yes	22.3	No	0.0	No	12.800	3.200	0.000
TCVGH089	Yes	No	No	Yes	24.7	No	0.6	No	27.260	1.640	0.000
TCVGH107	No	No	No	Yes	0.6	Yes	8.5	No	0.130	7.150	0.000
Pre-TKI											
NTUH08	No	No	No	No	0.0	No	0.3	No	7.922	2.458	0.024
NTUH14	No	No	Yes	No	0.0	No	0.0	Yes	1.280	0.390	5.760
NTUH16	No	No	No	No	0.0	No	1.6	No	1.120	2.300	2.920
NTUH20	No	No	Yes	No	0.0	No	1.7	Yes	0.490	1.030	66.920
NTUH31	Yes	No	No	Yes	40.7	No	0.0	No	53.938	1.644	0.000
NTUH39	Yes	No	No	Yes	5.4	Yes	8.9	No	12.160	5.340	0.000
NTUH45	No	No	No	No	0.0	No	0.4	No	7.190	2.040	0.000
NTUH51	No	No	No	No	0.0	No	0.3	No	0.799	1.004	0.022
NTUH54	Yes	No	No	Yes	19.4	No	1.1	No	19.500	2.050	0.000
NTUH60	No	No	No	No	0.0	No	0.0	No	0.834	5.558	0.171
NTUH61	Yes	No	No	Yes	58.6	No	0.0	No	59.749	2.369	0.000
NTUH65	No	No	No	Yes	6.6	No	0.4	No	8.109	1.419	0.008
NTUH67	Yes	No	No	Yes	31.3	No	0.5	No	19.681	0.534	0.013
NTUH68	No	No	No	Yes	25.5	No	0.1	No	2.050	0.438	0.000
Post-TKI											
NTUH86	Yes	Yes	No	Yes	5.0	Yes	9.0	No	2.767	11.752	0.066
NTUH88	Yes	Yes	No	Yes	33.7	Yes	9.9	No	5.363	4.771	0.000

Abbreviations: *EGFR*, epidermal growth factor receptor; MALDI-TOF MS, matrix-assisted laser desorption ionization–time of flight mass spectrometry; NGS, next-generation sequencing; NTUH, National Taiwan University Hospital; TCVGH, Taichung Veterans General Hospital; TKI, tyrosine kinase inhibitor.



Cox Regression Model

Variable	Hazard Ratio	95% CI	P
L858R or Del19 without T790M	1.000		
L858R or Del19 with T790M	1.854	1.044 to 3.292	.035
Without L858R, Del19, and T790M	4.965	2.524 to 9.765	< .001



Cox Regression Model

Variable	Hazard Ratio	95% CI	P
L858R or Del19 without T790M	1.000		
L858R or Del19 with T790M	0.864	0.416 to 1.797	.697
Without L858R, Del19, and T790M	1.105	0.459 to 2.657	.824

Fig 2. Kaplan-Meier and T790M frequency estimates of (A) progression-free survival and (B) overall survival according to epidermal growth factor receptor–activating mutations or T790M identified by matrix-assisted laser desorption ionization–time of flight mass spectrometry in pre–tyrosine kinase inhibitor patients (n = 73).

pre-TKI EGFR T790M could act as a negative predictor for PFS in patients with NSCLC who harbor EGFR-activating mutations and receive TKI therapy.

DISCUSSION

The identification of *EGFR*-activating mutations in NSCLC and their association with excellent response to TKIs are the major developments in management that have led to the new paradigm of using TKIs as the standard treatment for patients with NSCLC with *EGFR*-activating mutations.[11,15] However, acquired TKI resistance ultimately develops in most patients around 10 to 16 months after TKI treatment,[17,18] of which 50% is a result of T790M. However, it is still unclear whether and how T790M is induced by EGFR TKI therapy.

The present study shows that MALDI-TOF MS is highly sensitive in detecting *EGFR*-activating mutations and T790M mutations with detection limits of 0.4% to 2.2%. MALDI-TOF MS identifies more T790M mutations than direct sequencing in TKI-naive patients with NSCLC (27 of 107 patients [25.2%] *v* three of 107 patients [2.8%], respectively) and in TKI-treated patients (pre-TKI: 23 of 73 patients [31.5%] *v* two of 73 patients [2.7%], respectively; post-TKI: 10 of 12 patients [83.3%] *v* four of 12 patients [33.3%], respectively), which are confirmed by NGS. Thus, T790M is an independent predictor of decreased PFS in patients with NSCLC with TKI treatment (multivariate Cox regression, $P < .05$).

The high prevalence of T790M mutations (25.2% to 31.5%) detected by MALDI-TOF MS raises a clinically important issue; in patients with de novo T790M, a new therapeutic strategy is imperative to ensure better treatment outcome for patients who have *EGFR*-activating mutation and who are EGFR pathway dependent. The detection rate of T790M is 2.8% in TKI-naive patients and 2.7% in pre-TKI patients by direct sequencing. Both rates are consistent with other reports using conventional direct sequencing methods.[18,19,23,29] This suggests that there is no selection bias in the study cohorts. The low prevalence of de novo T790M detected by direct sequencing may be a result of the low copy number of T790M present in patients' tumor specimens before TKI therapy. Allelic dilution further obscures the detection of de novo T790M,[22,23,25] which may be under the detectable limits of the method used.

However, T790M rates of 25.2% and 31.5% in TKI-naive and pre-TKI samples, respectively, were determined by MALDI-TOF MS and are further confirmed by NGS, implying that the incidence of T790M may be more prevalent than expected. Populations of T790M cells within the tumor may then gradually increase after EGFR TKI therapy because of selection pressure, at least partially.

The role of T790M in lung cancer progression, especially its presence before TKI treatment, is still being debated and lacks systematic exploration. Maheswaran et al[30] showed that T790M was associated with poor outcome on TKI therapy and suggested that de novo T790M might contribute to poor survival aside from drug resistance. The results also highlight the role of T790M in predicting PFS in patients with NSCLC with *EGFR*-activating mutation treated with TKIs. In addition, T790M alone may confer resistance to EGFR TKIs and lung cancer development both in vitro and in vivo.[26,31] Therefore, T790M can be present before treatment, and T790M tumor cells may undergo a relatively indolent progression in tumorigenesis.[23,32,33] An animal model has also shown that T790M results in tumors with longer latency than L858R *EGFR*-activating mutation.[31]

We systemically explored the role of pretreatment T790M on TKI treatment outcome. There is a significant difference between PFS of patients with and without T790M (Fig 2A). However, the TKI response rate of patients with T790M was not different from the rate of patients without T790M (Table 4). This was consistent with a previous study.[30] Currently, we cannot explain why patients with T790M still have 57% response rate. It may be possible that a low percentage of T790M does not affect the treatment response but still influences the response duration to EGFR TKIs.

Regarding EGFR TKI treatment, the prevalence of T790M before TKI therapy is 31.5% but increases to 83.3% after TKI therapy. This indicates that primary tumor samples of patients with NSCLC have significant de novo T790M and de novo TKI-resistant cells before TKI therapy. These TKI-resistant clones grow in the selective environment of TKI therapy because of their survival advantage. Early detection of T790M may have implications for new treatment strategies to overcome or delay TKI drug resistance in managing patients with NSCLC.

Table 4. Pre-TKI T790M Mutation Status and TKI Treatment Response in 76 Patients With NSCLC

Pre-TKI Patients*	Patients (n =76) No.	%	Responders to TKI (PR) No.	%	P†	Progression-Free Survival Media (months)	P‡	Overall Survival Media (months)	P‡
Age, years (mean, 64.47 ± 11.96 years)					.818		.736		.880
≤ 65	36	47.4	18	50.0		6.8		19.5	
> 65	40	52.6	22	55.0		6.3		15.5	
Sex					.498		.664		.995
Male	41	54.0	20	48.8		6.7		21.1	
Female	35	46.0	20	57.1		7.0		17.5	
Smoking					.231		.245		.209
No	50	65.8	29	58.0		7.0		21.1	
Yes	26	34.2	11	42.3		3.5		14.4	
EGFR wild type§	17‖		3	17.6	< .001				
EGFR-activating mutations¶	56		37	66.1			.030		.646
With T790M	23		13	56.5	.257	6.7		21.1	
Without T790M	33		24	72.7		10.2		15.5	

Abbreviations: *EGFR*, epidermal growth factor receptor; NSCLC, non–small-cell lung cancer; PR, partial response; TKI, tyrosine kinase inhibitor.
*_EGFR_ was analyzed before TKI treatment.
†Fisher's exact test.
‡Log-rank test.
§Patients without L858R or Del19.
‖Three patients with atypical *EGFR* mutations without designed corresponding probes for mass spectrometry detection were excluded.
¶Patients with L858R or Del19.

The prevalence of reported de novo T790M varies from 1% to 38%[14,29,30,34] and is dependent on the sensitivity of detection methods used or patient selection. To exclude false-positive results arising from more T790M detection by MALDI-TOF MS, several issues in this study, in addition to high sensitivity, have been identified. First, MALDI-TOF MS measures the absolute mass value of T790M that represents the intrinsic property of the molecule instead of its electrophoretic mobility or fluorescence intensity as used by other detection methods. Second, in the sensitivity test performed by plasmid serial dilution (Fig 1), the T790M mutation frequency test in 36 PBMC samples from the normal population has no false-positive results. Third, the high prevalence of this study is probably not a false-positive finding because T790M mutation results before TKI therapy are an independent predictor of poor PFS of patients. The prevalence of T790M mutations in patients with NSCLC reported in this study is consistent with results reported in circulating tumor cells in another study.[30] This also corroborates the existence of T790M tumor cells before treatment, albeit at a low frequency.

NGS, currently the most powerful detection method with the highest resolution, has been used to validate the T790M results detected by MALDI-TOF MS in patients with NSCLC. All T790M mutations detected by MALDI-TOF MS can be identified and confirmed by NGS. The detection frequencies of T790M of both methods exhibit high correlation (Fig 2), which supports the premise that MALDI-TOF MS is a highly sensitive method that can address the challenge of small quantity of T790M in the tumor specimen as a result of allelic dilution. Regarding the methodology, it not only exhibited high sensitivity like other methods[35] and had similar pretreatment T790M detection rate by peptide nucleic acid–locked nuclec acid clamp, [34] but it also had high flexibility as a result of multiplex designation and the ability to conduct quantitative detection. MALDI-TOF MS provides an alternative strategy when direct sequencing fails to detect T790M. It also has the advantages of requiring less tumor sample (< 10 ng) in clinical practice.

Several second-generation TKIs and other new therapies designed to overcome TKI resistance are currently under active clinical development. In preclinical studies, irreversible EGFR TKIs (HKI-272 [neratinib], BIBW-2992 [afatinib], or PF-0299804) have been shown to inhibit cancer cells with T790M. However, clinical trials failed to demonstrate the efficacy of those TKIs in patients with T790M.[36-38] A recent trial of afatinib plus cetuximab has shown efficacy in patients with acquired EGFR TKI resistance (including patients with and without T790M).[39] Despite the promise posed by these new agents, it is still necessary for physicians to accurately identify patients with T790M who can benefit from treatment. Patients can develop TKI resistance as a result of the activation of alternative pathways such as c-MET.[40,41] Combination therapy with EGFR and other alternative pathway (eg, c-Met, Her3) inhibitors can also be investigated.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

Employment or Leadership Position: None **Consultant or Advisory Role:** James Chih-Hsin Yang, AstraZeneca (C), Boehringer Ingelheim (U), Roche (C); Jin-Yuan Shih, AstraZeneca (C) **Stock Ownership:** None **Honoraria:** James Chih-Hsin Yang, AstraZeneca, Roche; Jin-Yuan Shih, AstraZeneca, Roche, Boehringer Ingelheim **Research Funding:** None **Expert Testimony:** None **Other Remuneration:** None

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Acknowledgment

We thank the National Research Program for Genomic Medicine C5 microarray core facility for technique support and the Department of Medical Research of the National Taiwan University Hospital for the DNA direct sequencing support.