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Genetic alterations in epidermal growth factor receptor-tyrosine kinase inhibitor-naïve non-small cell lung carcinoma

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Abstract. Epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs) are an approved first-line therapy against unresectable or advanced non-small cell lung cancer (NSCLC) harboring *EGFR* gene activating mutations. However, the majority of tumors develop acquired resistance against EGFR-TKIs and some tumors exhibit natural resistance. A number of resistance mechanisms against the latest third-generation EGFR-TKIs have been reported, including tertiary *EGFR* C797S mutation and several gene alterations activating EGFR or other signaling pathways. The current study aimed to identify the frequency of natural EGFR-TKI resistance in pretreatment NSCLC and to predict the therapeutic effect of EGFR-TKIs. A total of 246 EGFR-TKI-naïve NSCLC patients harboring known *EGFR* gene mutations were identified. The presence of *EGFR* C797S and T790M mutations were determined using the peptide nucleic acid-locked nucleic acid PCR clamp method. *ERBB2*, *MET*, *EGFR*, *ALK*, *BRAF*, *FGFR1*, *MYC*, *RET*, *CCND1*, *CCND2*, *CDK4*, *CDK6*, *MDM2* and *MDM4* gene amplification, which can lead to resistance against any generation EGFR-TKIs, was determined using the multiplex ligation-dependent probe amplification assay. No concurrent C797S mutation with known *EGFR* mutations were identified. T790M mutation was identified in 12 patients (4.9%). *ERBB2* or *MET* gene amplification was found in some patients (0.0-0.4%). *MDM2* gene amplification was associated with tumor recurrence and shorter progression-free survival (PFS) for first- or second-generation EGFR-TKIs. *De novo EGFR* C797S mutation was not identified. Other resistance mechanisms against EGFR-TKIs were indicated in some patients with EGFR-TKI-naïve NSCLC. *MDM2* gene amplification, which can lead to altered cell cycle, was associated with tumor recurrence and shorter PFS in EGFR-TKI therapy.

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Key words: epidermal growth factor receptor-tyrosine kinase inhibitors-naïve non-small cell lung cancer, natural resistance, C797S, *MDM2* amplification

Introduction

Precision molecular targeted agents in non-small cell lung cancer (NSCLC) have improved survival of patients harboring driver gene mutations. Epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitor (TKI) improves progression-free survival (PFS) of NSCLC patients with *EGFR* mutations compared with traditional platinum-based doublet chemotherapy (1,2). Furthermore, osimertinib, a third-generation EGFR-TKI, is promising as first-line treatment for EGFR mutant NSCLC (3,4). Although good responses to EGFR-TKI therapy have been shown, tumor cells can acquire resistance through several methods, in particular, secondary gene mutations that cause structural changes in the ATP binding site of the EGFR tyrosine kinase domain. *EGFR* T790M mutation occurs in almost half of patients following first- or second-generation EGFR-TKI therapy (5), and *EGFR* C797S mutation is the most common mechanism of acquired resistance against third-generation EGFR-TKIs (6).

Approximately 0.4-8% of NSCLC patients harboring *de novo* or germline T790M mutations are resistant to first- or second-generation EGFR-TKIs (7). However, the frequency of *EGFR* C797S gene mutation remains unclear. To the best of our knowledge, only one case of an NSCLC patient harboring concurrent C797S and L858R mutations prior to receiving EGFR-TKI treatment has been reported (8).

Several other mechanisms of resistance against all generation EGFR-TKIs have been identified including tertiary gene mutations other than *EGFR* C797S mutation (9-11), activation of bypass signaling by gene amplification (e.g., *ERBB2* (12) and *MET* (13,14), driver gene mutations (e.g., *RAS*, *RAF* and *PIK3CA*) (3,15), gene alteration in cell cycle genes (14), and transformation to mesenchyme, small cell carcinoma (SCC), or squamous cell carcinoma (SqCC) (2,16,17). These described EGFR-TKI resistance mechanisms may also be expressed

during the pre-TKI NSCLC state (6,15) and can be a challenge for cancer treatment of NSCLC patients with *EGFR* mutations.

In this retrospective study, we assessed potential resistance against third-generation EGFR-TKI therapy, such as *EGFR* C797S mutation, and gene amplification in EGFR-TKI-naïve surgical specimens from patients harboring known *EGFR* mutations.

Materials and methods

Patient selection. Consecutive patients who underwent initial lung resection or surgical tumor biopsy in Fukushima Medical University Hospital and were diagnosed with NSCLC harboring a known *EGFR* gene activating mutation (e.g., exon 19 deletion, L858R, T790M, S768I, G719X and L861Q) at the time samples were collected, and whose specimens were available for gene examination described below, were included in this study. Patients who had received systemic treatment or irradiation therapy before surgery were excluded.

Ethics statement. This study was conducted with approval of the ethics board at Fukushima Medical University (approval no. 2955). Human rights and welfare of participants were protected in accordance with the Declaration of Helsinki, and written informed consent was obtained from participants.

Preparation of genomic DNA. Tumor DNA was extracted from macro-dissected tumor tissue of formalin-fixed paraffin-embedded surgical specimens using the QIAamp DNA FFPE Tissue kit (Qiagen) according to the manufacturer's instructions. Tumor DNA quantity was assessed using the Qubit dsDNA HS Assay kit (Thermo Fisher Scientific, Waltham, MA, USA) and Qubit 2.0 Fluorometer (Thermo Fisher Scientific).

Peptide nucleic acid-locked nucleic acid PCR clamp method. Premix Ex Taq (Takara Bio Inc.), 200 nM of primer, 100 nM of mutation LNA probe, 100 nM of total probe, 250-2,500 nM of clamp probe, and sample DNA were mixed in a total reaction volume of 25 μl and analyzed as described previously (18). Real-time PCR was performed in 50 cycles using Light Cycler480 II (Roche; denaturation: 5 sec at 95˚C; annealing and extension: 30 sec at 62˚C). *EGFR* C797S and T790M mutations were judged using LightCycler 480 software (Roche).

Multiplex ligation-dependent probe amplification (MLPA) Assay. Gene amplification was analyzed according to the standard protocol for MLPA (19) using SALSA MLPA Probemix P175 Tumor Gain (MRC-Holland). *ERBB2*, *MET*, *EGFR*, *ALK*, *BRAF*, *FGFR1*, *MYC*, *RET*, *CCND1*, *CCND2*, *CDK4*, *CDK6*, *MDM2* and *MDM4* gene amplification was assessed. Fragments of PCR products were analyzed using the 3130xl Genetic Analyzer (Thermo Fisher Scientific) and amplification was judged by Coffalyser Data analysis software (MRC-Holland).

Statistical analysis. Statistical analysis was performed using SPSS 21.0 (IBM; SPSS). Continuous variables were compared by two-tailed t-tests or one-way ANOVA, and categorical variables were compared by the chi-squared test or Fisher's exact test. Multivariate analyses using a binary logistic regression model were performed to evaluate independent predictors, and hazard ratio (HR) and confidence interval (CI) were calculated. PFS was estimated using the Kaplan-Meier method, and survival curves were compared using log-rank tests. P-values of less than 0.05 were considered statistically significant. Some of the statistical analysis was performed by AC Medical Inc. (Tokyo, Japan).

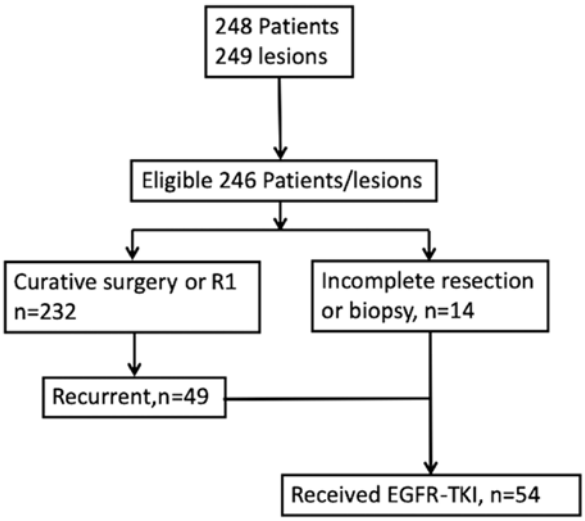

Figure 1. Flowchart of the patient selection process.

Results

Patient characteristics. Between January 2007 and December 2015, we identified 248 patients, of which 246 patients were eligible for this study and included in the analyses. Of the 232 patients who had undergone complete or microscopically incomplete resection, recurrence was found in 49 patients. Surgery with incomplete resection or tumor biopsy was performed in 14 patients. Of the 63 advanced or recurrent NSCLC patients, 54 patients received EGFR-TKI therapy (Fig. 1). Demographic data and clinicopathological characteristics of patients are presented in Table I: Age at surgery or biopsy ranged from 37 to 90 years (median age: 67 years). There were 159 (64.6%) women, 166 (67.4%) never smokers, 240 (97.6%) patients with adenocarcinoma, and 193 (78.4%) patients with pathological stage I (Table I).

EGFR C797S and T790M mutations. No patients harbored concurrent C797S mutation with known *EGFR* mutations. T790M mutation was identified in 12 patients (4.9%); 5 patients had the deletion in exon19 or L858R and 7 patients had T790M mutation alone (Table I).

Gene amplification. *EGFR* gene amplification was found in five patients. All patients with *EGFR* gene amplification had advanced disease or recurrence (9.8 vs. 0.0%, P<0.01). *ERBB2* gene amplification was found in only one patient (0.4%) who developed recurrence. No patients harbored *MET* gene amplification. *MDM2* gene amplification was found in 17 patients (7.1%) and *CDK4* gene amplification in

Table I. Clinicopathologic, genetic and histologic characteristics of patients.

A, Clinicopathologic characteristic

Characteristic	Total (n=246)	Received EGFR-TKI therapy(n=44)
Sex		
Male	87 (35.4)	14 (31.8)
Age (y)		
Median (range)	67.0 (37-90)	65.5 (37-82)
Smoking status		
Never	166 (67.4)	30 (68.2)
Former	67 (27.2)	11 (25.0)
Current	13 (5.3)	3 (6.8)
Pathological subtypes		
Adeno	240 (97.6)	42 (95.5)
AdSq	2 (0.8)	2 (4.5)
Sq	4 (1.6)	0 (0.0)
p-Stage at initial surgery		
I	193 (78.4)	
II	16 (6.5)	
III	24 (9.7)	
IV	12 (4.9)	

B, *EGFR* mutation

Characteristic	Total (n=246)	Received EGFR-TKI therapy(n=44)
C797S	0 (0.0)	0 (0.0)
Ex19del	86 (35.0)	21 (47.7)
Ex19del+T790M	1 (0.4)	0 (0.0)
L858R	134 (54.5)	20 (45.5)
L858R+G719S	1 (0.4)	1 (2.3)
L858R+T790M	4 (1.6)	0 (0.0)
T790M	7 (2.8)	0 (0.0)
G719A	3 (1.2)	0 (0.0)
G719C	1 (0.4)	0 (0.0)
L861Q	1 (0.4)	1 (2.3)
G719A+S768I	2 (0.8)	0 (0.0)
G719A+L861Q	2 (0.8)	1 (2.3)
G719C+L861Q	1 (0.4)	0 (0.0)

C, Gene amplification

Characteristic	Total (n=246)	Received EGFR-TKI therapy (n=44)
ERBB2	1 (0.4)	1 (2.3)
MET	0 (0.0)	0 (0.0)
EGFR	5 (2.0)	3 (6.8)
ALK	0 (0.0)	0 (0.0)
RET	0 (0.0)	0 (0.0)

Table I. Continued.

C, Gene amplification

Characteristic	Total (n=246)	Received EGFR-TKI therapy (n=44)
BRAF	0 (0.0)	0 (0.0)
FGFR1	1 (0.4)	0 (0.0)
MYC	2 (0.8)	1 (2.3)
CCND1	2 (0.8)	2 (4.5)
CCND2	1 (0.4)	1 (2.3)
CDK4	15 (6.0)	4 (9.1)
MDM2	17 (6.8)	6 (13.6)
MDM4	2 (0.8)	1 (2.3)

Data are presented as n (%) unless otherwise indicated. AdSq, adenosquamous; Sq, squamous; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor.

14 patients (5.8%); duplication of both *MDM2* and *CDK4* was observed in 11 patients (4.6%). *MDM2* gene amplification was significantly associated with tumor recurrence (16.7 vs. 5.1%, P=0.032). Patients with tumors with any gene amplification had significantly more advanced disease or developed recurrence compared with patients without recurrence after surgery (33.3 vs. 8.9%, P=0.002) (Fig. 2).

We next evaluated associations between clinicopathological parameters and/or gene amplification and progress of first- and second-generation EGFR-TKI treatment. Fifty-four patients had received EGFR-TKI therapy, and 44 patients underwent gene amplification analysis and were followed up. Patients who developed central nervous system metastasis (HR, 2.259, 95% CI, 1.150-4.436) and had *MDM2* gene amplification (HR, 3.405, 95% CI, 1.209-9.591) exhibited significantly shorter PFS (Figs. 3,4). Patients who had *EGFR* gene amplification (HR, 0.656, 95% CI, 0.195-2.210), *ERBB2* gene amplification (HR, 0.801, 95% CI, incalculable), *CDK4* gene amplification (HR, 0.194, 95% CI, 0.660-7.802) (Figs. 3,4), *CCND1* gene amplification (HR, 2.823 95% CI, 0.653-12.203), *CCND2* gene amplification (HR, 0.801, 95% CI, incalculable), *CDK4* gene amplification (HR, 0.194, 95% CI, 0.660-7.802), and *MDM4* gene amplification (HR, 0.465, 95% CI, 0.063-3.441) (data not shown) showed no significant difference in PFS (Figs. 3,4).

Discussion

We herein showed that in our study cohort no EGFR-TKI-naïve NSCLC patients harbored concurrent *EGFR* C797S mutation with known *EGFR* gene mutation. Amplification of several genes was found before EGFR-TKI therapy, and *MDM2* gene amplification was associated with resistance to first-generation EGFR-TKIs.

C797S mutation, which leads to acquired resistance to third-generation EGFR TKIs, occurs in 5.3-40.0% of NSCLC patients following osimertinib treatment (2,3,6,11,20) and is the most common resistance mechanism against osimertinib.

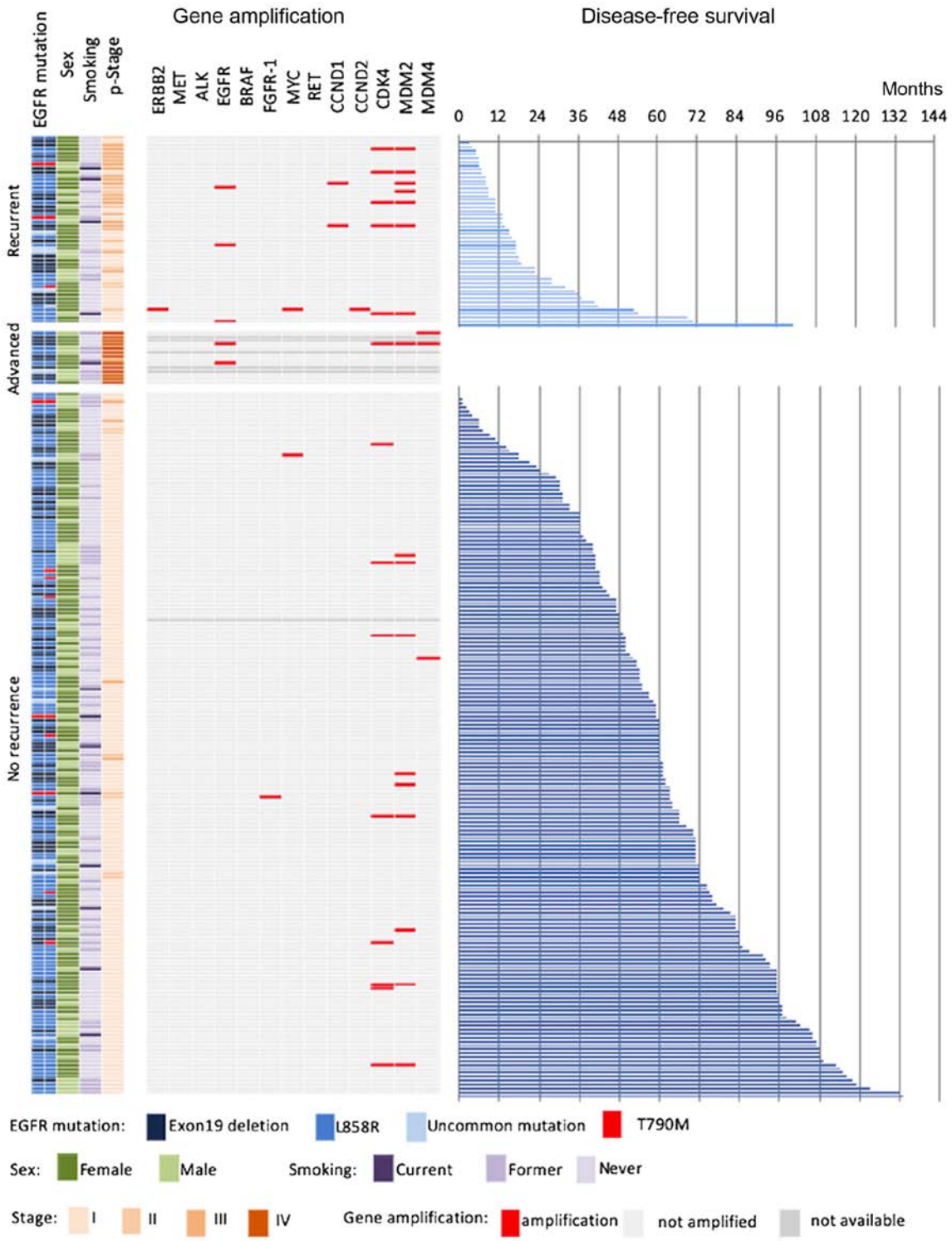

Figure 2. Correlation between gene amplification and disease-free survival. *EGFR* gene amplification was found more frequently in advanced or recurrent cohort than cohort without recurrence. There was no *MET* gene amplification and *ERBB2* gene amplification (0.4%) was indicated in the study cohort. MDM2 gene amplification was associated with the tumor in a recurrent or advanced state. EGFR, epidermal growth factor receptor.

No concurrent C797S mutation was found in this study, and, to our knowledge, only one case of *de novo* somatic L858R and C797S mutations has been reported (8). C797S mutation is an important challenge for *EGFR* mutant NSCLC therapy. A recent report suggests that therapeutic efficacy is dependent on allelic context of common *EGFR* mutations, C797S and T790M. If T790M and C797S mutations occur on separate alleles, then combination therapy comprising first- and third-generation EGFR-TKIs can restore EGFR inhibition *in vitro* (21). Unfortunately, these mutations almost always occur on the same allele (22). Conversely, combination therapy composed of osimertinib, bevacizumab, and brigatinib is effective for NSCLC with T790M and C797S mutations occurring on the same allele (23). Combination therapy comprising an allosteric inhibitor and cetuximab was also shown to be effective in a murine model of NSCLC driven by *EGFR* L858R,

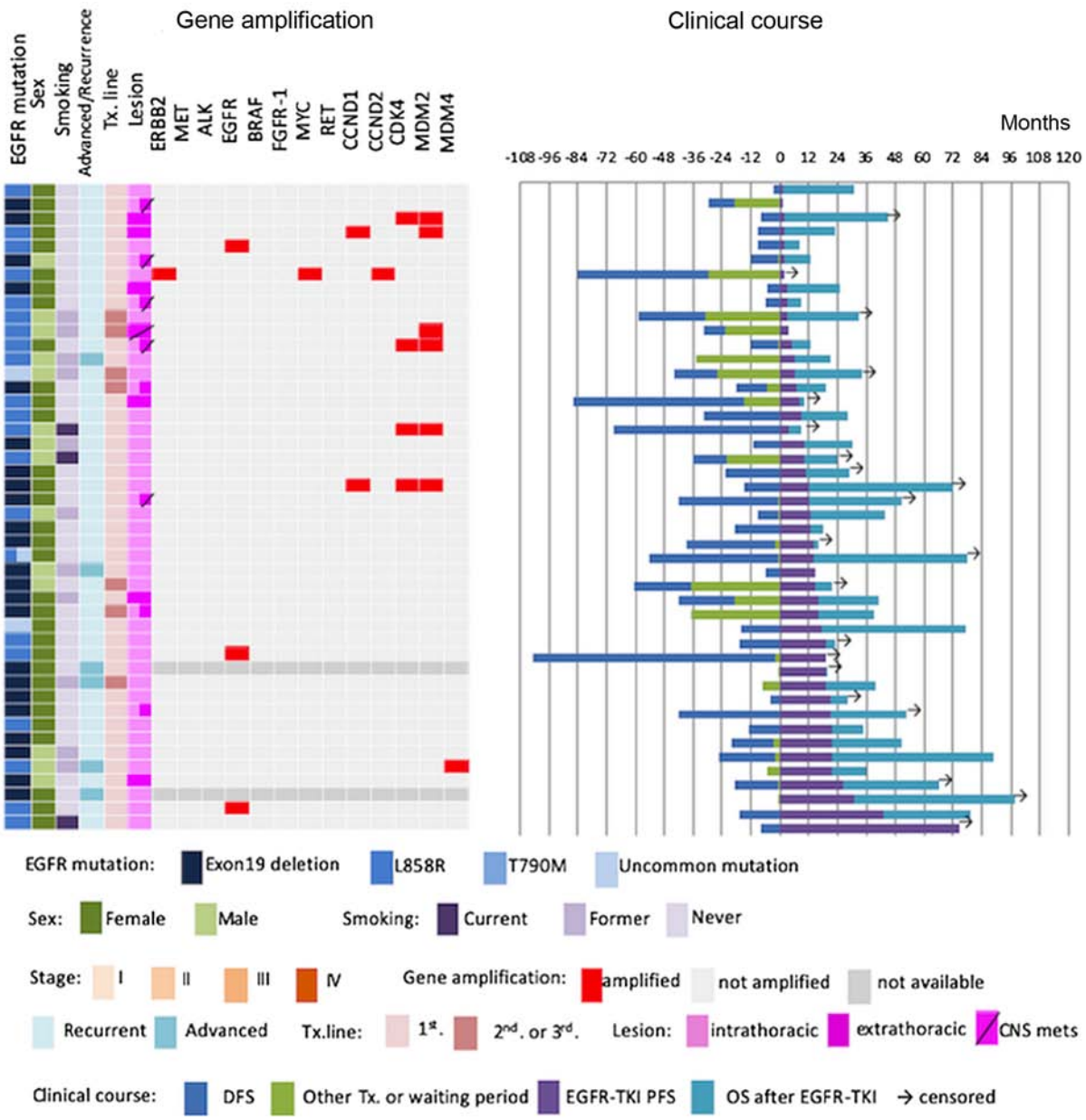

Figure 3. Correlation between gene amplification and progression-free survival for first- and second-generation EGFR-TKI therapy. EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; OS, overall survival; DFS, disease free survival.

T790M, and/or C797S mutation (24). Furthermore, other tertiary mutations of *EGFR* [L792X (11), G796D (9), L718Q, and L844V (5)] in the EGFR tyrosine kinase domain were reported, and novel therapies for tumors with these mutations are required.

Other resistance mechanisms against third-generation EGFR-TKIs involving activation of signaling pathways have been reported. Lin *et al* (2) and Ramalingam *et al* (3) carried out plasma-based analyses after development of osimertinib resistance in NSCLC. The following gene alterations were noted: *EGFR* C797S mutation in 5.2-16.7%, *KRAS* mutation in 2.6-7.7%, *BRAF* mutation in 7.7%, *PIK3CA* mutation in 2.6%, *MEK* mutation in 2.6%, *JAK2* mutation in 2.6%, *MET* amplification in 2.6-50%, *KRAS* amplification in 2.6%, *ERBB2* insertion in 2.6%, transformation to SCC in 9.1%, and transformation to SqCC in 4.5% of patients (2,3). Furthermore, analysis of matched samples of pre- and post-administration of first- or second-generation EGFR-TKIs showed acquired resistance in addition to T790M mutation in lung adenocarcinoma, *MET* amplification in 8%, *ERBB2* amplification in 5%, and *EGFR* amplification in 16% of patients (15). Transformation to SCC was noted in 2.6-5.0% of patients (15), and another report demonstrated SqCC rarely occurred (17).

The genetic alterations described above occurred in some pretreatment EGFR mutant NSCLC tumors and patients with these mutations can exhibit resistance to osimertinib therapy. Concurrent gene alterations in EGFR-TKI-naïve, *EGFR* mutant NSCLC have been reported. Yu *et al*, conducted next-generation sequencing analysis of tissue samples and revealed *EGFR* mutant NSCLC had concurrent alteration of *TP53* mutation in 60%, *RB1* mutation in 10%, *PIK3CA* mutation in 12%, *CTNNB1* mutation in 18.9%, *EGFR* amplification in 22%, *MDM2* mutation in 12%, *CDK4* mutation in 10%, *ERBB2* amplification in 8.4%,

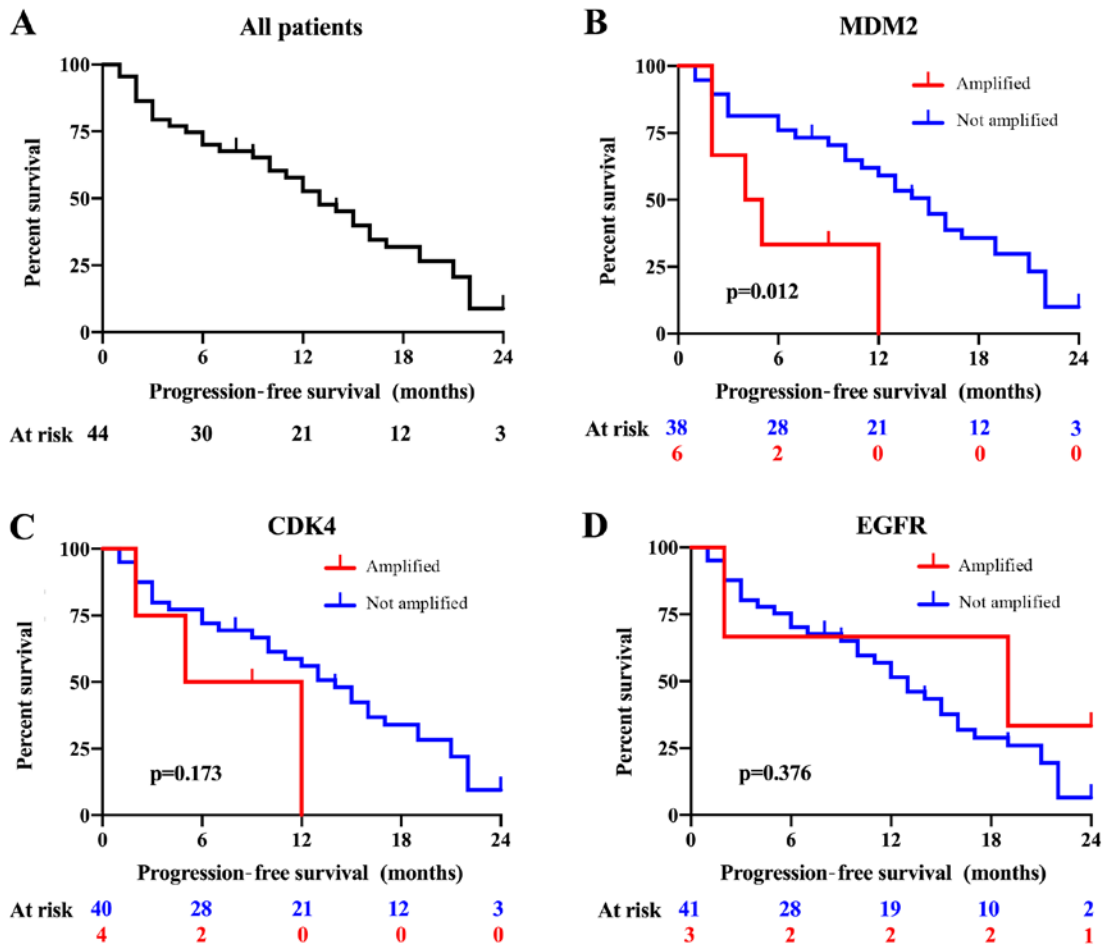

Figure 4. Survival curves for progression-free survival (PFS) in accordance with patients' genetic or clinical features. (A) Unadjusted survival curve and (B) adjusted survival curves for MDM2 amplification, (C) CDK4 amplification, and (D) EGFR amplification. MDM2 gene amplification was associated with shorter progression-free survival for first- or second-generation EGFR-TKI therapy significantly. EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor.

and *MET* amplification in 2% of patients before EGFR-TKI treatment (15).

Gene amplification of *ERBB2*, *MET*, *MDM2* or *CDK4* alone leads to poor prognosis for NSCLC regardless of *EGFR* gene mutation (25-27). Furthermore, Le *et al* (11) and Blakely *et al* (14) reported that cell cycle gene alteration, such as *CDK4* or *CDK6*, shortens PFS following osimertinib therapy. Patients with *EGFR* mutant NSCLC accompanied by *TP53* mutation, *ERBB2* amplification, or *MET* amplification before first- or second-generation EGFR-TKI treatment exhibited a shorter time to progression and also showed resistance to third-generation EGFR-TKIs (12,13,28).

In this study, *MDM2* gene amplification was shown to be associated with shorter PFS for first- or second-generation EGFR-TKIs. MDM2 is a proto-oncogene that is often coexpressed with CDK4 in liposarcoma (29). MDM2 binds to TP53 to downregulate transcription, leading to proteasome degradation by ubiquitination (30).

The limitations of this study are as follows: First, we did not evaluate *EGFR* mutations associated with acquired resistance to EGFR-TKIs other than C797S (e.g., L792X, G796X and L718Q), exon 20 insertion, mutation in driver genes *KRAS*, *BRAF* and *PIK3CA*, or transformation to mesenchyme or other pathological subtypes. Furthermore, the study cohort was limited to NSCLC patients harboring known *EGFR* gene mutations. Therefore, whether C797S mutation alone could be an oncogenic mechanism remains unclear. Second, this study did not evaluate single nucleotide polymorphisms or germline gene mutations. As only seven patients had *EGFR T790M* mutation, they could have germline mutations (7) rather than somatic mutations.

Finally, because our survival analysis was conducted in a small cohort, it is possible that amplification of genes other than *MDM2*, such as *MDM4* and *ERBB2* evaluated in this study, could be biomarkers for EGFR-mutant NSCLC.

In conclusion, no concurrent EGFR-C797S with known EGFR mutant NSCLC was identified in our study cohort. This result confirms the efficacy of third-generation EGFR-TKIs; however, it is important to remain aware of how genetic alterations can affect EGFR-TKI responses. Therefore, further studies in a large cohort are required to completely elucidate resistance mechanisms against third-generation EGFR-TKIs.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TY, SM and HS designed the study. TY, HM, HT, MW, YO, TI, MF, NO, YM, TH, JO, MHo, MHi and YS collected and analyzed the data. TY and HS wrote and revised the manuscript. All the authors approved the final manuscript.

Ethics approval and consent to participate

The current study was conducted with approval of the ethics board at Fukushima Medical University (approval no. 2955). Human rights and welfare of participants were protected in accordance with the Declaration of Helsinki, and written informed consent was obtained from participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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