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Identification of Genomic Alterations Acquired During Treatment With EGFR-TKIs in Non-small Cell Lung Cancer

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671Abstract. *Background/Aim: Patients with non-small cell lung cancer (NSCLC) treated with epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) eventually develop resistance to these drugs. Although various mechanisms of such resistance have been identified, the mechanism in many cases remains unknown. Materials and Methods: Whole-exome sequencing was performed for tumor tissue from 15 patients with NSCLC who developed EGFR-TKI resistance. Tumor specimens obtained before EGFR-TKI treatment were also analyzed for four patients and normal white blood cell samples for six patients in order to detect genomic alterations that occurred during treatment. Results: The mutational signature and mutational load acquired during EGFR-TKI treatment varied among patients, with common EGFR-TKI resistance mechanisms including the T790M secondary mutation of EGFR and MET amplification being acquired together with many other genomic alterations. Our results provide insight into the mutational landscape acquired during the development of EGFR-TKI resistance in NSCLC.*

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The development of cancer is governed by the accumulation of many somatic genetic alterations, some of which are driver mutations (1, 2). Recent advances in biomedical research have provided important insight into the molecular basis of cancer and have led to breakthroughs in therapeutic interventions (3-5). Mutations in the epidermal growth factor receptor (*EGFR*) gene and rearrangements of the anaplastic lymphoma kinase (*ALK*) gene that result in constitutive up-regulation of the tyrosine kinase activity of the encoded proteins have been identified as drivers of non-small cell lung cancer (NSCLC). Tyrosine kinase inhibitors (TKIs) that target these mutant kinases–including gefitinib (5) and erlotinib (6) for EGFR, and crizotinib for ALK (3, 7), have shown marked efficacy for the treatment of NSCLC positive for the corresponding genetic alterations, with treatment decisions being currently based on testing for these genetic changes (8, 9). However, all patients with NSCLC who are initially sensitive to TKIs eventually develop resistance to these drugs that is mediated by various mechanisms (10-13).

Well-characterized mechanisms underlying the resistance of NSCLC to EGFR-TKIs include the T790M secondary mutation of *EGFR* (11-13); the activation of alternative signaling pathways mediated by amplification of MET proto-oncogene, receptor tyrosine kinase (*MET*) or overexpression of human epidermal growth factor receptor (HER) family proteins (14, 15); aberrant downstream signaling of EGFR mediated by KRAS proto-oncogene, GTPase (*KRAS*) mutation or loss of phosphatase and tensin homolog (*PTEN*) (16-18); EGFR-TKI-induced apoptosis associated with Bcl2 like 11 (*BCL2L11*) deletion polymorphism (19); and transformation to small cell carcinoma (20, 21). Although the

T790M secondary mutation of *EGFR* is rarely present before EGFR-TKI treatment, it has been detected in about half of such treated patients. Detection of this mutation has, thus, become an important determinant of treatment strategies, such as the administration of third-generation EGFR-TKIs that target *EGFR*T790M (21-24), and attempts are underway to develop additional agents to overcome EGFR-TKI resistance (24-26). Despite the identification of various mechanisms of EGFR-TKI resistance, in many cases they remain obscure. Furthermore, genomic alterations that might occur in addition to the characterized resistance-conferring changes are largely unknown. For example, it remains unclear whether other genetic changes accompany the appearance of the T790M secondary mutation of *EGFR* and, if so, whether such additional changes occur selectively with the T790M mutation or also accompany the genetic alterations underlying other resistance mechanisms.

The recent development of next-generation sequencing (NGS) technology has allowed the identification of somatic mutations at the whole-genome level. Whole-exome sequencing (WES), in which captured coding regions of the genome are subjected to sequencing, is a cost-effective method for the detection of disease-causing variants and discovery of drug targets. In order to characterize further the mechanisms of acquired resistance to targeted therapy in NSCLC, we performed WES on tumor DNA from patients with NSCLC with acquired resistance to EGFR-TKIs.

Materials and Methods

Patients and specimens. Fifteen Japanese patients with NSCLC were included in the study. All patients were enrolled and examined between January 2013 and December 2015 and were treated daily with either erlotinib or gefitinib. Patient specimens were collected and studied with the approval of the Ethical Review Board for Human Genome/Gene Research at each participating institute. Informed consent was obtained from all participants. In four patients (cases 1-4), cancer specimens were obtained both before EGFR-TKI treatment and after treatment failure. In six patients (cases 5-10), cancer specimens obtained after EGFR-TKI treatment failure as well as normal white blood cell (WBC) samples were analyzed. In the five remaining patients (cases 11-15), only cancer specimens obtained after EGFR-TKI treatment failure were studied.

WES library preparation and sequencing. Genomic DNA was extracted from each specimen, and libraries were generated with the use of a SureSelect XT Human All Exon V5+lncRNA enrichment kit (Agilent, Santa Clara, CA, USA). The libraries were sequenced on an Illumina HiSeq 2000 instrument to generate 100- or 133-nucleotide paired-end reads for each sample.

WES analysis. High-quality reads were obtained after trimming the low-quality bases from the 3' end and the adapter sequences from the 5' end with the use of the NGS QC Toolkit (27). The resulting reads were aligned to the reference human genome (hg19) (28) with the use of the Burrows-Wheeler Aligner (29). The alignment information was stored in BAM format files, and duplications were marked with the use of Picard (http://broadinstitute.github.io/picard). Realignment and recalibration were performed to obtain reliable alignment results with the use of Genome Analysis Toolkit (GATK) (30).

Somatic single-nucleotide variations (SNVs) in TKI-resistant tumors, TKI-sensitive tumors, and WBCs were detected with GATK. *De novo* SNVs were selected by subtraction of those identified in TKI-sensitive tumors or WBCs from those identified in TKI-resistant tumors. A series of filters was then applied to define SNVs. For somatic mutations, the mutant allelic frequency had to be ≥10% and mutations with low base quality (<20) or low mapping quality (<20) were discarded. Other thresholds for determination of somatic point mutations included a sequencing depth for both tumor and matched normal sample of ≥20, a distance between two adjacent mutations of ≥10 bp, and occurrence outside of simple repeat regions. Allelic frequencies in the Exome Aggregation Consortium data (http://exac.broadinstitute.org), Exome Sequencing Project 6500 data (http://evs.gs.washington.edu/EVS), and The Human Genetic Variation Database (http://www.genome.med.kyoto-u.ac.jp/SnpDB) had to be ≤0.1%. SNVs that overlapped with single-nucleotide polymorphisms in dbSNP version 138 (31) were also removed. Nonsynonymous missense mutations called by WES were evaluated with SIFT (http://sift.jcvi.org) and Polyphen2 (http://genetics.bwh.harvard.edu/pph2) for their potential impact on protein function. Mutect was also used with default options (32) to detect *de novo* SNVs in cases 1 to 10. The SomaticIndelDetector tool included in GATK was used to detect *de novo* insertions-deletions (indels) by comparison of specimens as described above, with the command for filtering option being "T_COV<20||N_COV<10||T_INDEL_F<0.1||T_INDEL_CF<0.7." ANNOVAR (33) was used to annotate the variant results, and EXCAVATOR (34) was used to detect copy number variants (CNVs) by comparison of TKI-resistant tumors with control samples (TKI-sensitive tumors or WBCs).

Data availability. Sequencing data have been deposited under the accession number JGAS00000000102 in the Japanese Genotype-Phenotype Archive (JGA, http://trace.ddbj.nig.ac.jp/jga).

Results

Clinical characteristics of the study patients. All patients were Japanese and included 10 women and five men with an age range of 47 to 79 years (Table I). Ten patients (66.7%) were never-smokers and five (33.3%) were former smokers. Twelve patients had stage IV adenocarcinoma and the remaining three had recurrent adenocarcinoma at the start of EGFR-TKI therapy. Eight patients were treated with gefitinib, and seven received erlotinib. The median duration of EGFR-TKI therapy was 347 days, ranging from 80 to 2,557 days. Cancer specimens were obtained after EGFR-TKI treatment failure for all patients. Cancer specimens were also obtained by biopsy before TKI treatment in four patients (cases 1-4), and WBCs were obtained in six patients after TKI treatment (cases 5-10) as control samples.

Mutational signatures. The most frequent base substitution was C-to-T (G-to-A) in all tumor specimens of the study patients, with the second most frequent being A-to-G

(T-to-C) with the exception of EGFR-TKI-resistant specimens in cases 1 and 8 (Figure 1). Hydrolytic deamination of cytosine or 5-methylcytosine (5mC) results in C-to-U and 5mC-to-T mutations, respectively, with subsequent misrepair leading to replacement of uracil with thymine and a consequent C-to-T transition. Frequent 5mC-to-T substitutions were previously implicated in the appearance of the T790M (ACG→ATG) mutation of *EGFR* (35). The C-to-T mutation was also previously found be strongly positively correlated with age (1), which is consistent with the age range (47 to 79 years) of the patients in the present study. The point mutations acquired during EGFR-TKI treatment varied among patients. For example, C-to-T and C-to-A mutations increased markedly during EGFR-TKI treatment in cases 1 and 2 but not in cases 3 and 4 (Figure 1). Patients 1 and 2 were never-smokers, suggesting that some other stress that induces point mutations at such sites might be related to EGFR-TKI resistance.

T790M mutation of EGFR and MET amplification are accompanied by a large number of additional mutations. The *EGFR* mutations detected before TKI treatment are shown in Table I. All patients had NSCLC positive for activating mutations of *EGFR*, with nine and six patients harboring exon 19 deletions and L858R in exon 21, respectively. The TKI resistance-conferring T790M mutation in exon 20 was detected in four patients (cases 1, 4, 11, and 15) after treatment failure, and was also detected before treatment initiation in case 4. We also detected a large number of CNV candidates in the patients (cases 1-10) for whom control specimens were available (Table I). *MET* amplification, another common mechanism of EGFR-TKI resistance, was detected in two patients (cases 2 and 8) (Table I).

Comparison of WES data for tumor specimens obtained before EGFR-TKI treatment and after development of resistance in cases 1 to 4 allowed the identification of genomic alterations acquired during treatment. The number of *de novo* SNVs detected in cases 1 and 2 was high (117 and 50 genes, respectively), whereas that in cases 3 and 4 was low (0 and 1 gene, respectively) (Table I). In case 4, the T790M mutation was already present before treatment onset. The number of *de novo* indels in cases 1 to 4 was relatively low at 2 to 7, and none of these indels was present in more than one of these patients [an indel in zinc finger and SCAN domain containing 18 (*ZSCAN18*) was detected in cases 3 and 8] (Table I). The number of CNV candidate regions was especially large in case 2, with this patient also being positive for *MET* amplification (Table I).

For cases 5 to 10, in which cancer specimens obtained after the development of EGFR-TKI resistance were compared with normal WBC samples to identify genomic alterations acquired before treatment failure, the number of genomic alterations also varied. The numbers of *de novo*

Table I. *Clinical characteristics and whole-exome sequencing results for the study subjects.*

Case	Age (years)	Gender	Smoking status	Stage	*EGFR* mutation	Control specimen	TKI-resistant specimen	TKI treatment duration, days	*EGFR* T790M mutation before/after treatment	*MET* amplification before/after treatment	SNVs, n	Indels, n	CNVs, n (total length)
1	66	F	Never	rIIIB	del19	Yes	Pleural effusion	946	No/yes	No/no	117	7	14 (49.8 Mb)
2	74	F	Never	rIIIA	L858R	Yes	Pleural effusion	330	No/no	No/yes	50	7	32 (531.1 Mb)
3	66	F	Never	rIIIB	del19	Yes	Pleural effusion	80	No/no	No/no	0	2	10 (21.7 Mb)
4	63	M	Former	IV	L858R/E709G (exon 18)	Yes	Pleural effusion	836	Yes/yes	No/no	1	3	9 (27.1 Mb)
5	78	F	Never	IV	del19	Yes (WBC)	Pleural effusion	2,557	No	No	0	3	2 (0.2 Mb)
6	73	M	Former	IV	L858R	Yes (WBC)	Pleural effusion	108	No	No	0	2	9 (3.7 Mb)
7	65	M	Former	IV	del19	Yes (WBC)	Pleural effusion	1096	No	No	0	0	0 (0.0 Mb)
8	59	F	Former	IV	del19	Yes (WBC)	Pleural effusion	1404	No	Yes	182	10	7 (132.4 Mb)
9	47	F	Never	IV	del19	Yes (WBC)	Autopsy	103	No	No	26	3	13 (48.4 Mb)
10	79	F	Never	IV	del19	Yes (WBC)	Pleural effusion	1,109	No	No	1	2	2 (1.9 Mb)
11	64	F	Never	IV	L858R	NA	Pleural effusion	133	Yes	No	85*	8*	NA
12	67	M	Never	IV	del19	NA	Pleural effusion	363	No	No	43*	9*	NA
13	60	F	Never	IV	L858R/E709K (exon 18)	NA	TBLB	320	No	No	134*	8*	NA
14	66	M	Former	IV	del19	NA	TBLB	1,357	No	No	78*	10*	NA
15	79	F	Never	IV	L858R	NA	TBLB	329	Yes	No	89*	20*	NA

F: Female; M: male; *EGFR*: epidermal growth factor receptor; TKI: tyrosine kinase inhibitor; *MET*: MET proto-oncogene, receptor tyrosine kinase; SNVs: single-nucleotide variations; indels: insertions-deletions; WBC: white blood cells; r: recurrent; del19: exon 19 deletion; NA: not available; TBLB: transbronchial lung biopsy. SNVs and indels were de novo with the exception of cases 11 to 15. *Number of SNVs and indels in tumor specimens in cases 11 to 15.

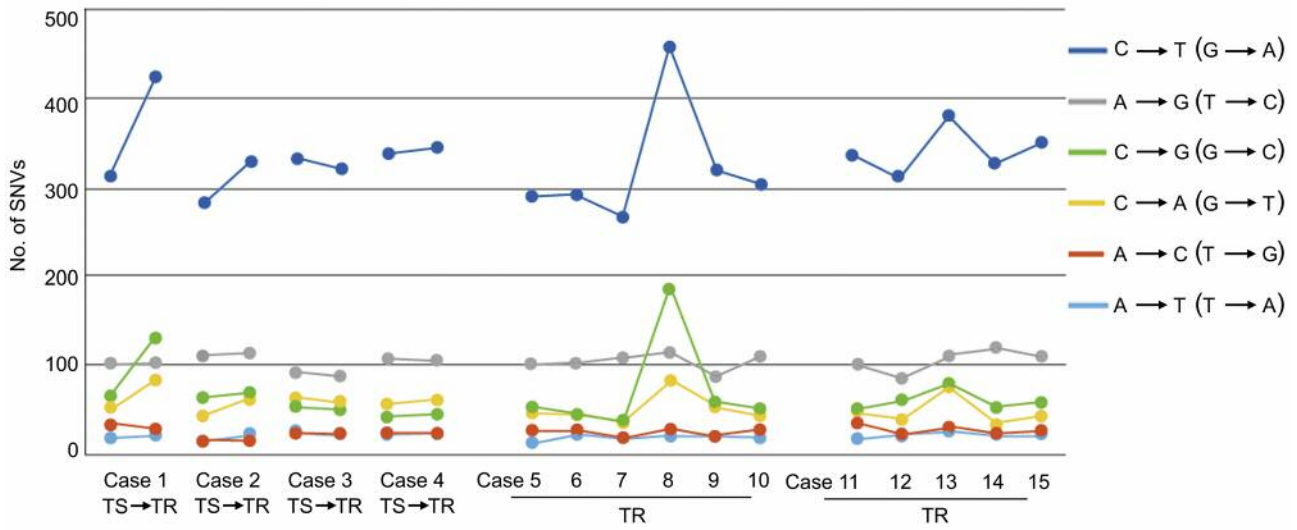

Figure 1. *Mutational signatures. The number of single-nucleotide variations (SNVs), including synonymous substitutions, in tumors of the study patients is plotted. The most frequent base substitution was C-to-T (G-to-A) in all 15 cases, and the second most frequent mutation was A-to-G (T-to-C) with the exception of the tyrosine kinase inhibitor(TKI)-resistant (TR) tumors of cases 1 and 8. TS: TKI-sensitive.*

SNVs and indels in case 8 were high (182 and 10 genes, respectively), with the number of genomic alterations identified in the other cases being low (0-26 SNVs and 0-3 indels) (Table I). The number of genes located in CNV regions was also high in case 8 (1,109 genes) and included *MET* (Table I).

The variable mutational load and our relatively small sample size made it difficult to identify candidate genes that might be the cause of EGFR-TKI resistance with statistical significance. We, therefore, referred to previous studies that analyzed mutations in EGFR-TKI-resistant tumor specimens (36, 37) in order to identify overlapping mutated genes among the studies and examine similarity in variability of mutational load. Although both previous studies lacked EGFR-TKI-sensitive specimens as controls, they detected a variable mutational load among the cases, and some of the genes with SNVs overlapped with those in our data set.

The T790M secondary mutation of *EGFR* or *MET* amplification were identified in cases 1, 2, and 8, with these patients having a high mutational load, suggesting that these frequent EGFR-TKI resistance mutations are acquired together with a large number of additional genomic alterations. Gene ontology analysis of genes affected by SNVs in cases 1 and 2 with the Database for Annotation, Visualization, and Integrated Discovery tool (38) identified terms such as "Cell adhesion" and "Pathways in cancer". With regard to the relation between mutational load and duration of EGFR-TKI treatment, for cases with a high mutational load (≥25 *de novo* SNVs; cases 1, 2, 8, and 9) the number of SNVs tended to be positively correlated with the duration of TKI treatment (r=0.99) (Figure 2). In contrast, other cases continued to show a low mutational load even after long-term TKI treatment (>500 days).

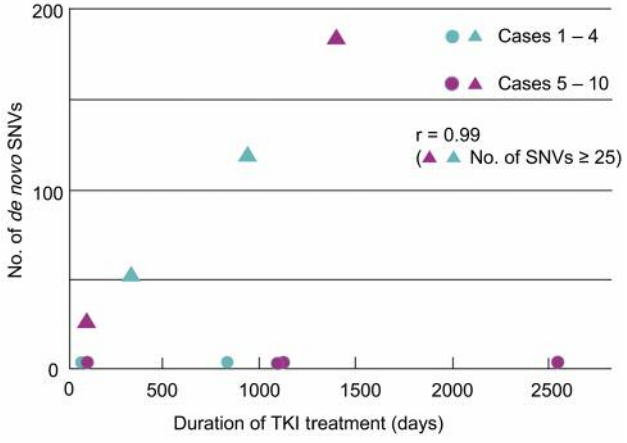

Figure 2. *Duration of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) treatment and mutational load. The number of de novo single-nucleotide variations (SNVs) and the duration of EGFR-TKI treatment for cases 1 to 10 are shown in a scatter plot. Cases with a high mutational load are indicated by triangles (≥25 de novo SNVs; cases 1, 2, 8, and 9) and the number of de novo SNVs was positively correlated with the duration of TKI treatment (r=0.99). On the other hand, cases with a low mutational load are indicated by circles (≤2 de novo SNVs; cases 3-7 and 10) and showed various durations of TKI treatment.*

Discussion

EGFR-TKI resistance is a pressing clinical problem in the treatment of NSCLC, with the identification of resistance mechanisms being essential for the development of new strategies to overcome treatment failure. The next generation of EGFR-TKIs and specific antibodies to related molecules are under development (39-41), but further characterization of the mechanisms of EGFR-TKI resistance is needed. In the present study, we performed WES on clinical specimens of patients with NSCLC who underwent a second biopsy after failure of EGFR-TKI treatment. We identified *de novo* genomic alterations acquired during EGFR-TKI treatment by comparing these WES data with those obtained for tumor specimens collected before TKI treatment or for WBC samples.

The mutational signatures of the cancer specimens showed several similarities and differences. The predominant base substitution was C-to-T (G-to-A), with the C-to-A (G-to-T) mutation, which is prominent in smoking-associated cancer (1), being relatively infrequent in all cases. However, both C-to-T and C-to-A mutation frequencies increased during EGFR-TKI treatment in cases 1 and 2, which acquired the T790M mutation of *EGFR* and *MET* amplification, respectively. These mutation frequencies did not change substantially during EGFR-TKI treatment in cases 3 and 4, suggesting that a specific endogenous or exogenous stressor that induces C-to-T and C-to-A mutations might be associated with these common TKI resistance mechanisms.

The frequencies of genomic alterations varied among cases, with the T790M mutation of *EGFR* and *MET* amplification occurring together with a large number of other mutations. Given the high frequency of these EGFR-TKI resistance mutations, it might be expected that they occur preferentially without multiple other mutations during EGFR-TKI treatment. However, we identified multiple mutations in addition to T790M of *EGFR* or *MET* amplification in two cases (cases 1 and 2), whereas fewer mutations were detected in patients who did not develop these common resistance mechanisms during EGFR-TKI treatment (cases 3 and 4).

Nivolumab, an antibody to the programmed cell death-1 immune-checkpoint protein, is a recent breakthrough in NSCLC therapy, with clinical studies having suggested that both former and current smokers respond well to this agent (42). One potential explanation for this latter finding is that smoking-associated lung cancer is associated with a high mutational load that may lead to the production of a greater number of tumor neoantigens and increased tumor immunogenicity. In the present study, we found that the mutational load differed among patients with various mechanisms of TKI resistance. Cases 1 and 2, for example, which acquired the T790M mutation of *EGFR* and *MET* amplification, respectively, showed a high mutational load, whereas cases 3 and 4, for which a well-characterized mechanism of TKI resistance was not identified, had a low mutational load. The efficacy of nivolumab for patients with NSCLC who have experienced EGFR-TKI treatment failure might thus differ depending on the mechanism of TKI resistance. Among cases 11 to 15, however, for which only cancer specimens obtained after the development of EGFR-TKI resistance were available, the number of SNVs in cases 11 and 15, which were positive for the T790M mutation of *EGFR*, was not substantially higher than that in the cases without this mutation. Although we were not able to identify *de novo* mutations acquired during EGFR-TKI treatment for cases 11 to 15 because of the lack of control specimens, these results suggest that the T790M mutation might not always be associated with a high mutational load, as in cases 1 and 2. A larger patient population will thus be necessary to reveal statistically significant associations among genomic alterations acquired during EGFR-TKI treatment. Another limitation of our study is the variability in sampling sites. Most of the cancer specimens obtained after failure of EGFR-TKI treatment comprised pleural effusion and differed from those obtained before treatment. It is possible that the type of biopsy specimen might affect the accuracy of mutation detection. The adoption of strict sampling criteria is thus warranted in future studies.

Our WES-based study has, thus, identified somatic mutations acquired during EGFR-TKI treatment and revealed a variable mutational load among patients. A high mutational load was preferentially identified in patients with common EGFR-TKI resistance mechanisms including the T790M mutation of *EGFR* and *MET* amplification. Furthermore, among those tumors with a high mutational load, C-to-T and C-to-A mutation frequencies increased during EGFR-TKI treatment. Our data will serve as an important resource for future studies and may facilitate further efforts to identify novel mechanisms of EGFR-TKI resistance and improve therapeutic options for patients with NSCLC.

Conflicts of Interest

K.N. has received research funding from MSD and Novartis Pharma as well as honoraria from AstraZeneca, Chugai Pharmaceutical, Eli Lilly Japan, Kyowa Hakko Kirin, Nippon Boehringer Ingelheim, Nippon Kayaku, and Ono Pharmaceutical. All other Authors declare no conflicts of interest in regard to this study.

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