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Significance of Polar Charged Amino Acids in Compound Mutations in EGFR-mutated Patients Treated With First-line Afatinib

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Abstract. *Background/Aim: Next-generation sequencing (NGS) has recently made it possible to investigate polar charged amino acids in compound mutations in the epidermal growth factor receptor (EGFR) mutations in non-small cell lung cancer (NSCLC). Several preclinical studies have suggested the involvement of polar charged amino acids in the treatment of EGFR mutations and EGFR-tyrosine kinase inhibitors (TKIs). With this background, a retrospective study was conducted aiming to clarify the prognostic significance of these amino acids in complex mutations in NSCLC patients with common EGFR mutations. Patients and Methods: EGFR gene mutations were investigated using nonoverlapping integrated read sequencing system (NOIR-SS) in pathological specimens of 20 EGFR-mutated NSCLC patients. For clinical information, the medical records were retrospectively investigated. We investigated prognostic significance of these amino acids in compound mutations in progression free survival (PFS) and overall survival (OS) in patients treated with first-line afatinib. Results: Among the 20 patients examined, 5 patients had polar charged amino acids in compound mutations and 15 had not. There were no statistically significant differences in the clinical background factors examined in these two groups of patients. In uni- and multivariate analysis, 'poor performance status' and 'polar charged amino acids in compound mutations' were significant favorable factors in OS. Conclusion: Patients with 'polar charged amino acids in compound mutations' might have favorable prognosis than those without them. Detailed examination of EGFR gene information might contribute to the understanding of TKI response duration.*

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The most common epidermal growth factor receptor (EGFR) mutations in non-small cell lung cancer (NSCLC) patients are exon 19 deletions and exon 21 L858R mutation (1). Other types of mutations are collectively referred to as uncommon or minor mutations (2, 3). There are many types of uncommon EGFR mutations (2-4) and, although rare, there are patients with both common and uncommon mutations at the same time (5, 6). These patients have been treated as those with compound mutations, which are defined as double nonsynonymous mutations in the EGFR gene (5, 6). As there are many types of compound mutations, each in a small number of patients, only a few studies have examined the treatment response and survival of patients with compound mutations as a whole group (5). Recently, the new technology of next-generation sequencing (NGS) allowed researchers to investigate in detail whether compound mutations co-exist in patients with common mutations (4, 7-9). Moreover, NGS allowed the investigation of the amino acids of compound mutations in detail (10). There are twenty types of amino acids, 9 of which are nonpolar and 11 are polar (10). These 11 polar amino acids are divided into 5 polar charged amino acids (arginine, histidine, lysine, glutamic acid, and aspartic acid) and 6 polar uncharged amino acids (10).

The majority of EGFR mutation-positive NSCLC patients respond to EGFR- tyrosine kinase inhibitors (TKIs) and are

expected to have a prolonged progressive free survival (PFS) and overall survival (OS) (1, 2). However, some patients have poor prognosis even if they have a common mutation (1, 2). Several preclinical studies have suggested the involvement of polar charged amino acids in interaction between EGFR gene and TKIs (11-16). Considering this evidence, we speculated that the presence or absence of polar charged amino acids in the compound mutations might be related to the prognosis of these patients.

In the present study, we investigated the significance of polar charged amino acids in compound mutations in the survival of EGFR mutated NSCLC patients. For this purpose, we compared the prognosis of patients with or without polar charged amino acids in compound mutations. We included only patients who received afatinib as a first-line TKI, as this TKI has been reported to provide a favorable response in patients with compound mutations.

Table I. *Characteristics of patients with or without polar charged amino acids in compound mutations.*

	Patients		
	With polar charged AAs (n=5)	Without polar charged AAs (n=15)	*p*-Value
	n	n	
Age*, years	69 (62-87)	68 (58-82)	0.304
Sex (male:female)	1	7	0.999
PS (0-1:2-3)	5:0	12:3	0.540
Stage (IIIA-C:IVA-B)	1:4	2:13	0.999
EGFR (Ex19 deletion:others)	4:1	6:9	0.613

AA: Amino acid; PS: performance status; EGFR: epidermal growth factor receptor. *Data presented as median (range)

Patients and Methods

Materials. Among the pathological specimens of EGFR gene mutation-positive patients collected since April 2009 in our tertiary hospitals, 109 patients were diagnosed pathologically with EGFR-mutated NSCLC. Out of these patients, 47 were included in the study since there were sufficient tissue specimens in their pathological material to enable genetic analysis. Patients with exon 20 insertions were excluded because of their different response to TKIs (17).

Analysis of EGFR mutations. EGFR mutations were examined using a non-overlapping integrated read sequencing system (DNA Chip Research Inc. Tokyo, Japan) (7, 8). Briefly, DNA was extracted from slices of formalin-fixed paraffin-embedded (FFPE) tissue blocks using a Maxwell® RSC DNA FFPE kit (Promega, Madison, WI, USA). Double-stranded DNA was quantified with the Qubit dsDNA HS Assay (Thermo Fisher Scientific, Waltham, MA, USA) on the Qubit 2.0 Fluorometer (Thermo Fisher Scientific). A total of 50 ng of DNA was fragmented by a Covaris focused-ultrasonicator (Covaris Inc., Woburn, MA, USA) following the manufacturer's instructions. The shearing time was optimized for FFPE material to obtain fragmented DNA with a peak around 200 bp. A molecular barcoded NGS library was constructed by the non-overlapping integrated read sequencing system method, as described previously (7, 8). A customized panel covering the entire region of the EGFR tyrosine kinase domain (exons 18-21) was used to amplify the target regions. The constructed library was loaded on an Ion 540 chip using the Ion Chef System (Thermo Fisher Scientific). Sequencing was performed on the Ion S5 platform. Mutation variants with a *p*-value less than 0.01 were called somatic mutations. Common single-nucleotide polymorphisms deposited in the Human Genetic Variation Database were removed from the set of called variants. A CV78 filter (7, 8) was applied to remove artefactual substitutions with no entry in the Catalog of Somatic Mutations in Cancer (v92) database.

Statistical analysis. The χ^2 test was used to compare nominal variables, while the nonparametric Mann-Whitney test was used to compare variables with asymmetrical or unknown distribution. Using univariate analysis, we investigated the association between patient background factors and PFS for the first-line TKIs and overall survival (OS). PFS for the first-line TKIs was calculated from the date of initiation of afatinib to the recurrence or any cause of death. Total PFSs was defined as 'PFS of afatinib' plus 'PFSs of second or later line TKIs. OS was defined as the interval between the initiation of first-line TKI and the date of death, or latest follow-up contact. PFS and OS were evaluated using patient data extracted from the database of each institution. The effects of clinicopathological factors on survival were analyzed using the log-rank test for univariate analysis and Cox proportional hazards model for multivariate analysis. In this study, multivariate analyses were performed using only the variables with a *p*-value of less than 0.2 in the univariate analysis. A *p*-value <0.05 was considered to indicate a significant difference.

Ethics. This study was approved by the institutional ethics committee of each institute (Project approval number: NO18-46). Written comprehensive informed consent for obtaining pathological specimens was obtained from each patient at the time of admission.

Results

We had planned to exclude patients with exon 20 insertions from this study because their response to TKIs is clearly different to that of other patients with uncommon mutations; however, none of the patients had this uncommon mutation. Among the 47 patients examined, 20 were treated with first-line afatinib. Five patients had polar charged amino acids in compound mutations (Group I) and the remaining 15 patients had nonpolar amino acids or polar uncharged amino acids (Group II). The median follow-up period for these 20 patients was 24 months (range=1-74). As shown in Table I, there was no statistically significant difference between Group I and Group II regarding the socio-clinical characteristics.

Since there was no difference in patient backgrounds between group I and group II, comparisons of survival

Table II. *Uni- and multi-variate analysis of first-line afatinib progression-free survival (PFS), PFS for tyrosine kinase inhibitors (TKIs), and overall survival (OS), in patients with epidermal growth factor receptor (EGFR) mutation.*

	Univariate analysis (*p*-value)			Multivariate analysis								
	Afatinib-PFS	PFS	OS	Afatinib-PFS			Total PFS			OS		
				OR	95% CI	*p*-Value	OR	95% CI	*p*-Value	OR	95% CI	*p*-Value
Age (<70 years:>70 years)	0.541	0.750	0.315									
Sex (male:female)	0.891	0.430	0.181	0.914	–0.94-1.12	0.863	0.496	–0.46-1.76	0.196	1.228	0.07-2.74	**0.037**
PS (0-1:2-4)	0.169	**0.002**	**0.001**	0.492	–0.67-1.44	0.310	0.248	–0.08-2.81	0.065	2.268	0.55-3.99	**0.010**
Stage (IIIA-C:IVA-B)	0.773	0.787	0.642									
EGFR mutation (exon 19 deletion:other)	0.082	0.770	0.689									
Amino acids (polar charged:other)	0.264	**0.003**	**0.030**	0.560	–0.75-1.90	0.392	0219	–0.05-3.08	0.057	1.737	0.11-3.37	**0.037**

PS: Performance status; OR: odds ratio; CI: confidence interval. Statistically significant *p*-values are shown in bold.

between these groups were performed for factors that might be associated with survivals. When comparing PFS for the first-line afatinib between the two groups, no statistically significant difference was found. However, good performance status (PS 0-1) (p=0.002), and polar charged amino acids in compound mutations (p=0003) were significant favorable factors for total PFS as well as for OS (p=0.001 and p=0.030, respectively), and female sex tended to be significant (p=0.181) (Table II). Based on the results of these univariate analyses, we next performed multivariate analysis using Cox proportional hazards model. None of the examined factors was significant predictor of PFS of the first-line afatinib, while good PS (PS 0-1) (p=0.065) and polar charged amino acids in compound mutations (p=0.057) were associated prolonged total PFS, at a level of significance of 10%. For OS, good PS (PS 0-1) (p=0.010), female sex (p=0.037) and polar charged amino acids in compound mutations (p=0.037) were significant favorable factors (Table II).

Discussion

Using NGS technology, we investigated the significance of polar charged amino acids of compound mutations in the survival of EGFR mutated NSCLC patients. In particular, we aimed to clarify the significance of polar charged amino acids in compound mutations in PFS for first-line TKI (afatinib), total PFS (for first or later line TKIs), and OS of EGFR mutated NSCLC patients. In this study, only patients who received afatinib, which has been known to be effective in patients with uncommon and compound mutations, were included. The major finding of this study was that 'good PS' and 'female sex' but also 'polar charged amino acids in compound mutations' were significant favorable factors for OS in uni- and multi- variate analyses, although the latter was not statistically significant favorable factor for first-line afatinib PFS and PFSs.

Morita *et al*. reported that the difference in survival post progression (SPP) could be reflected in the difference in OS, if there was a difference in SPP even though there was no difference in PFS (18). That is, there could be a difference in OS between the two groups if the effects of second or later-line treatment were not negligible. According to the results of the present study, polar charged amino acids in compound mutations were not associated with PFS in patients treated with first-line afatinib. However, it was a significant favorable factor for OS and tended to be significant for total PFS (PFS of patients treated with afatinib and other TKIs). These results suggest that polar charged amino acids in compound mutations might predict favorable prognosis for patients treated with chemotherapy and immune checkpoint inhibitors. In addition, even if afatinib as a first-line TKI was ineffective, subsequent effective TKIs and chemotherapy and good OS might be obtained. Finally, our results could suggest that patients with these compound mutations might progress slowly, regardless of the type of post-afatinib treatments.

Recently, advances in NGS technology using NGS for investigating the EGFR gene have made it possible to elucidate the existence of compound mutations in detail (7, 8). These advances have revealed that some patients with common EGFR mutations also have compound mutations (5, 6). Furthermore, since it became possible to precisely investigate the polarity and charge of amino acids in compound mutations, several preclinical studies have suggested the involvement of polar charged amino acids in

the interaction between EGFR gene and TKIs (11-16). Among them, the following three biological studies are particularly interesting (14-16). About two decades ago, Holbrook *et al*. showed that the mutation of negatively charged amino acid residues near Tyr992 to their uncharged analogues increased the rate of EGF receptor internalization (14). Recently, Hartman *et al*. reported that a specific amino acid context in EGFR and HER2 phosphorylation sites enabled selective binding to the active site of Src homology phosphatase 2 (15). In addition, Kiriwan *et al*. demonstrated the difference in EGFR binding preference between an EGFR-TKI (erlotinib) and a tripeptide, where erlotinib was stably bound in the ATP-binding pocket for 4-anilinoquinazoline class of inhibitors in relation to interaction with polar amino acid residues on the substrate-binding region (16). Their findings showed the involvement of polar amino acid residues at the TKI binding site and could be useful in further research for the development of new EGFR-TKIs (16). Although biological research has been carried out in this context, to the best of our knowledge, clinical research on the relationship between amino acids of EGFR binding site and response of TKIs has not been conducted. Advances in NGS technology are expected to dramatically increase studies in this area in the future.

Although interesting results were found, there were limitations in this study. This study was conducted retrospectively on a small number of patients. Moreover, the study based on clinical information of patients who had residual pathological specimens for which the EGFR gene could be investigated. In addition, patients with a long interval from sample collection to this NGS were included. Therefore, it is important to consider the effects of selection bias. It is known that gene mutations other than the EGFR gene, such as such as KRAS gene mutation and amplification, cMET gene amplification, and PI3K gene mutation, are involved in drug resistance to EGFR-TKI (19, 20); however, we could not examine their effects on EGFR-TKI resistance. No further analysis was possible on the difference in the involvement of polar charged amino acids in compound mutations between PSF and OS, while the underlying biological mechanisms were not studied. Nevertheless, clarifying the role of charged amino acids in compound mutations of the EGFR gene may lead to the development of new therapies. The results of this study would be expected to provide suggestions for future research in this area.

NSCLC patients that carry common mutations and have polar charged amino acids in compound mutations might have a better OS than those without polar charged amino acids. Further studies and detailed information on charged or uncharged amino acids in compound mutations may contribute to a better understanding of survival in patients with EGFR mutated NSCLC.

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Conflicts of Interest

The Authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Authors' Contributions

HS, YS, KM, YS and NH had full access to all of the data in this study and take responsibility for the integrity of the data and the accuracy of the data analysis. HS, YS and NH contributed substantially to the study design, data analysis and interpretation, and writing the manuscript.

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