Prognostic Value of Aquired Resistance-related Molecules in Japanese Patients with NSCLC Treated with an EGFR-TKI

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Abstract. *Background: Most patients with lung cancer experience relapse, although epidermal growth factor receptor (EGFR) of tyrosine kinase inhibitor (TKI) has an astounding effect on tumors with EGFR-activating mutations. It is therefore critical to determine the mechanisms of resistance against agents and the prognostic value of acquired resistance-related molecules to EGFR-TKI. Materials and Methods: Tumor specimens were obtained from 19 matched specimens before and after treatment with gefitinib. A retrospective multi-institutional study analyzed the correlation between patients' survival and acquired resistance-related molecules in non-small cell lung cancer (NSCLC) samples, that possessed sensitive EGFR mutations (7 cases: exon 19 deletion, and 12 cases: exon 21 point mutation). The status of the epidermal growth factor receptor (EGFR) and KRAS genes were investigated by polymerase chain reaction (PCR)-based analyses. Real-time PCR assays were also used to evaluate MET gene amplification. The expression of hepatocyte growth factor (HGF) and changes in the epithelial-mesenchymal transition (EMT) status including the expression of E-cadherin and γ-catenin as epithelial markers, and those of vimentin and fibronectin as mesenchymal markers, were evaluated by immuno-histochemistry. Results: Eight of the gefitinib refractory tumors exhibited a secondary threonine-to-methionine mutation at codon 790 in EGFR (T790M). All of the tumors had wild type KRAS gene expression. No MET amplification was detected in any of the samples. A strong expression of HGF was detected in eight of the specimens at post-treatment. A change in the EMT status between pre-and post-treatment was found in five cases. The 5-year survival rate of patients with and without T790M was 86.7% and 13.3%, respectively (p=0.020). The 5-year overall survival (OS) rate for patients with overexpresion and for those with weak expression of HGF was 75.0% and 22.2%, respectively (p=0.259). In addition, the 5-year OS rate for patients with unchanged and changed EMT status was 83.3% and 40.0%, respectively (p=0.123). Conclusion: The current results showed that the presence of T790M is associated with favorable survival. On the other hand, the patients with weak HGF expression and EMT change tend to have a poor survival. The current patients' selection might be changed by discrimination of acquired resistance-related molecules in patients with NSCLC treated with an EGFR-TKI.*

Molecular-targeted drug therapy has been promoted because the selection of patients by genetic markers can increase the therapeutic response for patients with non-small cell lung cancer (NSCLC) (1). However, despite an initial response to treatment with EGFR-TKIs in specific patients, the majority of patients eventually experience a progression of their disease (2, 3). Understanding the mechanisms of resistance to treatment can provide a method for overcoming such resistance.

Explanations for the resistance to EGFR-TKI include the T790M mutation in exon 20 of the EGFR, MET amplification, overexpression of HGF, changes in the EMT status, and others (4-8). However, few studies have investigated resistance-related genes in EGFR-TKI-resistant specimens from a translational viewpoint, because of the clinical difficulty of re-biopsy. Therefore, a detailed study using matched specimens from both pre- and post-treatment is essential. This is the first comprehensive analysis of prognostic markers for molecules related to the acquired resistance in such pre- and post-treatment specimens, in order to elucidate their prognostic value.

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Table I. *Summary of patients exhibiting acquired resistance to gefitinib.*

Case	Gender	Age (years)[a]	Smoking status	Stage[b]	Previous chemotherapy	Response to gefitinib	TTP (days)	Survival (days)
1	M	58	Never	IIIB	Yes	PR	191	2488+
2	M	55	Never	IIIB	No	PR	174	2165
3	F	54	Never	IIIB	Yes	SD	368	2961
4	F	70	Never	IA	Yes	PR	60	1629
5	F	65	Current	IIIB	No	PR	110	2073
6	M	53	Current	IIIA	Yes	PR	352	2410
7	F	84	Never	IIB	No	PR	295	619
8	F	57	Never	IIA	No	SD	210	3568+
9	F	76	Never	IV	No	SD	221	597
10	F	85	Never	IIIA	No	CR	210	575
11	F	52	Never	IIIB	No	PR	233	2222+
12	F	87	Never	IIIA	Yes	SD	88	136+
13	F	79	Never	IIIA	No	PR	166	359
14	F	70	Never	IV	Yes	PR	773	1113+
15	M	59	Never	IV	Yes	PR	792	613
16	F	76	Never	IV	No	PR	290	1234+
17	F	62	Never	IIIB	Yes	PR	254	826
18	M	63	Never	IB	Yes	CR	1041	1258
19	F	79	Never	IV	Yes	PR	259	734

[a]At beginning of gefitinib. [b]At first presentation, TTP: time-to-progression after gefitinib therapy. +Patients were alive at the time of analysis. CR: Complete response, PR: partial response, SD: stable disease.

Materials and Methods

Patients and their characteristics. The characteristics of the 19 patients are listed in Table I. There were five male and 14 female patients. The tumor stage was classified according to the new TNM Classification for Lung Cancer (9). Six patients developed recurrent disease after surgery for primary tumors and three cases underwent incomplete resection. Ten patients were advanced cases. Therefore, the pathological stage was adopted for the surgical cases, and the clinical stage for the 10 non-surgical cases. All cases of diseases were firstly controlled for gefitinib (Table I).

The Institutional Review Board approved the informed consent obtained for the use of the tumor tissue specimens, either from the patients or from their legal guardians. All patients received 250 mg gefitinib every day. The treatment was continued until the disease progressed. Prior chemotherapy had been administrated to 10 patients. The tumor samples were collected before treatment with gefitinib, from surgically resected specimens from primary tumors except for those which were from two metastatic lymph nodes. Refractory tumors were obtained from pulmonary metastases (5 cases), lymph node metastases (4 cases), skin metastases (2 cases), pleural effusions (4 cases), primary tumors (3 cases), and liver metastasis (1 case). All of the specimens were stained with hematoxylin and eosin for histopathological diagnosis or cytology, and were confirmed to be adenocarcinoma except for one adenosquamous carcinoma (case 14).

The objective response of the patients was evaluated using the response evaluation in solid tumors (RECIST) criteria, while Routine clinical and laboratory assessments and chest X-rays were performed bi-weekly and computed tomographic (CT) scans were performed one month after the start of gefitinib and every three months thereafter. Imaging studies (bone scans and brain imaging) were performed every three months after the initiation of gefitinib treatment. The response to the initial gefitinib treatment was complete response (CR) in 2 cases, partial response (PR) in 13 cases, and stable disease (SD) in 4 cases. The time to progression (TTP) ranged from 60 to 1,041 days. The mean follow-up period from the date of administration of gefitinib to the date of death or last known contact was 1,452 days, with a range from 136 to 3,568 days.

Analyses of gene expression status of resistance-related molecules in sensitive and resistant tumors. Genomic DNA was extracted from each tumor and *EGFR* mutations in exons 19-21 were examined by sequencing, using previously described methods (10). The *KRAS* mutations were investigated by PCR-based analyses (11). *MET* gene copy numbers were determined by real-time PCR assays (12). The status of *HGF* was also investigated by using previously described methods (11). The *EMT* status was also examined using a previously described method. Briefly, immunohistochemical (IHC) staining was used to analyze the protein expression of E-cadherin and γ-catenin as epithelial markers, and those of vimentin and fibronectin as mesenchymal markers. The up-regulation of mesenchymal markers or down-regulation of epithelial markers in acquired samples was defined as a change in the EMT (13). Fresh malignant cells in the pleural effusion were fixed in an alcohol-based liquid (CytoRich Blue preservatives: BD Diagnostics, Burlington, USA) by thin-layer preparations, followed by immunohistochemistry.

Statistical analyses. The Kaplan-Meier method was used to estimate the probability of survival, and survival differences were analyzed by using the log-rank test. Differences were considered to be

Table II. *Summary of gene expression status of resistance–related molecules in sensitive and resistant tumors.*

Case	Pre-treated specimen[a]	Post-treated specimen[b]	EGFR[c]	T790M	KRAS[d]	MET	*HGF*[e]	EMT[f]
1	T	Pulmonary metastasis	19/19	–/+	w/w	–/–	W/S	+
2	T	Pulmonary metastasis	19/19	–/+	w/w	–/–	W/S	+
3	T	LN	21/21	–/+	w/w	–/–	W/S	–
4	T	Liver metastasis	21/21	–/–	w/w	–/–	W/W	+
5	LN	Pleural effusion	19/19	–/+	w/w	–/–	W/ n.e	n.e
6	T	LN	19/19	–/+	w/w	–/–	W/S	–
7	T	Pleural effusion	21/21	–/–	w/w	–/–	W/ n.e	n.e
8	T	LN	21/21	–/+	w/w	–/–	W/S	–
9	T	T	19/19	–/+	w/w	–/–	W/S	–
10	T	Skin metastasis	21/21	–/–	w/w	–/–	W/W	+
11	LN	LN	21/21	–/–	w/w	–/–	S/W	–
12	T	Pleural effusion	21/21	–/–	w/w	–/–	S/W	n.e
13	T	Skin metastasis	21/21	–/–	w/w	–/–	W/S	+
14	T	T	19/19	–/+	n.e	–/–	W/W	–
15	T	Pulmonary metastasis	21/21	–/–	n.e	n.e	n.e/n.e	n.e
16	T	T	21/21	–/–	n.e	n.e	n.e/ S	n.e
17	T	Pulmonary metastasis	19/19	–/–	n.e	n.e	n.e/ W	n.e
18	T	Pulmonary metastasis	21/21	–/–	n.e	n.e	n.e/ W	n.e
19	T	Pleural effusion	21/21	–/–	n.e	n.e	n.e/n.e	n.e

[a,b]T: Primary lung tumor, LN: lymph node metastasis. [c]19: exon19 deletion, 21: exon21 L858R, [d]w: wild-type, n.e: not evaluated. [e]W: weak, S: strong. [f]The change in EMT status from the tumor before treatment with gefitinib to the lesion after treatment.

statistically significant for p-values <0.05. The data were analyzed using the Stat View software package (Abacus Concepts, Inc., Berkeley, CA, USA).

Results

Gene expression status of resistance–related molecules in sensitive and resistant tumors. All tumors exhibited *EGFR* mutations. Twelve showed a substitution of arginine for leucine at codon 858 (L858R) in exon 21 and seven had a deletion in exon 19 of *EGFR* in the pre-treated with gefitinib tumors (Table II). Eight of the gefitinib refractory tumors exhibited a secondary *T790M* mutation, which had not been detected in the tumors before the gefitinib treatment. All of the tumors had wild-type expression of the *KRAS* gene at codon 12 both before and after the treatment with gefitinib. No *MET* amplification was detected in any of the samples. Strong expression of *HGF* was detected in eight of the specimens at post-treatment. A change in the *EMT* status between pre-and post-treatment were found in five cases.

Influence of gene expression status of resistance–related molecules on overall survival. Neither chemotherapy prior to administration of EGFR-TKI, nor subsequent chemotherapy after treatments, was associated with any statistically significant difference in survival. The 5-year survival rate of patients with T790M and those without was 86.7% and 13.3%, respectively (p=0.020). The 5-year overall survival rate of patients with overexpression and for those with weak expression of HGF was 75.0% and 22.2%, respectively (p=0.259). In addition, the 5-year overall survival rate of patients with unchanged and those with changed EMT status was 83.3% and 40.0%, respectively (p=0.123) (Figure 1).

Discussion

There are complicated relationships among acquired resistance-related genes including the *EGFR* T790 mutation, the overexpression of *HGF*, and changes in the *EMT* status (7). However, the prognostic values of these factors remain unclear. This study uncovered three important findings.

Firstly, the presence of T790M in EGFR was associated with a favorable survival. A small fraction of tumor cells harboring the *T790M* mutation might be enriched during the proliferation after drug treatment (14) and the germline *EGFR* mutation *T790M* was found in a family with multiple cases of NSCLC (15). Moreover, the *T790M* mutation in the primary tumor was found significantly more frequently in advanced tumors than in early-stage tumors (14). These phenomena suggest the growth advantage of cells carrying *T790M*. However, contrary to expectations, T790M may also be a useful marker for predicting a favorable prognosis in Japanese patients treated by an EGFR-TKI, which was consistent with previous data and with data from another group with a relatively short median follow-up (16, 17), and with *in vitro* data (18). This might be due to a difference in the biological significance between the resected primary tumor and the unresectable tumor treated by gefitinib. Most of the

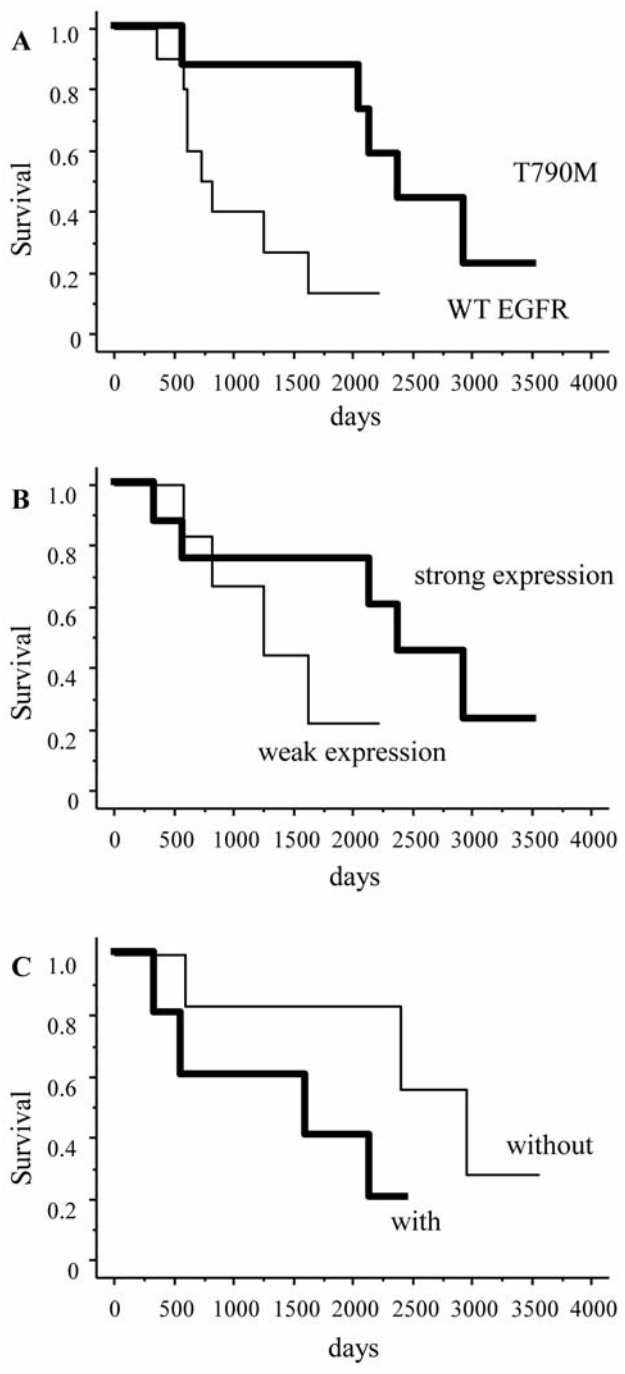

Figure 1. *Kaplan-Meier survival curves stratified according to the T790M of EGFR status (A) HGF expression (B) and EMT status (C). Survival was calculated from the date of gefitinib treatment to either the date of death or the last known contact.*

current post-treatment specimens were from metastatic or recurrent lesions. In fact, Molinari *et al*. reported that there are differences between primary tumors and metastases with respect to the EGFR pathway de-regulation mechanisms, implying a different response to EGFR targeted treatment (19). Other reasons might include the aggressive behavior in cases without T790M, possessing other gene alterations independent of the *EGFR* mutation, leading to poorer prognosis. Therefore, the absence of T790M after progression, is likely to indicate some other resistance mechanism, which might be associated with earlier development of new metastatic disease sites, of a poorer performance status, contributing to the shorter survival of these patients (17). In fact, disease flares sometimes develop following the discontinuation of TKI therapy (20), thus suggesting that a proportion of cells in an apparently resistant tumor cell population remain sensitive to EGFR inhibition (21).

Secondly, in this study patients with HGF overexpression had a tendency towards a more favorable prognosis than those who did not. A high pre-treatment serum HGF level has been associated with poor clinical outcomes in another study of patients with NSCLC treated with EGFR-TKI (22). This discrepancy could be related to the difference of sampling for specimens, such as pre- or post-treatment or the assays used for detection. In fact, the opposite survival curve was also found by analyzing pre-treatment tumor-biopsy specimens for the *T790M* mutation (23). Interestingly, five of seven tumors with *T790M* had HGF overexpression. On the other hand, only two of eight cases without *T790M* exhibited HGF overexpression. Therefore, HGF might interact with T790M in the EGFR signaling axis (8, 24). For that matter, pre-treatment plasma HGF levels have no correlation with tissue immunoreactivity for HGF (25).

Thirdly, EMT changes were associated with poor survival. These findings seem to be reasonable, because the EMT is an important contributor to the invasion and metastasis of epithelial cell-derived cancer (26). Interestingly, *in vitro* studies demonstrate that benzo(a)pyrene, a chemical fumed cigarette smoke seems to induce EMT in lung cancer cells (27). In fact, non-smokers with lung cancer have a more favorable prognosis than smokers (28).

In summary, these findings suggest that not only T790M but also HGF and a change in the EMT status might be associated with prognosis in Japanese patients treated with an EGFR-TKI. This analysis has the inherent limitations of a retrospective study and imbalances in the patients' characteristics cannot be excluded, given the small number of patients with limited biopsies. Nevertheless, the results may represent an important issue, since understanding the mechanisms of treatment resistance allows the possibility of establishment of personalised treatment.

Conflicts of interest

Dr. Uramoto and Dr. Tanaka have received research grants from NIPPON ZOKI, Taisho Pharmaceutical Co, Pfizer Inc, Mitsubishi Tanabe Pharma Corporation, Bristol-Myers Squibb, Sanofi, and Chugai Pharma. Dr. Yano has received a research grant from Chugai Pharma, and lecture fees from Chugai Pharma and Astrazeneca.

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