Epithelial–Mesenchymal Transition in EGFR-TKI Acquired Resistant Lung Adenocarcinoma

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Abstract. *Background: The epithelial to mesenchymal transition (EMT) may well play a part in determining the sensitivity to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI). However to date, no study has investigated the association between the EMT status and acquired resistance using cancer specimens. Patients and Methods: Immunohistochemical (IHC) staining was used to analyse the protein expression of epithelial and mesenchymal markers in tumour samples from lung adenocarcinoma patients. Mutations in the EGFR and K-ras gene were also examined. Results: All patients showed a positive expression of epithelial markers in sensitive tumours. Tumour in 4 (44.4%) out of 9 patients showed down-regulation of epithelial markers or up-regulation of mesenchymal markers. The change in the EMT status between pre-and post-treatment was shown in 2 cases each with and without the T790M mutation. Conclusion: EMT plays a role in approximately half of the cases of resistance to EGFR-TKI, independent of T790M mutation.*

Lung cancer remains the most common cause of cancer death worldwide (1). Non-small cell lung cancer (NSCLC) accounts for approximately 80% of all lung cancer cases and its outcome is poor due to aggressiveness of this type of cancer. The lack of curative treatment options for metastatic NSCLC emphasizes the need for a better understanding of its biological processes. Recently, selection of patients by gene markers has enabled molecular-targeted drug therapy to be proposed producing extraordinary results (2). Somatically sensitive mutations in the epidermal growth factor receptor *(EGFR)* gene in lung adenocarcinoma are associated with a dramatic clinical response to *EGFR* tyrosine kinase inhibitor (TKI) (3, 4). However, despite an initial response to treatment with *EGFR*-TKI in such patients, most patients eventually experience a progression of the disease (5). The discovery of the T790M mutation in exon 20 of *EGFR* was reported to explain the cause of gefitinib-resistance (5, 6). However, other mechanisms must be further explored because this mutation has been discovered in up to 50% of patients with acquired resistance (7). In addition, *MET* amplification and overexpression of hepatocyte growth factor (HGF) may also be the cause of partially acquired resistance to EGFR-TKI (8-10). Therefore, the overall causes of acquired resistance remain unclear.

Epithelial to mesenchymal transition (EMT) is characterized by the loss of epithelial cell junction proteins such as E-cadherin and gamma catenin, and the gain of mesenchymal markers such as vimentin and fibronectin (11). E-Cadherin modulates *EGFR* activation and signalling through its downstream targets (12). However, few studies have so far investigated EMT in EGFR-TKI-resistant specimens from a translational viewpoint. Moreover, the relationship between the EMT status and T790M are also unknown (12). Therefore, this study was an immunohistochemical (IHC) analysis of EMT-related molecules for refractory tumours after a sensitive response to treatment in order to elucidate the mechanism of acquired resistance to gefitinib.

Patients and Methods

Patients and their characteristics. The study included *9* lung adenocarcinoma patients, 3 male and 6 female, of whom 8 were non-smokers and 1 was a current smoker. Patients characteristics are listed in Table I. The tumour stage was classified according to the International Union against Cancer tumour-node-metastasis classification of malignant tumours (13). Seven patients had recurrent disease after the surgery for the primary tumours from 1995 to 2007. The pathological stage was adopted for the surgical cases, and the clinical stage for two non-surgical cases; two patients were non-surgical cases (cases 7 and 9). The tumour samples were collected prior to gefitinib treatment from the surgically resected specimens from 7 primary tumours, and on each from the biopsy specimen from an endobronchially invading tumour by

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Table I. *Summary of the patients showing acquired resistance to gefitinib.*

Case	Gender	Age[a] (years)	Smoking	Stage[b]	Specimen[c]	
					Before	After
1	M	58	Never	IIIB	Primary lung tumour	Pulmonary metastasis
2	M	55	Never	IIIB	Primary lung tumour	Pulmonary metastasis
3	F	54	Never	IIIB	Primary lung tumour	Lymph node
4	F	70	Never	IA	Primary lung tumour	Liver
5	M	53	Current	IIIA	Primary lung tumour	Lymph node
6	F	57	Never	IIA	Primary lung tumour	Lymph node
7	F	76	Never	IV	Primary lung tumour[d]	Primary lung tumour
8	F	85	Never	IIIA	Primary lung tumour	Skin metastasis
9	F	52	Never	IIIB	Lymph node	Lymph node

[a]Beginning of gefitinib therapy; [b]at first presentation; [c]specimens analysed before and after gefitinib therapy; [d]endobronchially invading tumour.

Table II. *Summary of EMT status by sensitive and resistant tumours.*

Case No.	EGFR	T790M	E-Cadherin		Gamma-catenin		Fibronectin		Vimentin	
		R	S	R	S	R	S	R	S	R
1	Exon19 deletion	+	+	+	**+**	**–**	–	–	**–**	**+**
2-	Exon19 deletion	+	+	+	+	+	–	–	**–**	**+**
		–	+	+	+	+	–	–	**–**	**+**
		–	+	+	+	+	–	–	**–**	**+**
		–	+	+	+	+	–	–	**–**	**+**
3	Exon21 L858R	+	+	+	+	+	–	–	+	+
4	Exon21 L858R	–	+	+	+	+	–	–	**–**	**+**
5	Exon19 deletion	+	+	+	+	+	–	–	+	–
6	Exon21 L858R	+	+	+	+	+	–	–	+	+
7	Exon19 deletion	+	+	+	+	+	+	+	+	+
8	Exon21 L858R	–	–	+	+	+	**–**	**+**	–	–
9	Exon21 L858R	–	–	+	+	+	–	–	–	–

S: Gefitinib-sensitive tumor resistant tumour. R: positive and negative expression, respectively. Bold indicates a change in the EMT status.

a transbronchial biopsy and a metastatic lymph node by a computed tomography (CT)-guided biopsy. The Institutional Review Board's approved informed-consent for the use of the tumour tissue specimens was obtained either from all the patients or from the patient's legal guardians. All patients received 250 mg gefitinib per day. The treatment was continued until the disease progressed. All of the tumours were pathologically confirmed to be adenocarcinoma. Prior chemotherapy had been administered in four patients. The objective response of the patients were evaluated using the Response Evaluation Criteria in Solid Tumors (RECIST) (14), and routine clinical and laboratory assessments and chest X-rays were performed bi weekly. CT scans were performed 1 month after the start of gefitinib and then every 3 months thereafter, and imaging studies (bone scan and brain imaging) were performed every 3 months after the initiation of gefitinib treatment. Refractory tumours were obtained from pulmonary metastasis (2 cases), lymph node metastasis (4 cases), and endobronchially invading tumour, skin metastasis, and liver metastasis (case 4 by autopsy). Four separate metastatic pulmonary tumours located in segments of 4, 5, 6, and 8 of the left lung were obtained from case 2 by video-assisted thoracoscopic surgery (10). Thus, 12 gefitinib-refractory specimens were available for analysis.

Analyses of EGFR and K-ras mutations. The *EGFR* mutations in exons 19-21 were examined by sequencing (3). A previously described restriction fragment length polymorphism (RFLP) method was used for the detection of the *K-ras* codon 12 mutations (15).

Analyses of E-cadherin, gamma-catenin, vimentin, and fibronectin by IHC staining. IHC staining was used to analyse the protein expression of E-cadherin and gamma-catenin as epithelial markers, and vimentin and fibronectin as mesenchymal markers in both pre- and post gefitinib treated tumour samples. Three-μm-thick sections sliced from paraffin-embedded specimen were prepared on glass slides. All the specimens were stained with haematoxylin and eosin for the histopathological diagnosis. The sections were then deparaffinised in a xylene series graded with ethanol. The sections were placed in 0.1 mol/l citrate

buffer (pH 6.0) and autoclaved at 121˚C for 10 min. They were treated with 3% H_2O_2 for 5 min to reduce the endogenous peroxidase activity. The primary antibody reaction used a mouse monoclonal anti-E-cadherin Ab (BD Bioscience, San Diego, CA, USA; diluted 1:2000), anti-gamma-catenin Ab (610254, BD Biosciences, Japan; diluted 1:200), anti-vimentin Ab (BD Bioscience; diluted 1:200), anti-fibronectin Ab (sc-8422, Santa Cruz Biotechnology, CA, USA; diluted 1:100), and incubated for 18 hours at 4˚C using a previously described method (16, 17). Thereafter, IHC staining was performed by the labelled, polymer method (Histofine Simple Stain MAX-PO kit, Nichirei, Tokyo, Japan) according to the manufacturer's instructions. The positive controls were processed using the normal colon epithelium for E-cadherin and gamma-catenin, and pleural malignant mesothelioma for vimentin and fibronectin, respectively. The negative controls were processed by the exclusion of the primary antibody. The stained specimens were categorised into six groups according to the positive staining and were assigned scores according to the proportion of positive tumour cells (0, none; 1, <10%; 2, <30%; 3, <50%; 4, <70%; and 5, <100%). The expression status of the tumour was categorised as a negative expression when the score was 0-3 and a positive expression when the score was 4-5. The slides were examined independently by two of the investigators (HU and TO) who were blinded to the clinicopathological data. A consensus was reached *via* their simultaneous examination using a double- headed microscope whenever any discrepancy was found between the data.

Statistical analysis. Student's *t*-test was used to assess the expression level of epithelial/mesenchymal markers by comparison between sensitive and resistant tumour. A statistical difference was considered to be significant if the *p*-value was less than 0.05. The data were analysed with the use of Abacus Concepts, Survival Tools for Stat View (Abacus Concepts, Inc, CA, USA).

Results

Response to the gefitinib and analyses of the EGFR mutation status. All of the tumours exhibited *EGFR* mutation. Four showed a deletion in exon 19 and another five had a substitution of arginine for leucine at codon 858 (L858R) mutation in exon 21 of *EGFR* in the gefitinib pre-treatment tumours (Table II). The response to the initial gefitinib treatment was complete response (CR) in 1 case, partial response (PR) in 5 cases, and stable disease (SD) in 3 cases. The time to progression (TTP) ranged from 60 to 368 days. Six of the gefitinib refractory tumours exhibited a secondary T790M mutation, which was not detected in the tumours before the gefitinib treatment. Only one of 4 pulmonary metastatic tumours in case 2 showed T790M (10). There were no other novel secondary mutations of the *EGFR* gene at exon 19-21. All the tumours showed wild-type *K-ras* gene at codon 12 before and after they were treated with gefitinib.

Analyses of the EMT status. Representative IHC staining is shown in Figure 1 and the results are summarised in Table II. Most cases (7/9) showed positive expression of E-cadherin in both the sensitive and resistant samples (Figures 1A and B). The expression of gamma-catenin showed similar results except for case 1. Interestingly, pre- and post-treatment specimens from case 1 showed positive and negative expression of gamma-catenin, respectively (Figures 1C and D). Conversely, pre- and post-treatment specimens from case 8 showed a negative and positive expression of fibronectin, respectively (Figures 1E and F). The pre- and post-treatment specimens from cases 1, 2, and 4 also showed negative and positive expression of vimentin, respectively (Figures 1G and

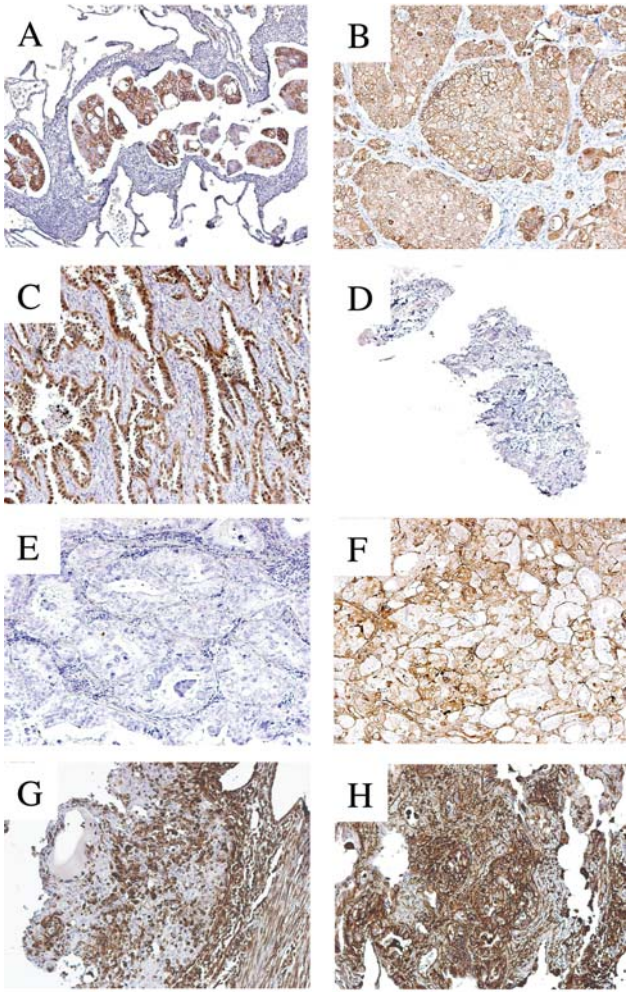

Figure 1. *Representative IHC staining. A: Positive expression of E-cadherin with brown stained membranes of pre-treatment tumour in case 8. B: Positive expression of E-cadherin with brown stained membranes of post-treatment tumour is shown in case 8. C: Positive expression of gamma-catenin with brown stained cytoplasm of a pre-treatment tumour in case 1. D: Negative expression of gamma-catenin of post-treatment tumour in case 1. E: Negative expression of fibronectin of pre-treatment tumour in case 8. F: Positive expression of fibronectin with brown stained membrane of post-treatment tumour in case 8. G: Negative expression of vimentin of pre-treatment tumour in case 2. H: Positive expression of vimentin with brown stained membrane and cytoplasm of post-treatment tumour in case 2. Original magnification is ×100 in all cases.*

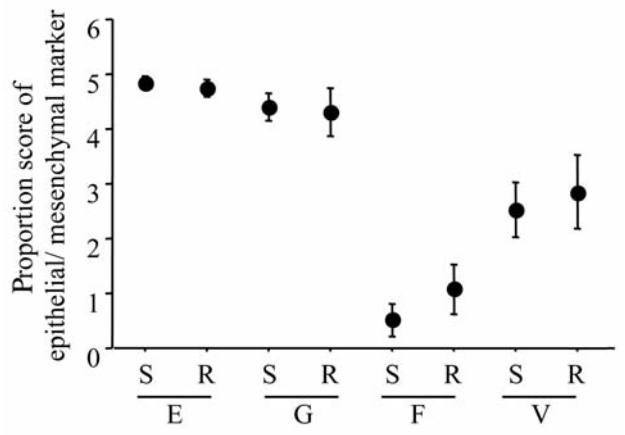

Figure 2. *The mean proportion scores in a comparison between refractory and sensitive tumours are shown. Error bar: mean±standard deviation. S: Gefitinib-sensitive tumour, R: gefitinib-resistant tumour, E: E-cadherin, G: gamma-catenin, F: fibronectin, V: vimentin.*

H). In total, 4 (cases 1, 2, 4, and 8; 44.4%) out of 9 cases showed the down-regulation of epithelial markers or up-regulation of mesenchymal markers. The change of EMT status between pre- and post-treatment was shown in two cases each with an exon19 deletion and one with a substitution of arginine for leucine at codon 858 (L858R) in exon21, and with and without T790M mutation.

The actual proportion score for the IHC with each marker is shown in Figure 2, since categorisation as positive or negative by IHC staining was an artificial classification. Epithelial markers had a tendency to slightly decrease in resistant than sensitive tumours. On the other hand, the mesenchymal markers had a tendency to slightly increase, although the differences were not statistically significant.

Discussion

The development of acquired resistance to *EGFR*-TKI is a major problem (4). The primary mechanism associated with acquired resistance may be ascribed to T790M mutation in exon 20 of *EGFR* (5, 6). The current results are consistent with previous findings in 6 out of the 9 cases and 6 out of 12 gefitinib-refractory tumours (7). In addition, *MET* amplification and overexpression of HGF have also been reported as causes of partially acquired resistance (8, 9). Neither other novel secondary mutations of *EGFR* nor *MET* gene amplification were observed in 10 pre- and post-treatment matched cases (10). Furthermore, no overexpression of HGF was observed in resistant tumours without the T790M mutation, suggesting that mechanisms other than T790M, MET, and HGF status are involved in acquired resistance to EGFR-TKI (10). Therefore, additional markers are needed not only to select precisely the cases that will respond to EGFR-TKI, but also to eliminate unsuitable cases that might suffer frequent adverse events and incur high expenses without a clinical response. In addition, understanding the mechanisms of treatment resistance offers the potential development of other modalities to intervene by overcoming the acquired resistance.

EMT is defined by the combined loss of epithelial markers and the induction of mesenchymal markers (18). *In vitro* studies show that the mesenchymal phenotype is more resistant to EGF-TKI than the epithelial phenotype (19). Moreover, restoration of E-cadherin enhances the sensitivity to EGFR-TKI (20). All cases in the current study showed positive expression of gamma-catenin as epithelial markers in the sensitive samples, which was consistent with recent reports (21, 22). Therefore, epithelial markers might be surrogate markers, although *EGFR* genetic testing is the most powerful tool to select the patients who will like demonstrate a clinical benefit at the present time (2).

The EMT is associated with not only primary sensitivity but also with acquired resistance to EGFR-TKI *in vitro* (23). Therefore, a detailed study of the EMT status of lung adenocarcinoma and its association with EGF mutations using matched specimens from both pre- and post-treatment is essential. EMT was observed in approximately half of post-gefitinib treatment samples. Why the remaining half were not correlated with EMT is unclear. The discrepancy between the *in vitro* and *in vivo* results may be due to (i) disparity among experimental systems, (ii) micro-environments such as tumour-related macrophages and fibroblasts, and (iii) unknown mechanisms. Why does the EMT cause the resistance to EGFR-TKI? One possible explanation is that vascular epithelial growth factor receptor (VEGFR-1) plays a role in cancer progression through the induction of EMT (24). Recent findings showed E-cadherin expression was found less frequently in tumours with positive expression of VEGF in resected stage I NSCLC (17).

This study has three limitations in its interpretation: (i) it is unclear that up-regulation of one of two mesenchymal markers without down-regulation of epithelial markers can be defined as EMT; (ii) it cannot be distinguished whether the EMT process is a cause of gefitinib resistance or is the result of metastasis; and (iii) imbalances in the patients' characteristics cannot be excluded, given the small number of patients with limited biopsies for specimens of recurrent tumours. To overcome these limitations, further exploratory studies of the mechanisms that target the mesenchymal process are therefore urgently needed for patients that are resistant to EGFR-TKI.

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