

Fig. 1. A MHC stabilization assay of potential HLA-A24-binding peptides within CEA protein. The MHC stabilization assay was performed to check the binding affinity of CEA-derived peptides candidates using T2-A24 cells. Stabilization efficiency was rated by mean fluorescence intensity (MFI) increase, which was calculated as $MFI \text{ increase} = (MFI \text{ with the given peptide} - MFI \text{ without peptide}) / (MFI \text{ without peptide})$.

($2 \leq MFI \text{ increase} < 3$), low in six ($1 \leq MFI \text{ increase} < 2$) and not binder in one peptide ($MFI \text{ increase} < 1$). The CMVpp65 HLA-A24-binding peptide with high BIMAS score showed high efficiency for MHC stabilization, while CEA HLA-A24-binding

peptides with a BIMAS score above 100 did not always indicate high stabilization efficiency (Fig. 1).

CTL induction assay using CEA HLA-A24 peptides and serum CEA-positive cancer patient-derived PBMC. In Figure 2, six representative cases of positive CTL response are shown. These CTL responded to various CEA HLA-A24-binding peptides including peptides 3, 6, 8 and 9. Especially, CTL from Case015 demonstrated very large amounts of IFN- γ production, 9.7 ng/mL. A summary of CTL induction assays using cancer patient PBMC is shown in Table 2. The CTL derived from 29 cases responded to all CEA HLA-A24 peptides except peptides 2, 7 and 10. The frequency of positive responses was relatively high against CEA peptides 6 and 9. Among 29 cases, 12 patients including two who showed positive responses for more than two peptides, demonstrated significant IFN- γ responses against peptide-pulsed TISI cells (positive rate 41.4%). On the other hand, the positive response rate against CMVpp65 peptide as positive control was 55.2%.

Docking simulation assay for CEA HLA-A24-peptides binding to HLA protein. Eight CEA peptides and CMVpp65 were shown positive for docking simulation to HLA-A24 protein (Fig. 3). The peptide with highest affinity was CEA A24-3 ($\Delta G = -43.5 \text{ kJ/mol}$), but CEA 7 and 10 were not binding. Meanwhile, CMVpp65 A24 peptide showed high affinity (-32.2 kJ/mol).

Additionally, we prepared an example figure of our *in silico* docking simulation assay using CMVpp65₃₄₁₋₃₄₉ peptide, which showed a high docking affinity (Fig. 4).

Characterization of known HLA-A24-restricted peptides using various peptide-evaluation tools. Sixteen HLA-A24-restricted peptides including CMVpp65 and CEA HLA-A24 peptides 2, 6, 9 and 10 were investigated in binding activity using BIMAS,

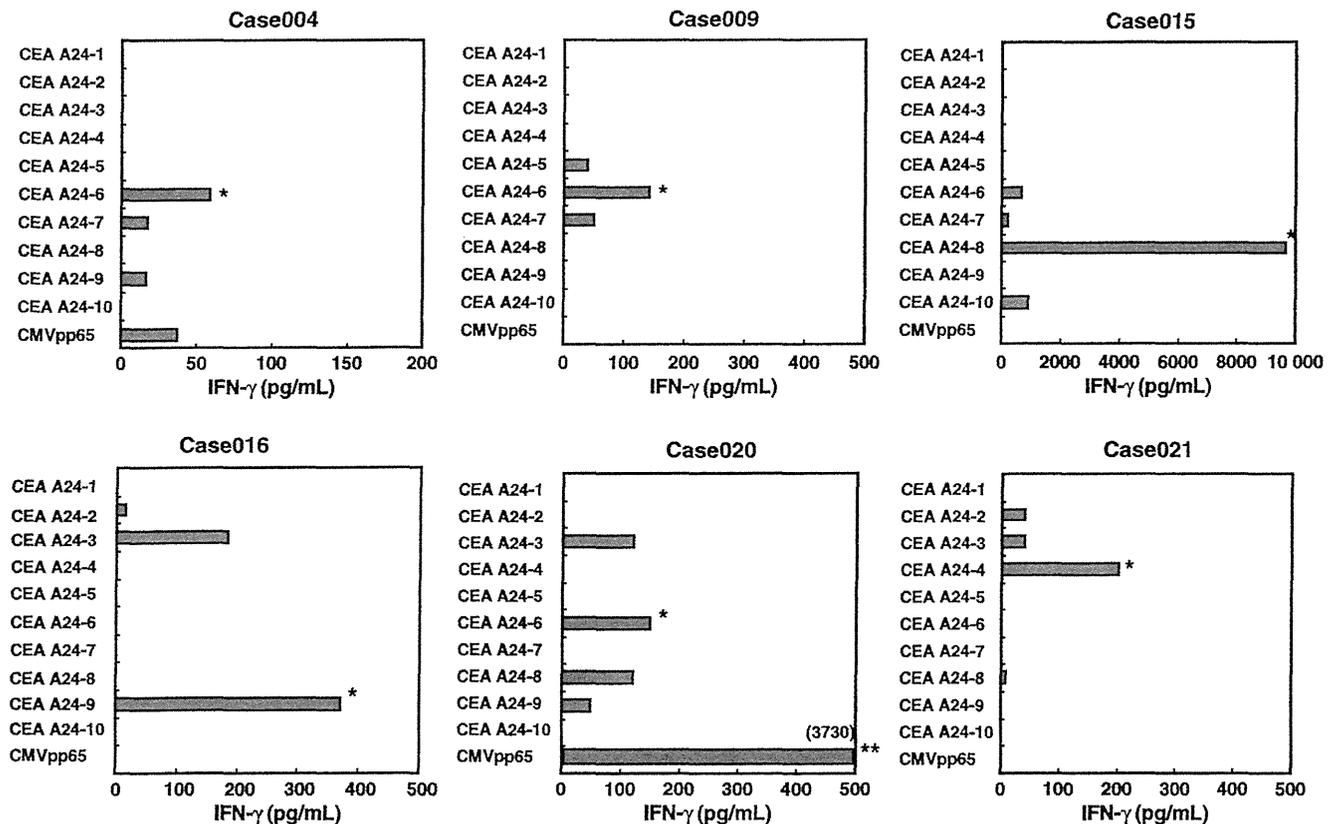


Fig. 2. The CTL inducing activity of CEA HLA-A24-restricted peptides. CTL induction cultures were successful in 29 of 32 cases of CEA-positive cancer patients. Six representative cases of positive CTL response against CEA peptides 4, 6, 8 and 9 are shown. Measuring IFN- γ levels in supernatant from co-culture of CD8⁺ T cells and peptide-pulsed TISI cells was performed in triplicate. * $P < 0.05$, ** $P < 0.01$.

Table 2. Frequency of positive CTL-inducing activities in CEA-positive cancer patients

Peptide name	Tested cases	Positive cases	Positive rate (%)
CMVpp65 A24	29	16	55.2
CEA A24-1	29	2	6.9
CEA A24-2	29	0	0
CEA A24-3	29	1	3.4
CEA A24-4	29	2	6.9
CEA A24-5	29	2	6.9
CEA A24-6	25	4	16
CEA A24-7	29	0	0
CEA A24-8	29	1	3.4
CEA A24-9	29	3	10.3
CEA-A24-10	29	0	0
SUM	29	12†	41.4

†Two cases showed positive CTL responses for more than 2 peptides.

MHC-stabilization, docking simulation and IFN- γ production assay (Table 3). The IFN- γ production assay was performed using PBMC from enrolled HLA-A*2402⁺ cancer patients who were not included in the CEA peptide-CTL induction cultures. BIMAS scores ranged 0.1–720. The MHC stabilization assay demonstrated that peptides with high, medium and low affinity were 5, 5 and 4, respectively. Docking simulation assay revealed that MAGE3, tyrosinase and HER2 peptides showed high affinities (<-14 kJ/mol) besides CEA and CMVpp65 peptides. The IFN- γ production assay indicated that six HLA-A24 peptides exhibited significant CTL induction activity. Taking these observations into consideration, three peptides (MAGE3, HER2-2, CMVpp65) alone showed a high score in all three parameters except BIMAS.

Correlation between peptide docking and other immunological parameters. Correlations between HLA-A24-restricted peptide docking and other immunological parameters including BIMAS, MHC stabilization and IFN- γ production, were evaluated using a correlation analysis. The MHC stabilization score was inversely correlated with the affinity calculated in docking simulation alone ($r = -0.589$, $P = 0.015$), not with BIMAS or IFN- γ production index (Fig. 5). On the other hand, BIMAS was not significantly correlated with any other parameters.

Discussion

HLA molecules are cell surface proteins that bind to antigen peptides and present them as HLA-peptide complexes to T-lymphocytes, thereby inducing a specific immune response. The binding site of HLA-class I molecules is formed by a deep cleft between two α -helices on top of an extended β -sheet structure. Only approximately bound peptides with a high affinity could trigger a potent immune response. Therefore, to identify



Fig. 4. Docking structure of CMVpp65₃₄₁₋₃₄₉ peptide and HLA-A24 protein. The two subunits of HLA-A24 protein (shown as ribbons in green and cyan) are identical to chains D and F of 2BCK in the Protein Data Bank, respectively, and the CMVpp65 peptide (shown as a space-filling model of atoms) was docked to the binding pocket of the HLA protein. This model was drawn by PyMOL (<http://www.pymol.org/>).

possible high-affinity peptides derived from tumor antigens is a very important theme for developing cancer vaccines that have therapeutic efficacy.

Recently, a large amount of data for crystal structures of HLA-peptide complexes has been accumulated, allowing bio-informaticians to predict molecular binding simulations.^(20,21)

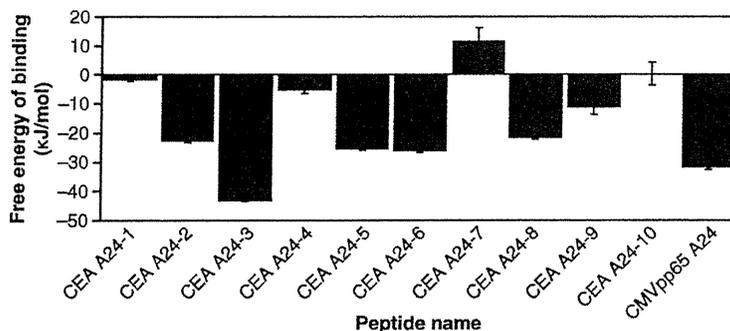


Fig. 3. Characterization of CEA HLA-A24-restricted peptides using peptide-HLA docking simulation assay. For each of 9-mer epitope candidate peptides, the mean and standard deviation of the three lowest binding free energies are shown.

Table 3. Peptide-HLA docking scores and other immunological factors of various HLA-A24-binding peptides

Peptide name	Amino acid sequence	Position	BIMAS score	MHC stabilization assay	Docking (kJ/mol)	IFN- γ production index
HIVgp120	RYLRDQQLL	584-592	720	1.60	13.5	1.02
MAGE1	NYKHCPEI	135-143	66	3.38	-0.7	1.37
MAGE2	EYLQLVFGI	156-164	90	2.27	-6.1	1.14
MAGE3	IMPKAGLLI	195-203	1.5	3.12	-14.9	4.72
Tyrosinase	AFLPWHRLF	206-214	18	2.50	-17.3	0.96
WT1-1M	CYTWNQMNL†	235-243	200	2.87	-9.2	0.83
WT1-2	CMTWNQMNL	235-243	4	1.07	-8.4	0.97
HER2-1	TYLPTNASL	63-71	360	3.23	-14.5	0.89
HER2-2	PYVSRLLGI	780-788	7.5	3.20	-15.9	2.54
EBNA3A	RYSIFFDYM	246-254	60	1.40	-11.4	1.28
CEA A24-2	TYACFVSNL	652-660	200	2.85	-22.7	0.94
CEA A24-6	LYGPDTPII	590-598	60	2.46	-26.4	1.03
CEA A24-9	TFQQSTQEL	276-284	39.6	1.00	-11.5	2.25
CEA A24-10	KTITVSAEL	492-500	18.5	0.01	0.2	0.97
CMVpp65	QYDPVAALF	341-349	168	3.45	-32.0	2.31
gp100	VWKTWGQYW	152-160	0.1	0.34	13.1	1.00

†Modified peptide from natural WT1₂₃₅₋₂₄₃ peptide.

(a)

Analysis	Correlation coefficient (<i>r</i>)	<i>P</i> value
BIMAS vs MHC	0.141	0.609
BIMAS vs docking	0.274	0.311
BIMAS vs IFN- γ	0.258	0.342
MHC vs docking	-0.589	0.015* (<i>P</i> < 0.05)
MHC vs IFN- γ	0.312	0.244
Docking vs IFN- γ	-0.263	0.332

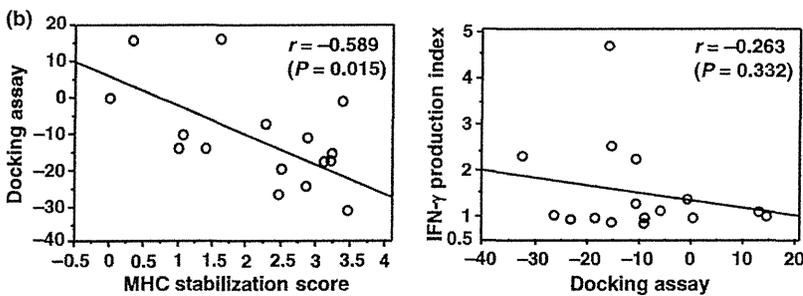


Fig. 5. Correlation of peptide-HLA binding predictions with immunological parameters. (a) Correlation of BIMAS score and peptide-HLA binding affinity predicted in docking simulation assay with MFI increase ratio in MHC stabilization assay and IFN- γ production index. MHC: MHC stabilization assay ratio; docking: peptide-HLA docking simulation assay; IFN- γ : IFN- γ production index. (b) Correlation of binding affinity predicted in docking simulation assay with MHC stabilization assay ratio or IFN- γ production index.

However, despite biophysical and biochemical analysis of HLA complexes, accurate prediction of antigen peptide binding to HLA molecule is still difficult.

In the present study, we developed an *in silico* docking simulation assay between the HLA-A24 molecule and an antigen peptide based on computational structural analysis, aiming to predict optimal docking peptides with high affinity leading to effective CTL activation. Conventional algorithms based on primary amino acid sequence have been successful so far in predicting antigenic peptides binding to the HLA class I pocket, but such algorithms have limited accuracy and provide no structural information.^(22,23)

Recently, the development of algorithms to predict the structure of MHC-peptide complex has been tried. Briefly, Logean and Rognan⁽²⁴⁾ used a binding free energy scoring function in a newly developed EpiDock, and Desmet *et al.*⁽²⁵⁾ developed a quantitative structure-based affinity scoring method for prediction of anchoring peptide side chains in peptide-HLA complex.

Unfortunately, these methods have not been applied to the intact HLA-A24 protein because of no such available structure in PDB at that time. We performed, for the first time, the epitope prediction for HLA-A24 protein using the 3D complex structure of the protein and epitope (PDB code 2BCK). In this study, we applied two software packages, AutoDock and MODELLER, to a novel *in silico* assay system of peptide binding affinity. MODELLER was performed to build the initial conformation of any given peptide, and AutoDock was used to assess the affinity (ΔG) between HLA-A24 protein and the given peptide. In general, AutoDock is originally available for predicting the binding mode of low-molecular compounds in the context of receptor-ligand docking, and is not always suitable for highly flexible ligands such as 9-mer peptides. In the case of HLA-peptide docking, however, the binding pocket and epitope conformation have been well investigated, and the conformation search parameters in simulation can be limited or narrowed down differently from so called "blind docking." We have shown that a local search simulation around the binding pocket of HLA-A24

protein reproduced the affinities between the protein and A24-restricted peptides. More importantly, all of these procedures are performed by combination of free software (AutoDock and MODELLER), suggesting that our *in silico* simulation assay is easily available to many bioinformaticians with the same interests. Recently, Fuhrmann *et al.*⁽²⁶⁾ reported a receptor–ligand docking simulation method based on a new Lamarckian genetic algorithm which can treat a large number of degrees of freedom.⁽²⁶⁾ Thus, the further improvement of an *in silico* binding assay system may be possible for not only HLA class I epitope peptides (approximately 10-mer) but also class II epitope peptides (approximately 20-mer). Additionally, we have performed MHC stabilization and CTL induction assays regarding various CEA and other HLA-A24-restricted peptides and investigated the correlation of docking simulation assay with other immunological parameters, the MHC stabilization score and IFN- γ production index.

Cytotoxic T-lymphocyte induction experiments using PBMC from CEA-positive cancer patients demonstrated that CEA A24-6 and 9 were mainly positive for CTL inducing activity. These peptides were shown to have moderate to high affinity in a docking simulation assay. These observations may support the negative selection theory that intermediate binders can be sometimes good CTL inducers in cancer patients because CTL precursors responding to high affinity peptides have been depleted in the thymus by negative selection, and such CTL may be exhausted after exposure to specific cancer antigen peptides.⁽²⁷⁾

Unfortunately, CEA A24-3, which has the highest affinity in a docking assay, did not show strong CTL activation. Similarly, CEA A24-1, with the highest affinity in MHC stabilization, showed marginal binding score in a docking simulation, which indicates the discrepancy of affinity between docking simulations and other immunological binding assays. In our current method, unlike peptide epitopes, we did not permit the flexibility of the epitope-binding pocket structure of the HLA protein because of long computational time. The MHC stability assay suggests that CEA A24-1 is a good binder (Fig. 1), but its docking affinity is not high (Fig. 3). Since CEA A24-1 is the second largest peptide (1082.2 Da) among those in Table 1, it might not stably occupy a rigid binding pocket of HLA-A24 protein in a docking simulation. Actually, CEA A24-7, the largest peptide (1136.2 Da), has shown the lowest docking affinity. Although a strong correlation was not observed between the epitope's molecular weight and docking affinity ($r^2 = 0.16$), the rigid pocket simulation condition for HLA protein may have affected the docking conformation and affinity. A docking simulation that allows a pocket structure to be flexible is a challenge for the future, and it may be solved by advances in computer technology.

Most interestingly, CEA₆₅₂ peptide, which is well known for specific CTL epitopes, did not show any positive CTL inducing activity in all patient PBMC. On the other hand, CEA A24-6, which showed the highest frequency of CTL induction, has not been reported as a CTL epitope previously, but few CTL induction studies using CEA-positive cancer patient PBMC have been done. Therefore, there might be some difference in CTL culture environments including immunological regulation between healthy donor and cancer patients, resulting in selection of antigenic peptides. Crosti *et al.*⁽⁷⁾ showed four CEA helper epitopes stimulating the proliferation of CD4⁺ T-lymphocytes from end-stage lung cancer patients. These results supported positively our results that CTL inducing activity for any CEA peptides with HLA-A24 restriction was seen in 12 of 29 cancer patients.

Nukaya *et al.*⁽⁹⁾ reported CEA₆₅₂-A24-restricted peptide using healthy donor PBMC-based CTL induction assay for the first time and demonstrated peptide-stimulated CTL efficiently killed CEA-positive cancer cell lines. The CEA₆₅₂ peptide has been

also used for DC-based cancer vaccine against CEA-positive gastrointestinal solid cancers. The clinical trial by Matsuda *et al.*⁽¹⁰⁾ using CEA-peptide treated DC vaccine showed that no significant clinical responses were seen, but four of seven patients developed *in vitro* CTL response against CEA peptide after vaccination. In the present study 44% of cancer patients demonstrated CTL activity against various CEA peptides; however, there was no response to CEA₆₅₂ peptide.

In another CTL induction assay using patient PBMC and 16 well-known HLA-A24 peptides including CEA peptides, six peptides were positive for specific CTL activation. With regard to these 16 peptides, we also obtained other immunological data. In Figure 5, correlations between HLA-A24 peptide docking simulation and other immunological parameters, including BIMAS, MHC stabilization and IFN- γ production, were investigated using a correlation analysis. The binding affinity predicted in docking simulation was inversely correlated with MHC stabilization score ($r = -0.589$, $P = 0.015$), whereas BIMAS was not significantly correlated with that or any other parameter. These observations suggested that our docking simulation assay might have potential advantage in efficiency of epitope prediction over BIMAS. However, our docking simulation assay is still not developed enough to compare with other predictive programs precisely, and the accumulation of more simulation data is needed.

Recently, novel conventional algorithms based on primary amino acid sequence like BIMAS have been developed. They show better prediction than BIMAS,^(28–30) but such algorithms have limited accuracy. Similarly, our *in silico* assay system has yet to predict genuine CTL epitope peptides with high affinity. Computational methods for modeling of peptide-HLA binding include threading, molecular dynamics and approaches based on HLA binding pocket recognition. Technical improvements for all methods should be requested to elevate the accuracy of CTL epitope prediction in future.

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Disclosure Statement

The authors have no conflict of interest.

Abbreviations

CEA	carcinoembryonic antigen
HLA	human leukocyte antigen
PDB	The Protein Data Bank
CTL	cytotoxic T cells
BIMAS	bioinformatics and molecular analysis section
PBMC	peripheral blood mononuclear cells
CMV	cytomegalovirus

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Disseminated calcifying tumor of the pleura: review of the literature and a case report with immunohistochemical study of its histogenesis

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Abstract Calcifying tumor of the pleura is a rare benign tumor, similar to the calcifying fibrous pseudotumor originally described in the subcutaneous and deep soft tissues of the extremities, trunk, and neck. Calcifying tumors of the pleura have also been reported infrequently as disseminated lesions. Here we report a case of disseminated calcifying tumor of the pleura, with some new findings obtained in this study, and review the literature of disseminated calcifying tumor of the pleura.

Key words Calcifying tumor · Calcifying fibrous pseudotumor · Pleura dissemination · Osteopontin

Introduction

In 1993, Fetch et al. first described a calcifying fibrous pseudotumor as a benign soft tissue tumor characterized by the presence of dense collagenization with psammomatous calcification and lymphoplasmacytic cell infiltrates.¹ Later, similar tumors were reported in the pleura and were termed calcifying fibrous tumor or pseudotumor. In the newly published *Classification of*

Tumours of the Lung, Pleura, Thymus and Heart by the World Health Organization, this lesion has been designated “calcifying tumor of the pleura” (CTP), which is synonymous with the previous calcifying fibrous pseudotumor.² Moreover, CTP have been reported as disseminated multiple lesions in the pleura.^{3–7} Herein we present an additional disseminated CTP case with new immunohistochemical findings accompanied by a literature review.

Case report

A 40-year-old Japanese man was referred to our hospital for further evaluation of a pleural mass initially found by a computed tomography (CT) scan of his chest. He had felt an uncomfortable feeling in his back. He had no history of smoking or asbestos exposure. A CT scan revealed a wide mass, 75 mm in maximum diameter, on the chest wall in the right dorsal area (Fig. 1). The mass appeared as diffuse high-density areas in the scan. Using integrated ¹⁸F-fluorodeoxyglucose positron emission tomography (FDG-PET)/CT imaging, the maximum standardized uptake (SUV_{max}) value of this mass was found to be 2.0, which was too low to consider the tumor as malignant. Percutaneous needle biopsy was performed, and the histological diagnosis was suggestive of CTP. Because the patient refused surgical treatment at first, he was carefully followed with CT scans for more than 2 years, but tumor size was unchanged. Finally, video-assisted surgery through a mini-thoracotomy was performed to remove the tumor.

At surgery, the largest tumor, 6.8 cm in diameter, was found to be suspended from two long pedicles and hanging down into the thoracic cavity from the chest

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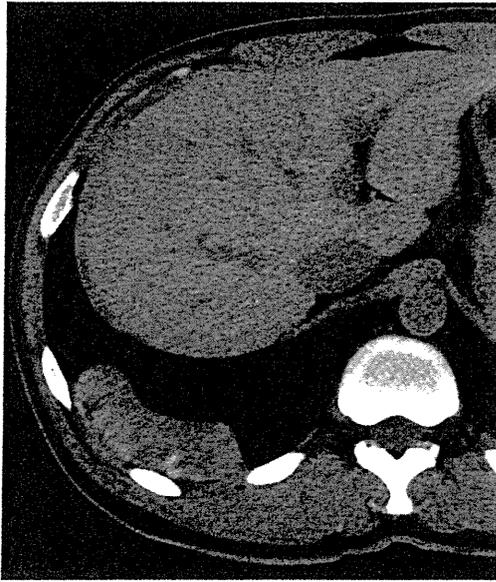


Fig. 1 Computed tomography (CT) scan image. A subpleural mass was seen in the posterior part of the lower part of the right lung

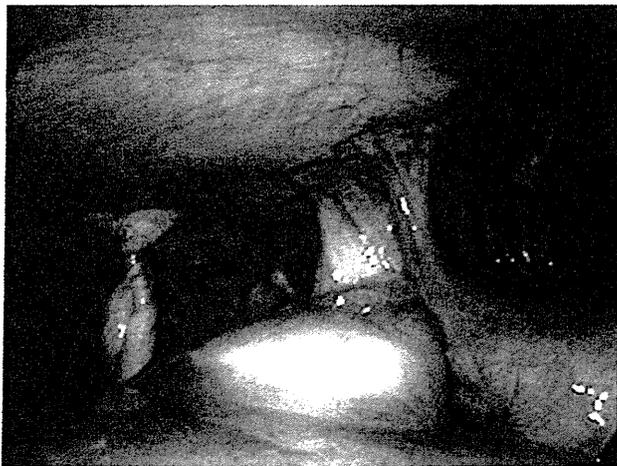


Fig. 2 Thoracoscopic view of disseminated calcifying tumor of the pleura (CTP). The largest tumor was found suspended with a long pedicle into the thoracic cavity from the chest wall. Another small tumor was also found hanging down beside the main tumor

wall (Fig. 2). Two other tumors, 2.0 cm in diameter, were also suspended from pedicles similar to the largest tumor. Many additional small nodules were present on the diaphragm, especially in the fatty tissue of the costophrenic angle. We resected all 17 visible lesions. The largest mass had a shiny surface and was lobulated in shape. All the lesions were firm, well circumscribed, but unencapsulated, and ash gray in color at the cut surface. The smallest nodule was about 2 mm in diameter.

The diagnosis was confirmed by histopathology using hematoxylin and eosin staining. Histologically, the central part of the larger tumors consisted of paucicellular, dense collagenous tissue with many psammomatous dystrophic calcifications. The peripheral areas of the largest tumor and many of the small tumors were composed of fibrogranulomatous tissue with plasma cell infiltrations and vascular proliferation. Moreover, there were many spindle-shaped or stellate fibroblast-like cells with large nuclei and abundant pale cytoplasm, sometimes forming multinucleated giant cells (Fig. 3a). Immunohistochemically, these cells were positive for vimentin and osteopontin, but negative for AE1/AE3, epithelial membrane antigen (EMA), desmin, HHF35, α -smooth muscle actin (ASMA), caldesmon, and anaplastic lymphoma kinase-1 (ALK-1), CD34, and CD68 (Fig. 3b). Osteopontin immunostaining was strongly positive for the psammoma bodies and many CD68-positive histiocytes (Fig. 3c,d).

The patient's postoperative course was uneventful, and no evidence of local recurrence or distant metastasis was found at 28 months after surgery.

Discussion

The previously reported cases of disseminated CTP and our case are summarized in Table 1.^{3–7} Mean age was 35.7 years (range, 23–52 years); there were two men and four women. Half these CTP cases, including our case, were reported from Japan. Most disseminated CTP is asymptomatic and found incidentally on radiographs. CT images of these tumors have been reported, but FDG-PET/CT images have not been previously reported. Our FDG-PET image revealed a very low SUV_{max} value, which suggested a benign lesion. With regard to its prognosis, no disease-related death has been reported for disseminated CTP, although its local recurrence has been observed.^{1,8,9} The disseminated CTP might be benign rather than malignant given the radiologic and clinical findings. Previous reports and our immunohistochemical study revealed that the spindle-shaped fibroblastic cells in CTP were positive for vimentin and negative for cytokeratin, EMA, ASMA, desmin, S-100 protein, CD34, and ALK-1. Although these findings do not clarify the histogenesis of this tumor, CTP might differ from benign spindle cell tumors such as solitary fibrous tumors and inflammatory myofibroblastic tumors.^{8–10}

We first studied immunohistochemical osteopontin expression in this CTP and found that the CTP was composed mainly of spindle-shaped or stellate cells, which were immunohistochemically positive for osteo-

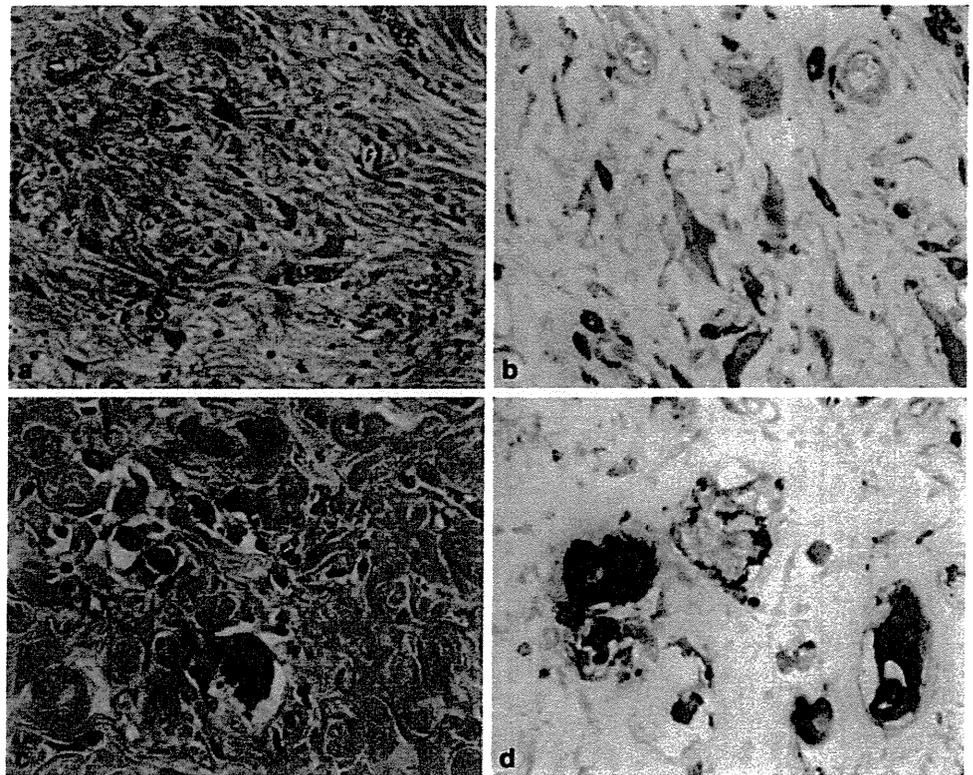


Fig. 3 Histopathological findings of disseminated CTP. **a** In the peripheral area of the tumor, the fibrous tissue contained inflammatory cells and large fibroblastic cells with pale cytoplasm. **b** Double immunohistochemistry showed that large fibroblastic cells were positive for only osteopontin (*brown*) and that histiocytic cells were positive for both osteopontin and CD68 (*dark blue*). **c** Psammomatous calcification was usually seen in the dense fibrous tissue

in the central area of the tumor, sometimes closely associated with inflammatory cells such as histiocytes and plasma cells. **d** Double immunohistochemistry showed that psammomatous calcifications were positive for osteopontin (*brown*), and large histiocytic cells (*blue-brown*) positive for both osteopontin and CD68 were present adjacent to the psammomatous calcifications

pontin. There are two types of osteopontin-positive cells: osteopontin-positive but CD68-negative fibroblast-like cells and osteopontin-positive and CD68-positive histiocytes. Osteopontin is a cytokine that is associated with inflammation and tissue remodeling.^{11,12} Signals mediated by inflammatory cells such as macrophages and neutrophils trigger expression of osteopontin in fibroblasts, which correlates with collagen production.¹¹ It is possible that the osteopontin-positive cells were fibroblasts leading to collagen deposition in the CTP. Moreover, psammoma bodies were strongly positive for osteopontin and sometimes were closely associated with CD68-positive histiocytes in our case. Osteopontin is also produced by macrophages and stimulates other macrophages.¹³ These macrophages are effector cells controlling ectopic calcification,¹⁴ and osteopontin plays a significant role in the development of psammoma bodies.¹⁵ These findings may suggest that the CPT is not

a true neoplasm, and that the multifocal and recurrent lesions appear during unusual inflammatory-reparative processes mediated by osteopontin expression. Osteopontin expression might be associated with the pathogenesis of disseminated CTP. Further studies are necessary, including clonality analysis of this tumor.

We followed up this case of disseminated CTP before and after surgery for the longest time reported to date. Although the long-term prognosis for patients with CTP is unclear, several studies have demonstrated that their clinical courses are good, even if they have disseminated lesions. Complete resection with a margin of normal tissue appears optimal, and should reduce the possibility of local recurrences, even among disseminated CTP. It is still unclear whether this treatment strategy is essential for patients with disseminated CTP. More knowledge about this is required to handle surprising disseminated lesions.

Table 1 Previously reported cases of disseminated calcifying tumors of the pleura

Case	First author (year) and country	Age/sex	Diagnostic procedure	Operation	Time before surgery	Follow-up (months)	Recurrence	Immunostaining positive	Immunostaining negative
1	Pinkard (1996) USA	23/F	Surgical specimen	Resection of all lesions	–	9	None	NR	NR
2	Hainaut (1999) Belgium	29/F	Surgical specimen	Resection of only the main tumor	–	NR	Alive with disease	NR	NR
3	Kawahara (2005) Japan	35/F	Surgical specimen	Resection of all lesions	2 years	NR	NR	Vimentin, factor XIIIa	SMA, CD34, S-100, desmin, AE-1/AE-3, ALK
4	Shibata (2008) Japan	52/F	Needle biopsy	Resection of only the main tumor	–	4	Alive with disease	Vimentin	CD34, factor XIIIa, SMA, desmin, CD68, beta-cell CLL/lymphoma 2
5	Suh (2008) Korea	35/M	Surgical specimen	Resection of all lesions	–	16	None	Vimentin, SMA, CD34	desmin, ALK
6	Present case Japan	40/M	Needle biopsy	Resection of all lesions	2 years	28	None	Osteopontin, vimentin, CD68	CD34, S-100, AE-1/AE-3, EMA, CEA

NR, not reported; SMA, smooth muscle actin; ALK, anaplastic lymphoma kinase; CLL, chronic lymphocytic leukemia; EMA, epithelial membrane antigen; CEA, carcinoembryonic antigen

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Surgical treatment for gastrointestinal metastasis of non-small-cell lung cancer after pulmonary resection

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Abstract

Purpose. Gastrointestinal metastasis is not common in recurrent non-small-cell lung cancer (NSCLC) patients. There is thus limited information on clinical outcome for these patients. This report presents the clinical characteristics and outcomes of patients with gastrointestinal metastasis after pulmonary resection.

Methods. The study retrospectively analyzed nine NSCLC patients with gastrointestinal metastases.

Results. Gastrointestinal metastases were observed in the small intestine ($n = 4$), colon or rectum ($n = 4$), and stomach ($n = 1$). All of the patients were symptomatic. The median survival after gastrointestinal recurrence was 10.8 months. Gastrointestinal surgery was performed in five patients, whereas no cancer treatment was indicated in the remaining four patients. Three patients who underwent surgery for a solitary metastasis survived for more than 2 years after surgery with no other recurrence.

Conclusion. Surgical resection of gastrointestinal metastasis is indicated not only for symptom relief but also for providing a potentially long-term survival if the patients are properly selected.

Key words Gastrointestinal metastasis · Non-small-cell lung cancer · Surgery

Introduction

Surgical resection is the most effective treatment for early-stage non-small cell lung cancer (NSCLC), and it can provide the maximum opportunity for cure and improved survival. Despite complete surgical resection, however, 50%–60% of patients with stage I–IIIA NSCLC relapse and die from their lung cancer.¹ Once the disease has recurred, it is seldom curable.² The most common sites of recurrence are the regional lymph nodes, lung, liver, bone, brain, and adrenal glands.³

The gastrointestinal tract is one of the target organs of metastatic disease in NSCLC patients. Gastrointestinal metastasis often produces serious symptoms that impair the patients' quality of life, such as intestinal obstruction, gastrointestinal discomfort, hemorrhage, and abdominal pain.⁴ It also may cause life-threatening events.

The principal strategy for metastatic disease in distant organs is chemotherapy because the disease is recognized as a systemic one. The physicians often hesitate to perform chemotherapy in patients with gastrointestinal metastasis because the treatment itself may increase the risk of perforation or bleeding. Meanwhile, surgical resection can be considered to prevent such a life-threatening event. According to previous reports, locoregional therapy such as surgery and radiotherapy yield excellent outcomes as well as symptom controls in a subset of patients with brain or adrenal gland metastases.^{5,6} Surgical treatment for gastrointestinal metastasis has not been well described.

This report presents the clinical characteristics and outcomes of the patients with gastrointestinal metastasis after pulmonary resection.

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Patients and methods

Patients

A total of 1552 patients underwent surgical treatment for NSCLC at the Osaka Medical Center for Cancer and Cardiovascular Diseases between January 1988 and December 2007. The institutional database included clinicopathological information and the postoperative clinical course, which includes the recurrence sites. The database was examined retrospectively, and the patients who had recurrence of the disease in gastrointestinal organs were identified to select the optimal patients for this study. Patients who underwent palliative or incomplete lung surgery for intrathoracic diseases were excluded. Patients were also excluded if they had any other metastatic disease before the gastrointestinal metastasis. However, patients with other metastatic foci were included if they were found at the same time as the gastrointestinal metastases or had been well controlled before the pulmonary resection, such as solitary brain metastasis. The performance status (PS) was evaluated according to Eastern Cooperative Oncology Group (ECOG) guidelines. The primary variables were patient age, sex, symptoms, stage at the time of pulmonary resection, histology, metastatic sites, and the interval between pulmonary resection and diagnosis of gastrointestinal metastasis.

Diagnosis of gastrointestinal metastasis

Patients were typically scheduled for clinic visits at 3-month intervals for the first 2 years after surgery. Patients suspected to have gastrointestinal recurrence during the follow-up period were instructed to undergo chest/abdominal computed tomography (CT), an endoscopic examination, and a histological examination with endoscopic guidance. In addition, the patients were examined before determining the treatment strategy if they had a metastatic lesion other than in the gastrointestinal tract; they were subjected to magnetic resonance imaging (MRI) of the brain and bone scintigraphy or ¹⁸F-fluorodeoxyglucose positron emission tomography (FDG-PET). The interval from the date of pulmonary resection to the date of the diagnosis of gastrointestinal recurrence was recorded. The survival time was measured from the date of the diagnosis of the gastrointestinal metastasis to the date of the most recent follow-up examination or the date of death.

Statistical analysis

The statistical analysis was performed using the StatView version 5.0 software package (Abacus Concepts,

Berkeley, CA, USA). The overall survival curves were estimated using the Kaplan-Meier technique.

Results

Patient characteristics

Gastrointestinal metastasis was recorded in nine patients (0.58%). The clinical information for each patient is summarized in Table 1. Two patients with solitary brain metastasis (cases 6, 8), that were well controlled after either stereotactic radiosurgery or surgical removal, were included. One patient (case 3) received adjuvant chemotherapy after pulmonary resection.

Symptoms and diagnosis of gastrointestinal metastasis

All of the patients had gastrointestinal symptoms (Table 1). Gastrointestinal metastases were observed in the small intestine ($n = 4$), colon or rectum ($n = 4$), and stomach ($n = 1$). More than two metastatic tumors in gastrointestinal organs were observed simultaneously in two patients (cases 4, 7). A histological diagnosis of recurrent NSCLC was obtained by endoscopic biopsy in three patients (cases 1, 6, 7), by surgical specimens in four patients (cases 2–5), or by autopsy (cases 7, 8). In most of the patients, the histological type was large-cell carcinoma or pleomorphic carcinoma. The diagnosis was not difficult because these histological types are rare as a primary gastrointestinal malignancy. One patient (case 9) was diagnosed in a different hospital by radiological examinations and underwent treatment there. Although histological examination was not obtained for this patient, the gastrointestinal metastasis from lung cancer may be a valid diagnosis according to the report from the hospital.

Multiple metastatic diseases were detected in six patients by systematic examination, but no intrathoracic recurrences (e.g., lymph node or pulmonary metastases) were observed in any of the patients.

Treatment

Gastrointestinal surgery was performed in five patients (cases 1–5). The interval from the pulmonary resection to the gastrointestinal metastasis was 1.2–19.6 months (median 6.9 months). Surgical procedure included resection of the intestinal metastasis with immediate anastomosis in all patients. The recurrent disease was completely removed in three patients (cases 1–3) who had a solitary metastasis. No cancer treatment was indicated owing to poor performance status in three (cases 6–8) of the four

Table 1 Clinicopathological findings in nine patients with gastrointestinal metastases

No.	Age ^a sex	His.	stage	GI metastatic site	Metastasis other than GI organs	Symptoms	PS	Treatment	Interval ^b (months)	Survival ^c (months)	Outcome
1	66/F	Pleo	IIIB	Colon	None	Melena	0	Surgery	6.7	40	AWD
2	68/M	Large	IIIB	Small intestine	None	Anemia	0	Surgery	9.5	93.6	AWD
3	70/F	Por Ad	IA	Rectum	None	Melena	1	Surgery	19.5	46.9	DOC
4	48/M	Large	IIB	Small intestine	Adrenal	Melena, vomiting	2	Surgery	9.4	14.9	DOD
5	83/M	Pleo	IIB	Colon	Liver	Abdominal mass	0	Surgery	6.9	3.7	DOD
6	51/M	Large	IV	Stomach	Skin (multiple)	Anemia	3	Supportive cares	2.2	0.4	DOD
7	57/M	Pleo	IIIB	Small intestine, Stomach	Liver, Kidney	Anemia, Ileus	2	Supportive cares	1.2	1.1	DOD
8	69/M	Por Ad	IV	Colon	Adrenal, brain	Unknown	2	Supportive cares	14.3	0.2	DOD
9	70/M	Large	IB	Small intestine	Adrenal, Pancreas	Melena	0	Supportive cares	4	10.8	DOD

GI, gastrointestinal; His, histology; por, poorly differentiated; Ad, adenocarcinoma; Large, large cell carcinoma; Pleo, pleomorphic carcinoma; AWD, alive without disease; DOD, dead of disease; DOC, dead of other causes

^aAge when GI metastasis was diagnosed

^bInterval from pulmonary resection to GI metastasis

^cSurvival after gastrointestinal metastases

patients who did not undergo surgery, and the fourth (case 9) had multiple metastases diagnosed within a short interval after pulmonary resection.

Symptom control and survival after gastrointestinal metastasis

The median survival of all nine patients after gastrointestinal recurrence was 10.8 months. The overall survival rates at 1 and 3 years after recurrence were 44.4% and 33.3%, respectively (Fig. 1). Three patients (cases 1–3) who underwent surgery for a solitary metastasis had not developed any other metastasis after surgery for more than 2 years. Two patients (cases 4, 5) who had undergone surgery to control the symptoms associated with gastrointestinal metastasis died of the disease within a year after surgery; however, the symptoms subsided after surgery, and the patients were able to enjoy oral food intake for several months. Interestingly, no additional gastrointestinal recurrence was observed in the patients who underwent surgery for the first recurrence. In contrast, four patients who did not undergo gastrointestinal surgery died less than a year after their gastrointestinal metastases were diagnosed.

Discussion

In one study, the most common site of first recurrence was the intrathoracic lesion in 44% of recurrent NSCLC

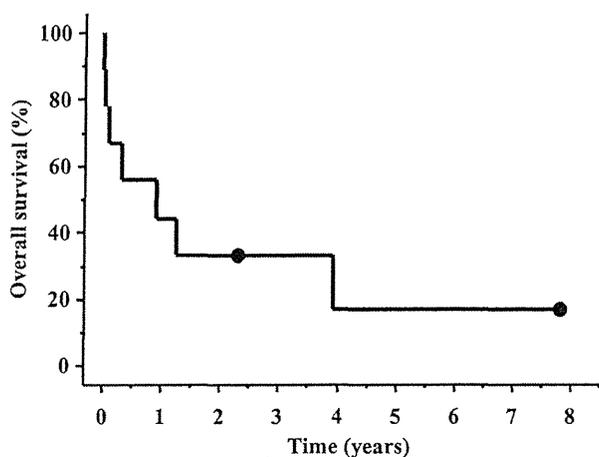


Fig. 1 Kaplan-Meier curve for the overall survival of patients with gastrointestinal metastasis from non-small-cell lung cancer

patients, the extrathoracic lesion in 44%, and combined intrathoracic and extrathoracic lesions in 12%.⁷ The common extrathoracic metastatic sites are brain (32%), bone (23%), liver (9%), and adrenal gland (6%). The occurrence rate of gastrointestinal metastasis was 0.19% of all lung cancer patients⁸ and 0.5% of patients who were treated surgically⁹ in previous studies. Gastrointestinal metastasis is seen in only a small proportion of the population of NSCLC patients with recurrence, but it is important in clinical practice. Gastrointestinal recur-

rence may cause abdominal symptoms such as pain or distention, gastrointestinal obstruction, and bleeding, which are symptoms not usually associated with thoracic disease. Gastrointestinal metastasis from lung cancer may occur at the late or terminal stage over the course of the disease.¹⁰ Therefore, most patients with gastrointestinal metastasis receive only palliative or supportive care.

The current study selected patients who were considered potential candidates for treatment of their gastrointestinal metastases. The occurrence rate was 0.58%. All patients were symptomatic, and the clinical symptoms due to gastrointestinal recurrence appeared within 20 months of pulmonary resection. The median interval from pulmonary resection to gastrointestinal metastasis was 6.9 months, and the median survival was 10.8 months in this study. The disease-free interval from complete resection to any kind of recurrence was reported to be 11.5 months in one study⁷ and 12.2 months in another.¹¹ Gastrointestinal metastasis may therefore tend to develop earlier than metastases at other sites.

Gastrointestinal metastases may occur with any kind of NSCLC histology. Large-cell carcinoma and squamous cell carcinoma are relatively common.^{12,13} There were three cases of pleomorphic carcinoma histology in the current series. This should be noted because pleomorphic carcinoma is a rare type of histology, being found in approximately 1.3% of all surgical patients with primary NSCLC in the database. The clinical behavior of pleomorphic carcinoma is known to be aggressive, with frequent distant metastases. Gastrointestinal organs should be taken into consideration as possible metastatic site in patients with pleomorphic carcinoma.

The most important treatment option for recurrent NSCLC is chemotherapy. Nevertheless, chemotherapy was not indicated for the gastrointestinal recurrence in any patients in this study. Chemotherapy was thought to increase the risk of gastrointestinal perforation and bleeding. In fact, a case of intestinal perforation that may have resulted from necrosis of the tumor caused by chemotherapy was described in a series of small bowel metastases from primary lung carcinoma.¹⁴

We considered surgery an option to manage gastrointestinal metastasis. There are two principal objectives of such surgery. One is to treat the disease, and the other is to relieve the symptoms associated with the metastasis. The clinical impact of surgical resection of a gastrointestinal metastasis has not been thoroughly discussed. Five patients underwent gastrointestinal surgery in this study. It should be emphasized that three patients are experiencing long survivals after resection without another recurrence. In addition, the gastrointestinal symptoms in the remaining two patients were well controlled after surgery. In contrast, four patients who did not undergo surgery had a shorter survival after the diagnosis of gastrointestinal metastasis. Notably, three died within only 2 months. Therefore, resection of gastrointestinal metastasis may provide benefits in terms of both symptom control and survival if the patients are properly selected.

The long-term survivors in this study had a metachronous solitary metastasis after a relatively long interval (≥ 6 months) between pulmonary resection and discovery of the gastrointestinal metastasis. There are two small clinical series that included patients who underwent resection of a gastrointestinal metastasis (Table 2). Three long-term survivors after resection were described in those reports. In contrast to the survivors in the current study, one of those patients had synchronous gastrointestinal metastases and two had multiple metastatic lesions outside the gastrointestinal tract. These conflicting data indicated that a disease-free interval or the number of metastatic foci was not always a predictor for long survival after resection. More clinical information is therefore necessary to identify indicators to select optimal patients for resection of gastrointestinal metastasis.

Conclusion

Although an initial recurrence of NSCLC after complete resection rarely develops in gastrointestinal organs, it

Table 2 Summary of previous and present reports in gastrointestinal metastases

Study	No. of cases	Surgery for GI metastasis (yes/no)	Interval between diagnosis of lung cancer and discovery of GI metastasis (mean)	Median Survival (median)	Survival duration after surgery	Long-survivors after GI surgery
Berger (1997)	7	6/1	11.2 months	120.0 days	7.5 months	Alive at 22 months ($M = 1$)
Kim (2009)	10	6/4	147.0 days	96.5 days	17.5 months	Alive at 33 and 63 months ($M = 2$)
Present report	9	5/4	6.9 months	10.8 months	27.7 months	Shown in Table 1

may nevertheless cause life-threatening symptoms and lead to shortened survival. Surgical resection of gastrointestinal metastasis is indicated not only for symptom relief but for providing the potential of a long-term survival if the patients are properly selected.

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Hepatocyte Growth Factor Expression in *EGFR* Mutant Lung Cancer with Intrinsic and Acquired Resistance to Tyrosine Kinase Inhibitors in a Japanese Cohort

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Introduction: This study was performed to determine the incidence rates of resistance factors, i.e., high-level hepatocyte growth factor (HGF) expression, epidermal growth factor receptor (EGFR) T790M secondary mutation, and *MET* amplification, in tumors with intrinsic and acquired EGFR tyrosine kinase inhibitor (TKI) resistance in *EGFR* mutant lung cancer.

Methods: Ninety-seven specimens from 93 *EGFR* mutant lung cancer patients (23 tumors with acquired resistance from 20 patients, 45 tumors with intrinsic resistance from 44 patients [nonresponders], 29 sensitive tumors from 29 patients) from 11 institutes in Japan were analyzed. HGF expression, *EGFR* T790M secondary mutation,

and *MET* amplification were determined by immunohistochemistry, cycleave real-time polymerase chain reaction, and fluorescence in situ hybridization, respectively.

Results: High-level HGF expression, *EGFR* T790M secondary mutation, and *MET* amplification were detected in 61, 52, and 9% of tumors with acquired resistance, respectively. High-level HGF expression was detected in 29% of tumors with intrinsic resistance (nonresponders), whereas *EGFR* T790M secondary mutation and *MET* amplification were detected in 0 and 4%, respectively. HGF expression was significantly higher in tumors with acquired resistance than in sensitive tumors ($p < 0.001$, Student's *t* test). Fifty percent of tumors with acquired resistance showed simultaneous HGF expression with *EGFR* T790M secondary mutation and *MET* amplification.

Conclusions: High-level HGF expression was detected more frequently than *EGFR* T790M secondary mutation or *MET* amplification in tumors with intrinsic and acquired EGFR-TKI resistance in *EGFR* mutant lung cancer in Japanese patients. These observations provide a rationale for targeting HGF in EGFR-TKI resistance in *EGFR* mutant lung cancer.

Key Words: EGFR-TKI, EGFR mutation, HGF, Acquired resistance, Intrinsic resistance.

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Epidermal growth factor receptor (EGFR)-activating mutations, in-frame deletion in exon 19, and L858 point mutation in exon 21 are selectively expressed in a population with lung cancer.^{1,2} *EGFR*-activating mutations are detected considerably more frequently in nonsmokers, females, adenocarcinomas, and patients from East Asia, including Japan.^{3–5} The reversible EGFR tyrosine kinase inhibitors (EGFR-TKIs) gefitinib and erlotinib show dramatic therapeutic efficacy, response rates of 70 to 80%, and significant prolongation of progression-free survival (PFS) compared

with standard first-line cytotoxic chemotherapy in patients with *EGFR* mutant lung cancer.^{6–9} However, patients almost always develop acquired resistance to EGFR-TKIs after varying periods.^{6,9,10} In addition, 20 to 30% of patients with *EGFR*-activating mutations show intrinsic resistance to EGFR-TKIs.⁴ Therefore, intrinsic and acquired resistance to EGFR-TKIs are major problems in management of *EGFR* mutant lung cancer.

Two genetically conferred mechanisms—*EGFR* T790M secondary mutation (T790M secondary mutation)^{11,12} and *MET* gene amplification¹³—induce acquired resistance to EGFR-TKIs in *EGFR* mutant lung cancer. In addition, we recently demonstrated the occurrence of hepatocyte growth factor (HGF)-induced resistance.¹⁴ HGF, a ligand of MET,¹⁵ induces EGFR-TKI resistance by activating MET, which restores phosphorylation of downstream MAPK-ERK1/2 and PI3K-Akt pathways,¹⁴ using Gab1 as an adaptor.¹⁶ HGF may be involved in both intrinsic and acquired resistance to EGFR-TKIs in *EGFR* mutant lung cancer.¹⁴

T790M secondary mutation, *MET* amplification, and high-level HGF expression were detected in clinical specimens from *EGFR* mutant lung cancer patients who acquired resistance to EGFR-TKIs,^{11–14,16–18} indicating the clinical relevance of all three resistance mechanisms in lung cancer. Although the number of cases in each study was limited (<30 cases/study), probably because of low availability of biopsy specimens from resistant tumors, *EGFR* T790M secondary mutation and *MET* amplification were estimated to have occurrence rates of 50%^{11,12,17,19} and up to 20%,^{13,16,17} respectively, in patients showing acquired resistance to EGFR-TKIs. Nevertheless, the incidence of HGF-induced resistance has not been determined. In addition, the incidence rates of these three resistance factors in intrinsic resistance (nonresponders) are unknown.

Here, we performed a large-scale study in 23 tumors with acquired resistance from 20 patients, 45 tumors with intrinsic resistance from 44 patients (nonresponders), and 29 sensitive tumors from 29 patients to determine the incidences of the three resistance factors not only in acquired resistance but also in intrinsic resistance (nonresponders) to EGFR-TKIs in Japanese patients with *EGFR* mutant lung cancer.

MATERIALS AND METHODS

Patient details are described in the Supplementary information (<http://links.lww.com/JTO/A197>).

Definition of Sensitivity to EGFR TKI

Here, tumors with *EGFR* mutation known to be associated with drug sensitivity (i.e., G719X, exon 19 deletion, and L858R) were obtained from patients before or after treatment with a single EGFR-TKI.⁹

Sensitive tumors were defined as those obtained from patients whose tumors showed a decrease in diameter of at least 30% (either documented partial response or complete response) associated with EGFR-TKI treatment in imaging studies (Response Evaluation Criteria in Solid Tumors [RECIST] version 1.0). Tumor specimens were obtained before EGFR-TKI treatment.

Tumors with acquired resistance were defined as described previously.⁹ Briefly, cases showing objective clinical benefit from treatment with an EGFR TKI as defined by either documented partial or complete response (RECIST) or significant and durable (>6 months) clinical benefit (stable disease as defined by RECIST) and systemic progression of disease (RECIST), while on continuous treatment with gefitinib or erlotinib within the last 30 days were defined as showing acquired resistance. Tumor specimens were obtained after systemic progression of disease.

As intrinsic resistance (nonresponders) has not been clearly defined, tumors without response to treatment with an EGFR TKI, i.e., either documented stable disease or progressive disease (RECIST), were defined as showing intrinsic resistance (nonresponders). Tumor specimens were obtained either before or after EGFR-TKI treatment.

Patients

Ninety-seven tumor specimens with EGFR mutations were obtained from 93 lung cancer patients, all of whom provided written informed consent, at 11 institutes in Japan. This study was approved by the Institutional Review Boards of each institute.

Patients' characteristics are shown in Table 1. Eighty-seven patients had adenocarcinomas, one had large cell carcinoma, two had squamous cell carcinoma, two had adenosquamous carcinoma, and one had undifferentiated non-small cell carcinoma. As the first EGFR-TKI, gefitinib and erlotinib were given to 82 and 10 patients, respectively, and the dual inhibitor of EGFR and VEGFR2, vandetanib,²⁰ was given to 1 patient.

Exon 19 deletion and L858R point mutation in exon 21 of *EGFR* were detected in 40 and 57 of the 97 tumors, respectively (Table 1). Two of these tumors had both exon 19 deletion and L858R point mutation. Two tumors without exon 19 deletion or L858R had G719X. Twenty-three tumors with acquired resistance were obtained from 20 patients after EGFR-TKI treatment. Forty-five tumors with intrinsic resistance (nonresponders) were obtained from 44 patients either before (41 tumors from 41 patients) or after (four tumors from three patients) EGFR-TKI treatment. Twenty-nine sensitive tumors were obtained from 29 patients before EGFR-TKI treatment.

Immunohistochemistry for HGF

Immunohistochemical staining was conducted on formalin-fixed, paraffin-embedded tissue sections (4 μm thick) of tumor specimens with microwave antigen retrieval in 0.01 M citrate buffer (pH 6.0). We used rabbit polyclonal antibody against HGF-α (IBL, Gunma, Japan) at 1:20 dilution as a primary antibody and EnVision/HRP Polymer Reagent (Dako, Glostrup, Denmark) and DAB (3,3'-diaminobenzidine tetrahydrochloride) Liquid (Dako) for detection.

Evaluation of HGF Expression

The percentages of cancer cells with positive cytoplasmic and/or membrane HGF immunoreactivity were evaluated (0 to 100%), and the modal intensity of the positively staining cells on a scale ranged from 0 to 3+ (0, complete

TABLE 1. Patient Characteristics

Number of Patients	Acquired Resistance (n = 20)	Intrinsic Resistance (n = 44)	Sensitive (n = 29)	Total (n = 93)
Age				
Median	59.5	65.5	65	64
Range	32–85	34–76	42–86	32–86
Gender				
Male	6	26	10	42
Female	14	18	19	51
Smoking history				
Former/current Smoker	3	21	11	35
Never smoker	17	23	18	58
Histological type				
Adeno	19	39	29	87
Large cell	0	1	0	1
Squamous cell	0	2	0	2
Undifferentiated non-small cell carcinoma, or adenosquamous	1	2	0	3
EGFR-TKI treatment				
Gefitinib	19	36	27	82
Erlotinib	1	7	2	10
Vandetanib	0	1	0	1
Number of Tumors	n = 23	n = 45	n = 29	n = 97
EGFR mutation status				
Exon 19 deletion	12	14 ^a	14 ^a	40
L858R	11	30	16	57
G719X	0	2	0	2

^a One patient's tumor had both exon 19 deletion and L858R point mutation.

absence of staining; 1+, weaker staining than normal bronchial epithelium; 2+, similar staining to normal bronchial epithelium; and 3+, clearly more intense staining than normal bronchial epithelium) (Supplementary Figure 1, <http://links.lww.com/JTO/A197>). The percentage and intensity were multiplied to give a scoring index (*H* score) ranging from 0 to 300, according to a previously reported method with minor modifications.¹⁶ Turke et al.¹⁶ reported that HGF expression was significantly higher in specimens with acquired resistance (mean \pm SD: 205 \pm 106) compared with pretreatment (126 \pm 112). On additional evaluation with specimens showing acquired resistance from patients whose tumors were obtained only after acquiring EGFR-TKI resistance, HGF expression was similar (176 \pm 126) to that of specimens with acquired resistance in patients with paired tumor specimens; they concluded that these findings with clinical specimens supported the suggestion that HGF mediated resistance to EGFR-TKIs. Therefore, we defined high-level HGF expression as *H* score \geq 200 in this study. Evaluation was performed independently by two investigators (KT and MN) blinded to individual clinical information.

Cycleave Real-Time Polymerase Chain Reaction Assay for T790M Mutation

Details of the cycleave real-time polymerase chain reaction (PCR) assay have been described previously.²¹

Briefly, tumor cell-rich areas in hematoxylin and eosin-stained sections were marked under a microscope, and tissues were scratched from the area of another deparaffinized unstained section. Pieces of the scratched tissue were incubated with 1 \times PCR buffer containing 100 μ g/mL proteinase K for 1 hour at 54°C. After heat inactivation at 95°C for 3 minutes, the solution was used directly as the template DNA for the assay. Then, exon 20 of the *EGFR* gene was amplified by real-time quantitative PCR assay on a SmartCycler (Cepheid, Sunnyvale, CA) using Cycleave PCR Core kits (TaKaRa Co. Ltd., Ohtsu, Japan) with a T790M-specific cycling probe and a wild-type cycling probe. This assay detected as few as 5% cancer cells with T790M mutation in a background of cells with wild-type T790M in *EGFR*.

MET Amplification

Formalin-fixed, paraffin-embedded tissue sections (4 μ m thick) were subjected to dual-color fluorescence in situ hybridization using a MET/CEP7 probe cocktail (Kreatech Diagnostics, Amsterdam, The Netherlands) according to the manufacturer's instructions. Staining was evaluated as reported previously.^{22,23}

Statistical Analysis

Statistical significance was determined by Student's *t* test. All statistical analyses were performed using GraphPad

TABLE 2. Expression of HGF, T790M Secondary Mutation, and *MET* Amplification in EGFR-TKI-Resistant Tumors Obtained from *EGFR* Mutant Lung Cancer Patients

	Acquired Resistance (n = 23)	Intrinsic Resistance (n = 45)	Sensitive (n = 29)
High-level HGF expression	14 (61%)	13 ^a (29%)	3 ^b (10%)
<i>EGFR</i> T790M secondary mutation	12 (52%)	0	0
<i>MET</i> amplification	2 (9%)	2 (4%)	0

^a High-level HGF expression was detected in the stroma in two patients.

^b High-level HGF expression was detected in the stroma in one patient.

Prism Ver. 4.01 (GraphPad Software, Inc., San Diego, CA). All tests were two sided, and $p < 0.05$ was taken to indicate statistical significance.

RESULTS

HGF Expression, T790M Secondary Mutation, and *MET* Amplification in Tumors with Acquired Resistance

Among 23 tumors with acquired resistance from 20 patients, *EGFR* T790M secondary mutation was detected in 12 tumors (52%) from 11 patients (60%) (Table 2). *MET* amplification was detected in two tumors (9%) from two patients (10%). As HGF is a soluble cytokine, evaluation of HGF is not as simple as that for genetically conferred T790M secondary mutation and *MET* amplification, which can be designated as plus or minus. As described in the Materials and Methods section, we defined high-level HGF expression as H score ≥ 200 in this study. High-level HGF expression was detected in 14 tumors (61%) from 13 patients (60%). In these 14 tumors, HGF was predominantly expressed in cancer cells.

The high HGF expression was simultaneously detected in 6 of 12 tumors positive for T790M secondary mutation (50%) (Table 3, Figure 1). High-level HGF expression was also detected simultaneously in one of two tumors positive for *MET* amplification (50%). These results suggested possible interactions among these three resistance factors, consistent with previous reports.^{16,17}

Expression of HGF, T790M Secondary Mutation, and *MET* Amplification in Tumors with Intrinsic Resistance (Nonresponders)

T790M secondary mutation was not detected in 45 tumors with intrinsic resistance from 44 patients (nonresponders), but *MET* amplification was detected in two tumors (4%) (Table 2). *EGFR* D761Y secondary mutation was detected in two tumors (4%) from one patient²⁴ (Supplementary Table 1, <http://links.lww.com/JTO/A197>). In contrast, high-level HGF expression in cancer cells was detected in 11 tumors (24%) from 11 patients. In addition, HGF was detected at high levels in stromal cells in two tumors (4%) from two patients (data not shown). In total, high-level HGF expression was detected in 13 tumors with intrinsic resistance

(29%). Notably, high-level HGF expression was simultaneously detected in one of two *MET* amplification-positive tumors (50%) (Table 2). These results suggested the involvement of HGF in intrinsic resistance to EGFR-TKIs in *EGFR* mutant lung cancer in Japanese patients.

Expression of HGF, T790M Secondary Mutation, and *MET* Amplification in Sensitive Tumors

Neither *EGFR* T790M secondary mutation nor *MET* amplification was detected in 29 sensitive tumors from 29 patients. High-level HGF expression was detected in two tumors (7%) (Supplementary Table 2, <http://links.lww.com/JTO/A197>). High levels of HGF were detected in stromal cells in one tumor (3%). In total, a high level of HGF expression was detected in three sensitive tumors (10%). Thus, although high HGF expression level was detected even in sensitive tumors, the incidence of high HGF expression was much lower in sensitive tumors than in those with acquired or intrinsic resistance. In addition, mean H score of HGF in tumors with acquired resistance was significantly higher than that in sensitive tumors ($p < 0.001$, Student's t test) (Figure 2). There was no significant difference in mean H score of HGF between tumors with intrinsic resistance (nonresponders) and sensitive tumors.

DISCUSSION

Our previous studies^{14,25,26} documented HGF-mediated resistance to EGFR-TKIs in *EGFR* mutant lung cancer, which was also confirmed by other groups.^{16,27} Here, we demonstrated that a high level of HGF expression was detected most frequently in tumors with intrinsic and acquired resistance to EGFR-TKIs in *EGFR* mutant lung cancer in Japanese patients. Our data indicated that although T790M secondary mutation and *MET* amplification are predominantly responsible for acquired resistance, HGF may be responsible not only for acquired resistance but also for intrinsic resistance to EGFR-TKIs.

The mechanism of intrinsic resistance to EGFR-TKIs is not well understood. To our knowledge, this is the first study with more than 40 clinical specimens indicating the incidence of resistance factors in intrinsic resistance to EGFR-TKIs in *EGFR* mutant lung cancer. Here, we found that a high level of HGF expression was most frequently (29%) detected in tumors with intrinsic resistance, compared with T790M secondary mutation (0%) and *MET* amplification (4%). It is noteworthy that although the high HGF expression level was detected in cancer cells in tumors with acquired resistance, HGF expression was detected in both cancer cells (10/12 tumors) and host stroma cells (2/12 tumors) in tumors with intrinsic resistance (nonresponders). HGF was reported to be produced by not only cancer cells but also stromal cells.¹⁵ Our data clearly indicated that both cancer cells and stromal cells are sources of HGF, which induces intrinsic EGFR-TKI resistance in *EGFR* mutant lung cancer. As HGF-induced resistance could be reversed by anti-HGF antibody and the natural HGF inhibitor NK4,^{25,27} highly produced HGF in

TABLE 3. Summary of Tumors with Acquired Resistance

ID	Gender	Histological Type	EGFR Mutation Status	Treatment	BOR	PFS	HGF	T790M	MET Amplification
KZ-1	M	Ad	Exon 19 del	Erlotinib	PR	254	60	—	+
KZ-2	F	Ad	L858R	Gefitinib	CR	1041	40	—	—
KZ-3	F	Ad	L858R	Gefitinib	PR	366	200	—	—
OK1—1	M	Ad	Exon 19 del	Gefitinib	PR	351	290	—	—
OK1—2							300	—	—
OK4—2	F	Ad	Exon 19 del	Gefitinib	PR	57	210	+	—
TS-1—3	F	Ad	L858R	Gefitinib	PR	180	90	—	—
TS-1—4							280	+	—
SG2	M	Ad	Exon 19 del	Gefitinib	PR	174	150	+	—
SG3	F	Ad	L858R	Gefitinib	SD	368	110	+	—
SG4	F	Ad	L858R	Gefitinib	PR	60	220	—	+
SG6	M	Ad	Exon 19 del	Gefitinib	PR	352	140	+	—
SG8	F	Ad	L858R	Gefitinib	SD	210	90	+	—
SG9	F	Ad	Exon 19 del	Gefitinib	SD	221	200	+	—
SG10	F	Ad	L858R	Gefitinib	CR	210	210	—	—
TB1—2	M	Ad	Exon 19 del	Gefitinib	PR	1770	230	+	—
TB2—2	F	AdSq	Exon 19 del	Gefitinib	PR	300	300	—	—
AC29—1	M	Ad	L858R	Gefitinib	PR	533	250	—	—
AC29—2							270	+	—
AC24	F	Ad	Exon 19 del	Gefitinib	PR	98	170	+	—
AC26	F	Ad	Exon 19 del	Gefitinib	SD	448	180	+	—
AC28	F	Ad	Exon 19 del	Gefitinib	PR	357	200	+	—
AC31	F	Ad	L858R	Gefitinib	PR	894	200	—	—

Ad, adeno; AdSq, adenosquamous; BOR, best overall response.

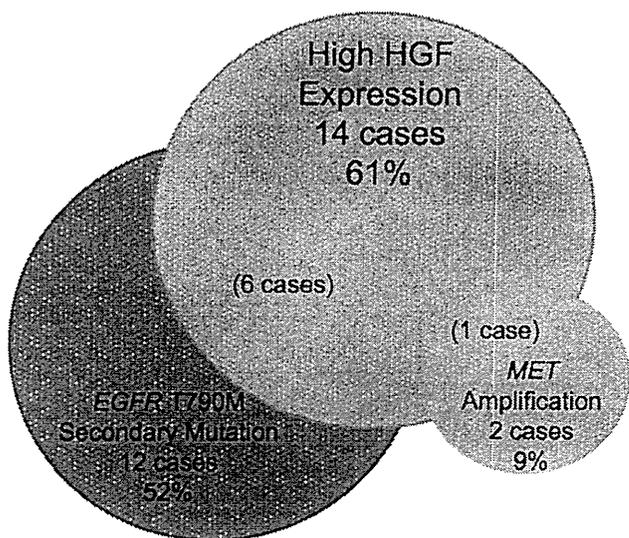


FIGURE 1. Incidences of high-level HGF expression, T790M secondary mutation, and MET amplification in 23 tumors with acquired resistance. Values in parentheses are the numbers of cases in which the tumors expressed two resistance factors simultaneously.

resistant tumors would be an ideal therapeutic target regardless of its origin.

It was of interest that a high level of HGF expression was detected in a small population of sensitive tumors. This

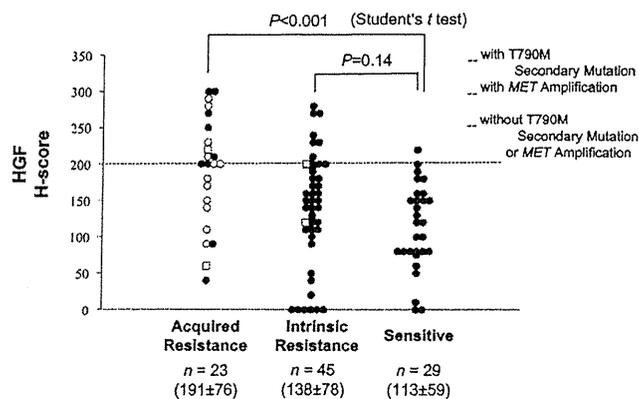


FIGURE 2. HGF expression score (H score) in EGFR-TKI-resistant tumors obtained from EGFR mutant lung cancer patients. Values in parentheses are mean ± SD of H score.

was consistent with a previous report¹⁶ indicating high-level HGF expression (H score ≥200) in several specimens from responders. Although the reason for the high level of HGF expression in tumors from responders is unclear at present, there are several possible explanations as follows. First, although HGF was expressed at high levels, natural inhibitors such as cleaved HGF and truncated MET, both of which inhibit binding of HGF to MET, may be generated in the tumors.^{28,29} Second, negative regulators of MET tyrosine kinase activity such as protein kinase C may be activated and negate the effect of HGF on induction of EGFR-TKI resis-

tance in these tumors.³⁰ As the amounts of each clinical specimen were limited, we would like to perform further analyses in future studies should sufficient amounts of specimens become available.

Recent studies indicated that multiple resistance factors can be induced simultaneously in a single cancer. For example, Qi et al.³¹ reported the simultaneous occurrence of *Met* mutation and activation of the EGFR pathway by ligand overexpression, similar to T790M mutation and HGF overexpression in EGFR mutant lung cancer, which caused resistance to Met-TKIs in gastric cancer. Katayama et al.³² also reported that *ALK* gene amplification and gatekeeper mutation in *ALK* occurred simultaneously and conferred resistance to ALK inhibitors in EML4-ALK lung cancer. In this study, T790M secondary mutation and the high HGF expression level were simultaneously detected at high incidence (50%) in tumors with acquired resistance. Irreversible EGFR-TKIs were thought to have potential to control acquired resistance caused by T790M secondary mutation, but clinical responses were rarely observed in clinical trials.^{33,34} We recently found that HGF induces resistance to not only reversible EGFR-TKIs but also irreversible EGFR-TKIs by activating the MET/PI3K/Akt pathway in *EGFR* mutant lung cancer cells with or without T790M secondary mutation.²⁶ Taken together, these observations suggest that HGF would be simultaneously expressed with T790M secondary mutation in tumors with acquired resistance and reduce the sensitivity to irreversible EGFR-TKIs in *EGFR* mutant lung cancer patients.

MET amplification has been detected in ~20% of tumors with acquired resistance to EGFR-TKIs in *EGFR* mutant lung cancer,^{13,16,17} while the incidence reported in Japanese patients is rare.^{14,18} Here, we detected *MET* amplification in two tumors (9%) with acquired resistance, suggesting that *MET* amplification can be detected in a significant proportion of tumors with acquired resistance even in Japanese patients. One case with high-level HGF expression and *MET* amplification (KZ-1) was treated with gefitinib and PFS was 254 days. The other case with low HGF and *MET* amplification (SG4) was treated with erlotinib and PFS was 60 days (Table 3). Although it is not possible to make definitive conclusions based on the data from only these two cases, the shorter PFS in the former case tentatively supports the observation that HGF accelerates expansion of preexisting clones with *MET* amplification.¹⁶ Notably, simultaneous expression of these two factors was also detected in one tumor with intrinsic resistance (nonresponder). However, the mechanism by which HGF is induced in *EGFR* mutant lung cancer is still not well defined. Further examinations are warranted to elucidate the interaction between HGF expression and *MET* amplification in *EGFR* mutant lung cancer.

Among 68 resistant tumors, high-level HGF expression, T790M secondary mutation, and *MET* amplification were not detected in one tumor with acquired resistance and 31 tumors with intrinsic resistance, indicating the involvement of other mechanisms of resistance in these tumors. *EGFR* D761Y secondary mutation in exon 20 was detected in two tumors from the same patient.²⁴ *EGFR* D761Y mutation

was originally identified in recurrent brain metastasis and was shown to induce intermediate-grade resistance to EGFR-TKIs.³⁵ In addition, rare secondary mutations (other than T790M and D761Y) or a preexisting resistance mutation in a minority of clones may also be involved in intrinsic resistance. Moreover, it was recently reported that a subpopulation of cancer cells that transiently exhibit a distinct phenotype characterized by engagement of IGF-1R activity, hypersensitivity to HDAC inhibition, and altered chromatin showed an intrinsic ability to tolerate exposure to EGFR-TKI.³⁶ Minor secondary mutations, a preexisting resistance mutation in a minority of clones, or chromatin-mediated drug resistance mechanisms may be involved in resistant tumors without high HGF expression, T790M secondary mutation, and *MET* amplification.

To overcome the HGF-induced resistance to EGFR-TKI in *EGFR* mutant lung cancer, double blockade of the EGFR pathway and HGF-MET pathway is therefore theoretically necessary.^{14,16,27} To inhibit mutant EGFR with or without T790M secondary mutation, EGFR mutant-specific inhibitors were developed in addition to irreversible EGFR-TKIs.³⁷ To inhibit HGF-MET signaling, several inhibitors, including anti-HGF antibody, NK4 (natural antagonist of MET), and MET-TKIs, were developed.^{16,25-27} Further studies are essential to determine optimal combined therapy with best efficacy and safety. In addition, a prospective study is required to determine whether immunohistochemical detection of HGF would be sufficiently reliable to identify patients with HGF-induced resistance to EGFR-TKIs. As levels of HGF in peripheral blood are correlated with clinical outcome to EGFR-TKIs in patients with non-small cell lung cancer,^{38,39} such noninvasive methods may facilitate individual therapy for overcoming HGF-induced resistance to EGFR-TKIs in *EGFR* mutant lung cancer patients.

Recent studies indicated at least three important roles of HGF in EGFR-TKI resistance in *EGFR* mutant lung cancer. First, HGF induces resistance to reversible EGFR-TKIs, gefitinib, and erlotinib, by restoring MET/Gab1/PI3K/Akt pathways.^{14,16} Second, HGF accelerates expansion of preexisting *MET*-amplified cancer cells and facilitates *MET* amplification-mediated resistance during EGFR-TKI treatment.¹⁶ Third, after acquiring resistance to reversible EGFR-TKIs, HGF induces resistance of lung cancer cells with T790M secondary mutation to irreversible EGFR-TKIs.²⁴ Here, we detected high-level HGF expression frequently in tumors with intrinsic and acquired resistance to EGFR-TKIs in *EGFR* mutant lung cancer in Japanese patients. These findings indicate the value of HGF as a therapeutic target for EGFR-TKI-resistant *EGFR* mutant lung cancer. Therefore, combined therapy with EGFR-TKIs and HGF-MET inhibitors in patients with HGF-induced resistance may improve the clinical outcome of *EGFR* mutant lung cancer.

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