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Figure legends

Figure 1. A, Flow cytometric profile of BEAMing. The wild-type *EGFR* fragment was mixed with 0.1% of the T790M *EGFR* fragment, and BEAMing was performed after PCR amplification. Horizontal axis, the fluorescence intensity of Alexa 647; vertical axis, the fluorescence intensity of Alexa 488. WT, signals from the wild-type *EGFR* fragment; MT, signals from the T789M fragment. B, Linear correlation between the inoculated amount of the T790M *EGFR* fragment and the BEAMing measurement. Horizontal axis, the fraction of the T790M fragment inoculated into the wild-type fragment; vertical axis, the fraction of the T790M-positive allele measured using BEAMing. C, Repeated measurements of the samples with 0.01% of the T790M fragment and those with no T790M fragment. The vertical axis indicates the fractions of the T790M-positive allele detected using BEAMing.

Figure 2. A, Distribution of the fraction of *EGFR* molecules with activating or resistant (T790M) mutations in the plasma DNA. Horizontal axis, the percentage of activating EGFR mutations; vertical axis, the number of patients. B, Relationship between the amount of recovered plasma DNA and the fraction of activating *EGFR* mutations. Horizontal axis, the amount of recovered plasma DNA (pg) corresponding to 400 µl of plasma; vertical axis, the percentage of activating *EGFR* mutations.

Table 1. Primer list.

	name	Sequence	modification	target
Primers for exon amplification from plasma DNA				
exon19	Tag119del	TCCCGCGAAATTAATACGACAAGTTAAAATTCCCGTCGCTATC		
	Tag219del	GCTGGAGCTCTGCAGCTAGACCCCCACACAGCAAAG		
exon20	Tag1T790M	TCCCGCGAAATTAATACGACGCATCTGCCTCACCTCCAC		
	Tag2T790M	GCTGGAGCTCTGCAGCTAAGCAGGTACTGGGAGCCAAT		
exon21	Tag1L858R	TCCCGCGAAATTAATACGACAGCCAGGAACGTACTGGTGA		
	Tag2L858R	GCTGGAGCTCTGCAGCTATGCCTCCTTCTGCATGGTAT		
Primers for emulsion PCR				
	Tag1 (forward)	TCCCGCGAAATTAATACGAC		
	Tag2 (reverse)	GCTGGAGCTCTGCAGCTA		
hybridization probes for detction of beads with successful amplification				
exon19	19del_BEAM_PE_b	AGCAAAGCAGAAACTCACATC	5`biotin	
exon20	T790M_BEAM_PE_b	CGGACATAGTCCAGGAG	5`biotin	
exon21	L858R_BEAM_PE_b	ATGCCTCCTTCTGCATGGTAT	5`biotin	
hybrization probe for BEAMing				
exon19	19del_35_49_647	GGAGATGTTTTGATAGCG	5`Alexa647	exon 19 E746-A750del
	19del_36_50_647	CGGAGATGTCTTGATAGC	5`Alexa647	exon 19 E746-A750del
	19del_40_57_647	TGGCTTTCGATTCCTTGA	5`Alexa647	exon 19 L747-S752del.P753S
	19del_AATTCC_647	TGTTGCTTCTCTTGGAATT	5`Alexa647	exon 19 E746-L747del.IP
	19del_36_55_T_647	GCTTTCGGAACCTTGATAG	5`Alexa647	exon 19 L747-S752del. E746V
	19del_35_53_ACT_647	GGAGAAGTTTTGATAGCG	5`Alexa647	exon 19 K745-E749del.A750K
	19del_39_56_CAG_647	TTTCGGCTGTTCCTTGAT	5`Alexa647	exon 19 L747-T751del.S752Q
	19del_39_48_C_647	GAGATGTTGGTTCCTTGAT	5`Alexa647	exon 19 L747-E749del.A750P
	19del_WT_488	TGTTGCTTCTCTTAATTCC	5`Alexa488	exon 19 wild type control
exon20	T790M_Mut_BNA_647	atgagctgcAtgatgag	5`Alexa647	T790M mutation
	T790M_WT_BNA_488	tgagctgcGtgatgag	5`Alexa488	wild type control for T790M
exon21	L858R_Mut_LNA_647	gtttggccCgcccaaaat	5`Alexa647	L858R mutation
	L858R_WT_LNA_488	gtttggccAgcccaaaat	5`Alexa488	wild type control for L858R
	T2582A_Mut_647	cacccagcTgtttggcc	5`Alexa647	T2582A mutation
	T2582A_WT_488	cacccagcAgtttggcc	5`Alexa488	wild type control for T2582A

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Table 2. Allele frequency of activating and resistant EGFR mutations.

Group 1 (patients with PD after EGFR -TKI treatment)

patient	Age	Sex	Histology	Stage	T790M (%)	Activating Mutation (%)	T790M/Activating Mutation (%)	Activating Mutation Type
1	56	M	adeno	4	0.029	0.058	50.8	L861Q
2	58	F	adeno	4	0.26	0.28	94.0	L858R
3	70	F	adeno	4	2.61	7.39	35.3	L858R
4	78	M	adeno	3A	0.08	0.13	65.0	L858R
5	65	M	adeno+Sq	4	0.63	1.10	57.6	L858R
6	20	M	adeno	4	0.14	1.03	13.3	exon 19 E746-A750del
7	41	F	adeno	4	4.28	10.3	41.6	exon 19 E746-A750del
8	59	F	adeno	4	9.54	42.7	22.3	exon 19 E746-A750del
9	53	M	adeno	4	0.16	0.19	83.6	exon 19 E746-A750del
10	49	F	adeno	4	ND	0.12	0.0	L861Q
11	75	F	adeno	4	ND	2.03	0.0	L858R
12	34	M	adeno	4	ND	0.33	0.0	L858R
13	64	M	adeno	4	ND	12.2	0.0	L858R
14	73	F	adeno	4	ND	0.046	0.0	L858R
15	66	F	adeno	4	ND	0.28	0.0	exon 19 L747-S752del.P753S
16	70	M	adeno	4	ND	11.5	0.0	exon 19 L747-S752del.P753S
17	44	F	adeno	4	ND	0.09	0.0	exon 19 L747-E749del.A750P
18	52	M	adeno	4	ND	0.74	0.0	exon 19 L747-E749del.A750P
19	74	F	adeno	4	ND	0.33	0.0	exon 19 E746-A750del
20	63	F	adeno	4	ND	ND	NA	L858R
21	65	F	adeno	4	0.10	ND	NA	exon 19 E746-A750del
22	51	F	adeno	4	ND	ND	NA	exon 19 E746-A750del
23	62	F	adeno	4	ND	ND	NA	exon 19 E746-A750del

Group 2 (patients not treated with EGFR-TKI)

patient	Age	Sex	Histology	Stage	T790M (%)	Activating Mutation (%)	T790M/Activating Mutation (%)	Activating Mutation Type
24	68	M	adeno	4	ND	0.17	0.0	L858R
25	45	F	adeno	4	ND	0.23	0.0	L858R
26	85	F	adeno	2B	ND	0.25	0.0	L858R
27	67	F	adeno	4	ND	0.079	0.0	L858R
28	58	F	adeno	4	ND	0.013	0.0	L858R
29	39	F	adeno	3B	ND	36.4	0.0	L858R
30	36	F	adeno	4	ND	0.11	0.0	exon 19 L747-S752del.E746V
31	56	M	adeno	4	ND	6.47	0.0	exon 19 L747-T751del.S752Q
32	55	F	adeno	3A	ND	6.24	0.0	exon 19 L747-E749del.A750P
33	65	F	adeno	3B	ND	11.8	0.0	exon 19 E746-A750del
34	76	F	adeno	4	ND	1.06	0.0	exon 19 E746-A750del
35	63	F	Sq	4	ND	0.73	0.0	exon 19 E746-A750del
36	63	M	adeno	4	ND	0.030	0.0	exon 19 E746-L747del.IP
37	72	F	adeno	4	ND	ND	NA	L858R
38	70	F	adeno	4	ND	ND	NA	L858R
39	63	M	adeno	4	ND	ND	NA	L858R
40	80	F	adeno	4	ND	ND	NA	L858R
41	70	F	adeno	4	ND	ND	NA	L858R
42	72	M	adeno	4	0.03	ND	NA	exon 19 L747-S752del.P753S
43	47	M	adeno	4	ND	ND	NA	exon 19 E746-A750del
44	54	F	adeno	4	ND	ND	NA	exon 19 E745-E749del.A750K

ND, not detetcted; NA, not applicable.

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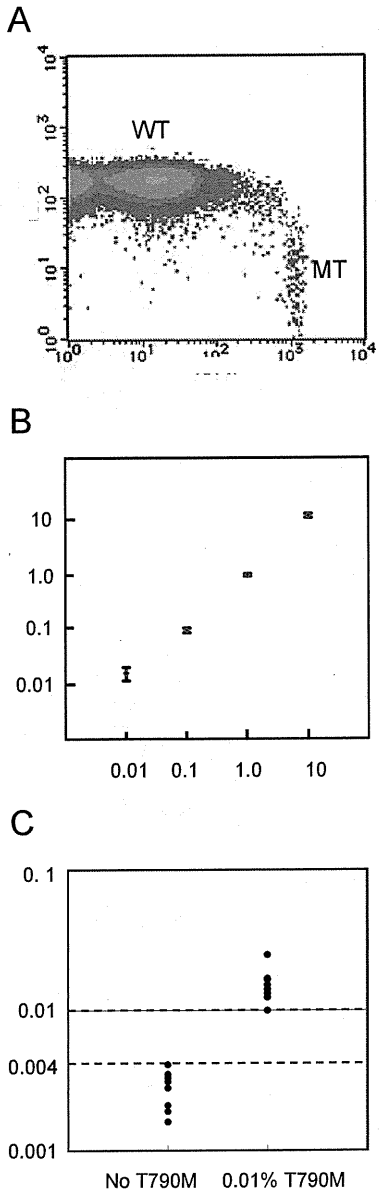

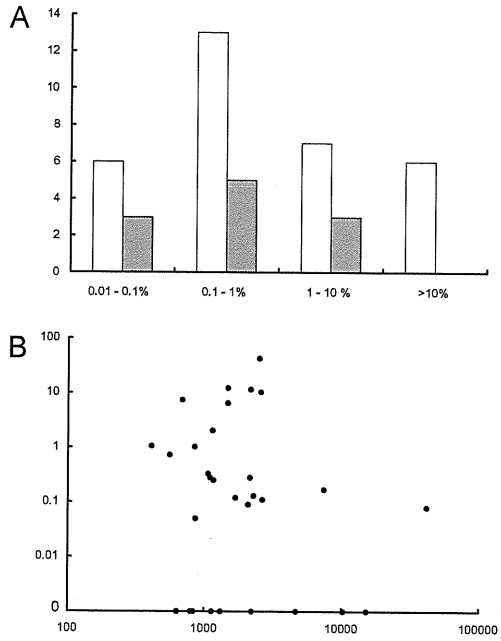

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Institutional report - Thoracic oncologic

Intrathoracic chemo-thermotherapy with radiofrequency waves after extrapleural pneumonectomy for malignant pleural mesothelioma

Toshiteru Tokunaga*, Masahiko Higashiyama, Jiro Okami, Jun Maeda, Ayako Fujiwara, Ken Kodama

Department of Thoracic Surgery, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan

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Abstract

Although multimodal treatment is advocated for malignant pleural mesothelioma (MPM), a standard therapeutic regimen has not been established. This study evaluated the outcome of our aggressive treatment including extrapleural pneumonectomy (EPP) and postoperative intrathoracic chemo-thermotherapy (PICT). Moreover, we assessed the association between the clinical effect and an in vitro chemosensitivity test. Eleven patients with MPM underwent treatment including EPP followed by PICT using 8 MHz radiofrequency waves. In vitro chemosensitivity was examined using the collagen gel droplet embedded culture drug-sensitivity test (CD-DST). Complete resection was performed in nine patients. More than two courses of PICT with sufficient heating were completely performed in seven patients. There was no perioperative mortality. Grade 3 or 4 toxicity was not recognized. The median overall survival was 19 months, and the median local relapse-free survival was 17 months. Local recurrence was recognized in four patients (36.4%). Of these patients, three had received incomplete PICT. Four patients with complete PICT including a CD-DST-sensitive chemoagent did not develop local recurrence. Of three patients who received complete PICT including a CD-DST-resistant chemoagent, one tumor recurred locally. The present multimodal treatment including EPP and PICT is promising in local control for MPM. Furthermore, CD-DST may provide clinically useful information for MPM.

Keywords: Intrathoracic chemo-thermotherapy; Malignant pleural mesothelioma; Radiofrequency waves

1. Introduction

Malignant pleural mesothelioma (MPM) is a rare thoracic malignancy arising from pleural mesothelial cells, and is characterized by local progression involving the entire pleura [1]. Various multimodal approaches including surgical resection have been attempted for MPM [2]. However, there is still no standard therapy.

Previously, we reported that treatment consisting of surgical resection and postoperative intrathoracic chemo-thermotherapy (PICT) using radiofrequency (RF) waves for lung cancer with pleural dissemination or carcinomatous pleuritis due to other organ malignancies was effective in preventing local recurrence and improving survival [3–6]. Expecting an efficacy similar to that previously reported, we performed extrapleural pneumonectomy (EPP) followed by PICT for patients with resectable MPM who had sufficient organ function. The present study reports the outcomes of patients undergoing our aggressive treatment regimen including EPP and PICT for MPM. Moreover, we examined the association between the clinical effect and the data from an in vitro chemosensitivity test using the collagen gel drop embedded culture drug-sensitivity test (CD-DST).

*Corresponding author. 1-3-3 Nakamichi, Higashinari-ku, Osaka 537-8511, Japan. Tel.: +81-6-6972-1181; fax: +81-6-6981-8055.
E-mail address: tokunaga-to@mc.pref.osaka.jp (T. Tokunaga).

2. Methods

From 1995 to 2008, surgical diagnosis or resection was performed for 41 patients with MPM in the department of thoracic surgery of our institution. Of this total, 11 patients with MPM underwent EPP followed by PICT. We performed this treatment for patients with potentially resectable tumor and adequate organ function. The operation consists of an en bloc resection of the entire pleura, lung and diaphragm, with or without resection of pericardium. The pericardial defects are reconstructed with a polytetrafluoroethylene patch (Gore-Tex patch; W.L. Gore and Associates, Flagstaff, AZ, USA). The patients' clinical characteristics are shown in Table 1. There were six males and five females with an average age of 54.8 years. Staging was determined according to the criteria proposed by the International Mesothelioma Interest Group [7].

PICT was administered two to four weeks after EPP, as previously described. Briefly, cisplatin (CDDP; 50–100 mg) or carboplatin (CBDCA; 450 mg) was injected into the thoracic cavity through the chest drainage tube, which had been left in place at the time of the surgery. Immediately thereafter, RF hyperthermia was performed through the chest wall for 60 min with an 8 MHz RF capacitive heating machine (Thermotron RF8; Yamamoto Vinitor Co., Ltd., Osaka, Japan) to heat the thoracic cavity. In every case, the peripleural temperature was directly monitored with

a thermosensor inserted through the chest drainage tube. During this treatment, we sought to maintain an effective peripleural temperature of 41.5 °C or higher for at least 40 min. At the end of the treatment, the chemotherapeutic drugs were removed as completely as possible from the thoracic cavity.

We generally performed two courses of this PICT treatment, and if possible three courses, as previously shown for lung cancer. Adjuvant hemithoracic radiotherapy with 54 Gy was performed after PICT in the most recent four patients. Additionally, two of these patients received preoperative systemic chemotherapy consisting of CDDP-based combinations. Imaging follow-up and/or symptom follow-up using computed tomography scanning of the chest cavity with EPP was performed every six months in each case to screen for local recurrence.

We performed in vitro chemosensitivity test to CDDP and CBDCA with CD-DST, as reported previously by Kobayashi et al. [8]. Briefly, MPM specimens obtained by surgery were digested in dispersion collagenase enzyme, and the dispersed cancer cells were incubated in a collagen gel-coated flask. Only the viable cells adhering to the collagen gel layer were collected and added to reconstructed type I collagen solution (Cellmatrix Type CDTM; Nitta Gelatin Inc, Yao, Japan). Three drops of these mixtures were placed in each well of a six-well multiplate, and CDDP (0.2 μg/ml) and CBDCA (0.2 μg/ml) were added to each well. The plates was then incubated for 24 h at 37 °C (normothermic CD-DST) and 1 h at 43 °C (hyperthermic CD-DST), respectively.

After removal of the medium containing the anticancer drugs, each well was incubated with serum-free culture medium (PCN-1; Nitta Gelatin Inc, Yao, Japan) for seven days. Thereafter, neutral red was added to stain colonies in the collagen gel droplets, which were finally fixed with formalin. The in vitro chemosensitivity effect was expressed as a ratio of the total colony volume (T) of the treated group to that of the control group (C) (T/C ratio). A T/C ratio of 60% or less was regarded as in vitro sensitive, as previously described [9].

In the present study, we evaluated the efficacy of our approach for MPM on local control and survival. Furthermore, we analyzed the relationship between the clinical effect and the CD-DST data. Survival curves were constructed using the Kaplan–Meier method. Survival was calculated from the date of surgery. Local recurrence was defined as relapse in the pleura. Intrathoracic lymph node metastases were excluded from local recurrence.

This retrospective investigation was approved by the institutional review board of Osaka Medical Center for Cancer and Cardiovascular Diseases. Written informed consent was obtained from each patient.

3. Results

Postoperative outcomes are shown in Table 1. The tumor histology was epithelioid type in four patients, biphasic type in four patients, and sarcomatoid type in three patients. Ten patients had stage III disease, and one had stage IV disease because of invasion into the descending aorta. Complete resection was performed in nine (81.8%) of the 11 patients. The reason for the incomplete resection was invasion into the chest wall in one patient (case 10) and invasion into the descending aorta in one patient (case 11). There was no perioperative mortality.

Postoperative complications occurred in two patients: atrial fibrillation and Horner syndrome occurred in cases 8 and 10, respectively. The number of courses of PICT was two in six patients and three in two patients according to schedule, but one in three patients (cases 7, 8 and 9) because of the patients' refusal. Two patients (cases 9 and 10) received incomplete heating treatment because of pain just under the RF applicator. More than two courses of PICT with sufficient heating were completely performed in seven patients. After PICT, nausea occurred in five patients due to drug toxicity. Of these patients, four developed vomiting. None of the patients experienced grade 3 or 4 toxicity due to PICT. There were no fatal treatment-related complications in any of the patients. Especially, there were no serious complications related to radiotherapy in the recent four patients.

The median follow-up period was 19 months (range nine to 63 months). The median survival time (MST) was 19 months, as shown in Fig. 1. The one-year and two-year survival rates were 63.6% and 18.2%, respectively. Two patients remain alive without recurrence at 23 and 63 months, respectively. The remaining nine patients have died of recurrence. The median local relapse-free survival was 17 months, as shown in Fig. 2. The one-year and two-year local relapse-free

Table 1. Characteristics and postoperative outcomes of patients with malignant pleural mesothelioma

Case	Age	Gender	Histology	Stage	Preoperative chemotherapy	Complete resection	Postoperative complications	PICT			Post RT	Local Rec	Survival
								Courses	Side effect	Completion			
1	69	M	Sarcomatoid	III (T3N0)		Yes		2		Yes	–	–	21 m, Dead
2	43	M	Biphasic	III (T3N0)		Yes		3		Yes	–	–	19 m, Dead
3	69	M	Biphasic	III (T3N2)		Yes		3	Nausea vomiting	Yes	–	+	12 m, Dead
4	42	F	Epithelioid	III (T3N2)		Yes		2		Yes	–	–	11 m, Dead
5	50	F	Biphasic	III (T3N2)		Yes		2		Yes	–	–	9 m, Dead
6	64	M	Sarcomatoid	III (T3N0)		Yes		2	Nausea vomiting	Yes	–	–	17 m, Dead
7	64	F	Epithelioid	III (T3N1)		Yes		1	Nausea vomiting	No	–	+	20 m, Dead
8	43	M	Epithelioid	III (T2N2)	CDDP+PEM	Yes	Atrial fibrillation	1	Nausea	No	+	–	23 m, Alive
9	60	F	Sarcomatoid	III (T2N2)		Yes		1	Pain	No	+	+	30 m, Dead
10	53	M	Biphasic	III (T3N0)	CDDP+GEM	No	Horner syndrome	2	Pain	No	+	+	11 m, Dead
11	46	F	Epithelioid	IV (T4N0)		No		2	Nausea vomiting	Yes	+	–	63 m, Alive

CDDP, cisplatin; F, female; GEM, gemcitabine; M, male; PEM, pemetrexed; PICT, postoperative intrathoracic chemo-thermotherapy; Rec, recurrence; RT, radiotherapy.

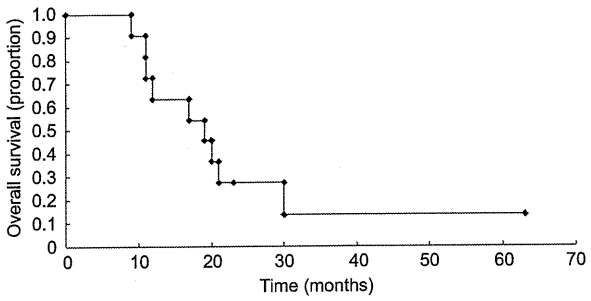

Fig. 1. Overall survival for all patients.

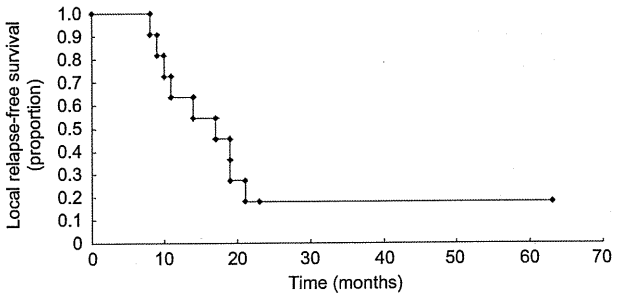

Fig. 2. Local relapse-free survival for all patients.

survival rates were 54.5% and 9.1%, respectively. Local recurrence was seen in only four patients (36.4%). Of these four patients, three received incomplete PICT (cases 7, 9 and 10). The local recurrence rate for the seven patients with complete PICT was 14.3%, whereas that for the four patients with incomplete PICT was 75%.

Normothermic CD-DST analysis was performed for 10 patients. For nine of these patients, hyperthermic CD-DST was also investigated. CD-DST was successfully performed on all specimens. Table 2 shows the association between the PICT regimen and the CD-DST data. PICT including a CD-DST-sensitive chemoagent was performed for four patients. In these patients, PICT was performed completely and local recurrence was not recognized. The remaining six patients received PICT including a CD-DST-resistant chemoagent. Of these patients, three patients (cases 7, 9 and 10) underwent incomplete PICT, and three (cases 2, 3 and 5) underwent complete PICT. The former developed local recurrence. On the other hand, of the latter, one patient (case 3) developed local recurrence.

4. Discussion

RF waves have been previously utilized for the treatment of superficial and deep-seated tumor [10, 11]. Generally, it has been considered difficult to achieve a sufficiently high temperature in the pleural cavity with RF hyperthermia because of wave distortion and the cooling effect of the respiration and circulation. However, by measuring the temperature in the pleural cavity, we showed that heat accumulated in the vicinity of pleura [3]. Particularly in patients undergoing pleuropneumonectomy, heat energy could be easily accumulated.

In the present study, we report the results of our aggressive treatment including EPP followed by PICT using RF waves for MPM. There has not been any report regarding intrathoracic chemo-thermotherapy (ICT) using RF waves after EPP for MPM. The median local relapse-free survival was 17 months. Local recurrence was detected in four patients (36.4%). However, of seven patients who received complete PICT, only one (14.3%) developed local recurrence. Moreover, of six patients who received complete PICT without adjuvant radiotherapy, only one (16.6%) developed local recurrence. Therefore, our PICT method seems to improve local control of MPM. The overall MST in this treatment was 19 months. Although the prognostic value was not demonstrated because there was no control group, considering that all patients were in the advanced stage, this outcome seems to be promising.

Another intracavitary heating method, perfusion with warm water, can be conducted. Although this method can heat the entire thoracic cavity, a high-dose of chemoagent is needed because of dilution. Recently, Tilleman et al. attempted treatment consisting of EPP followed by hyperthermic intraoperative intracavitary CDDP perfusion for MPM [12]. They showed that the ICT could reduce local recurrences. However, the MST was 13.1 months. Furthermore, the hospital mortality was 4.3%, and grade 3 or 4 morbidity

Table 2. Association between PICT regimen and in vitro sensitivity data

Case	PICT regimen			In vitro data T/C value (%)				(S or R)
	1	2	3	Normothermic		Hyperthermic		
				CDDP	CBDCA	CDDP	CBDCA	
1	CBDCA 450 mg	CDDP 100 mg		30	51	NA	NA	S
2	CBDCA 450 mg	CDDP 100 mg	CBDCA 450 mg	78	82	72	84	R
3	CDDP 100 mg	CBDCA 450 mg	CDDP 100 mg	115	127	130	138	R
4	CBDCA 450 mg	CBDCA 450 mg		35	61	22	39	S
5	CBDCA 450 mg	CBDCA 450 mg		56	79	88	84	R
6	CBDCA 450 mg	CBDCA 450 mg		34	48	27	25	S
7	CBDCA 450 mg			82	92	86	92	R
8	CBDCA 450 mg			NA	NA	NA	NA	NA
9	CBDCA 450 mg			83	91	87	96	R
10	CBDCA 450 mg	CBDCA 450 mg		74	82	80	82	R
11	CBDCA 450 mg	CDDP 50 mg		114	83	70	56	S

CBDCA, carboplatin; CDDP, cisplatin; NA, not applicable; PICT, postoperative intrathoracic chemo-thermotherapy; T/C, total colony volume/control colony volume; S, sensitivity; R, resistance.

occurred in 48.9%. Compared with this report, our treatment was performed safely.

Some investigators have attempted adjuvant radiotherapy to prevent local recurrence [13, 14]. Rusch et al. reported that high-dose hemithoracic radiotherapy combined with EPP dramatically reduced local recurrence and was associated with prolonged survival in early-stage MPM [13]. Recently, Bonnette showed that adjuvant high-dose hemithoracic radiotherapy after surgical resection decreased the local recurrence rate from 35% to 13% [14]. Based on those outcomes, we additionally performed adjuvant radiotherapy for recent cases. More recently, Xia et al. attempted treatment combined with ICT using RF waves and radiotherapy for unresectable MPM [15]. They reported a response rate of 27.3% and an MST of 27.1 months, and concluded that their therapy was an effective approach comparable to other multimodal treatments.

In our study, the local recurrence rate was 28.6% (two out of seven) in the group without radiotherapy, whereas it was 50% (two out of four) in the group with radiotherapy. Therefore, it was not known whether radiotherapy was effective in local control. However, of four patients receiving radiotherapy, three survived longer than the patients without radiotherapy. In particular, one patient who received complete PICT and adjuvant radiotherapy is alive without recurrence more than five years later despite incomplete resection. Therefore, adjuvant therapy including ICT using RF waves and radiotherapy may be hopeful.

In vitro chemosensitivity tests have been widely tried for clinical applications for various malignant tumors. Recently, we have investigated the in vitro sensitivity status of MPM tissues using CD-DST [9]. In this report, the clinical effects on some patients with MPM undergoing chemotherapy for primary or recurrent disease were also analyzed in comparison with the CD-DST data. CD-DST data for the chemoagents were marginally correlated with the disease control status of the chemotherapy. In the present study, four patients with complete PICT including a CD-DST-sensitive chemoagent did not develop local recurrence. Interestingly of these four patients, two (cases 4 and 11) showed sensitivity to hyperthermic CD-DST in spite of having resistance to normothermic CD-DST. On the other hand, three patients with incomplete CD-DST-resistant chemoagent treatment developed local recurrence. In addition of three patients who received complete PICT including a CD-DST-resistant chemoagent, one experienced local recurrence. The T/C ratio in this patient was more than 100%, while that in the other two patients was <100%. Thus, important information may be obtained using CD-DST. Especially, hyperthermic CD-DST data may correlate with the response to ICT.

5. Conclusions

In conclusion, these data suggest that our multimodal treatment including EPP and PICT using RF waves is a promising therapy for MPM, with an acceptable level of adverse effects, and especially offers good local control. To confirm the effectiveness of our procedure, a randomized study is necessary in the near future.

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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

Seiji Yano, MD, PhD, Tadaaki Yamada, MD, PhD,* Shinji Takeuchi, MD, PhD,* Keisei Tachibana, MD, PhD,† Yuko Minami, MD, PhD,† Yasushi Yatabe, MD, PhD,‡ Tetsuya Mitsudomi, MD, PhD,§ Hidenori Tanaka, MD, PhD,‖ Tatsuo Kimura, MD, PhD,‖ Shinzoh Kudoh, MD, PhD,‖ Hiroshi Nokihara, MD, PhD,¶ Yuichiro Ohe, MD, PhD,¶ Jun Yokota, MD, PhD,# Hidetaka Uramoto, MD, PhD,** Kosei Yasumoto, MD, PhD,** Katsuyuki Kiura, MD, PhD,†† Masahiko Higashiyama, MD, PhD,‡‡ Makoto Oda, MD, PhD,§§ Haruhiro Saito, MD, PhD,‖‖ Junji Yoshida, MD, PhD,¶¶ Kazuya Kondoh, MD, PhD,## and Masayuki Noguchi, MD, PhD†*

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

*Division of Medical Oncology, Cancer Research Institute, Kanazawa University, Takara-machi, Kanazawa; †Department of Pathology, Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, Ibaraki; ‡Department of Pathology and Molecular Diagnosis; §Department of Thoracic Surgery, Aichi Cancer Center Hospital, Nagoya, Aichi; ‖Department of Respiratory Medicine, Graduate School of Medicine, Osaka City University, Sumiyoshi-ku, Osaka; ¶Division of Internal Medicine and Thoracic Oncology; #Division of Biology, National Cancer Center Hospital, Tokyo; **Second Department of Surgery, University of Occupational and Environmental Health, Kitakyushu; ††Department of Hematology, Oncology, and Respiratory Medicine, Okayama University Graduate School of Medicine, Okayama; ‡‡Department of Thoracic Surgery, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Osaka; §§Department of Thoracic Surgery, Kanazawa University Hospital, Takara-machi, Kanazawa; ‖‖Department of Thoracic Oncology, Kanagawa Cancer Center, Yokohama; ¶¶Department of Thoracic Oncology, National Cancer Center Hospital East, Kashiwa, Chiba; and ##Department of Thoracic, Endocrine Surgery and Oncology, Institute of Health Bioscience, The University of Tokushima Graduate School, Tokushima, Japan.

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Address for correspondence: Seiji Yano, Division of Medical Oncology, Cancer Research Institute, Kanazawa University, 13-1 Takara-machi, Kanazawa, Ishikawa 920-0934, Japan. E-mail: syano@staff.kanazawa-u.ac.jp

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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 amplification[13]—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 ± SD: 205 ± 106) compared with pretreatment (126 ± 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 ± 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 ≥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× PCR buffer containing 100 μg/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 μm 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%)
EGFR T790M secondary mutation	12 (52%)	0	0
MET 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 patient[24](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.

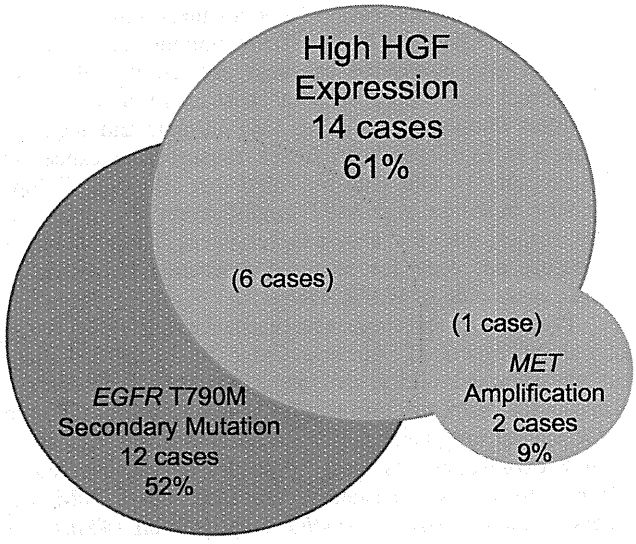

FIGURE 1. Incidences of high-level HGF expression, T790M secondary mutation, and *MET* amplification in 23 tumors with acquired resistance. Values in parentheses are the numbers of cases in which the tumors expressed two resistance factors simultaneously.

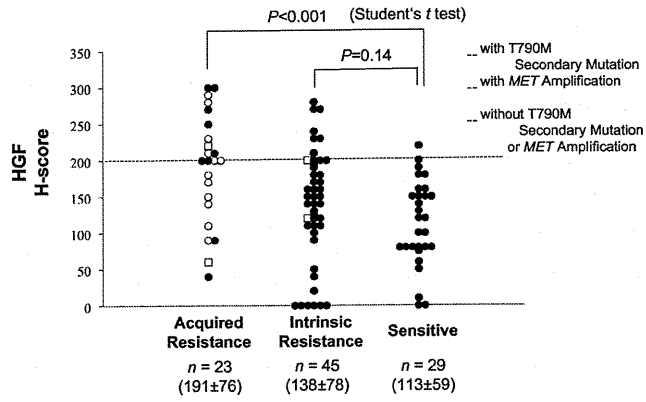

FIGURE 2. HGF expression score (*H* score) in EGFR-TKI-resistant tumors obtained from *EGFR* mutant lung cancer patients. Values in parentheses are mean ± SD of *H* score.

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 was consistent with a previous report[16] 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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