

procedure and has significant advantages over other methods.

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Analysis of Epidermal Growth Factor Receptor Gene Mutation in Patients with Non-Small Cell Lung Cancer and Acquired Resistance to Gefitinib

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Abstract Purpose: Non-small cell lung cancers carrying activating mutations in the gene for the epidermal growth factor receptor (EGFR) are highly sensitive to EGFR-specific tyrosine kinase inhibitors. However, most patients who initially respond subsequently experience disease progression while still on treatment. Part of this "acquired resistance" is attributable to a secondary mutation resulting in threonine to methionine at codon 790 (T790M) of EGFR.

Experimental Design: We sequenced exons 18 to 21 of the *EGFR* gene to look for secondary mutations in tumors with acquired resistance to gefitinib in 14 patients with adenocarcinomas. Subcloning or cycleave PCR was used in addition to normal sequencing to increase the sensitivity of the assay. We also looked for T790M in pretreatment samples from 52 patients who were treated with gefitinib. We also looked for secondary *KRAS* gene mutations because tumors with *KRAS* mutations are generally resistant to tyrosine kinase inhibitors.

Results: Seven of 14 tumors had a secondary T790M mutation. There were no other novel secondary mutations. We detected no T790M mutations in pretreatment specimens from available five tumors among these seven tumors. Patients with T790M tended to be women, never smokers, and carrying deletion mutations, but the T790M was not associated with the duration of gefitinib administration. None of the tumors had an acquired mutation in the *KRAS* gene.

Conclusions: A secondary T790M mutation of *EGFR* accounted for half the tumors with acquired resistance to gefitinib in Japanese patients. Other drug-resistant secondary mutations are uncommon in the *EGFR* gene.

Activating mutations in the gene for the epidermal growth factor receptor (EGFR) are present in a subset of pulmonary adenocarcinomas. Tumors with *EGFR* mutations are highly sensitive to gefitinib and erlotinib, small-molecule EGFR-specific tyrosine kinase inhibitors (1-3). These mutations occur in the tyrosine kinase domain of the *EGFR* gene. Deletion mutations in exon 19 and the substitution of leucine with arginine at codon 858 (L858R) account for ~90% of all these mutations (4). *EGFR* mutations are more prevalent in women,

never smokers, patients of Asian ethnicity, and those with adenocarcinoma histology (4). These features are the same as those of patients whose tumors have elevated sensitivity to EGFR-specific tyrosine kinase inhibitors. The response rates of lung cancers with an *EGFR* mutation are as high as 80% (5). Responses are often dramatic, and several reports have shown that patients with *EGFR* mutations survive significantly longer after gefitinib treatment than patients without mutations (6). However, it is also common for patients to show disease progression after presenting with an initial marked response to EGFR-specific tyrosine kinase inhibitors. The mean duration of the initial response is about 3 to 7 months (7, 8).

Recently, it has been reported by two groups that a secondary threonine-to-methionine mutation at codon 790 (T790M) of the *EGFR* gene is related to the acquired resistance to gefitinib and erlotinib (9, 10). Crystal structure modeling has shown that residue T790 is located in the ATP-binding pocket of the catalytic region of EGFR, and it seems to be critical for the binding of erlotinib and gefitinib (9). Substitution of the threonine at codon 790 with a bulkier residue, such as methionine, would result in steric hindrance to the binding of these two drugs. A secondary T790M mutation has been identified in one tumor (9) and in three of six tumors (10) with acquired resistance to gefitinib.

Imatinib is a tyrosine kinase inhibitor specific for BCR-ABL, KIT, and platelet-derived growth factor A, which is used to treat

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chronic myelogenous leukemia (CML) and gastrointestinal stromal tumor. Analogous secondary mutations in the kinase domains of these genes are considered to constitute one of the mechanisms of acquired drug resistance (11–14). The structural similarity between ABL and EGFR tyrosine kinases is fairly high, and the most common mutation related to acquired resistance is a threonine-to-isoleucine mutation at codon 315 (T315I), corresponding to T790M in the EGFR gene (15). In CML, 20 to 30 other mutations of the ABL gene have been identified as responsible for acquired resistance to imatinib (12, 16–19), so secondary EGFR gene mutations other than T790M are possible (Fig. 1).

Secondary mutations of the ABL gene have also been detected in pretreatment samples from some CML patients, although the fraction of mutant cells was very low (16, 20). The existence of a similar mechanism is expected for non-small cell lung cancer. Furthermore, we and others have reported that the T790M mutation of the EGFR gene exists as a major mutation independently of gefitinib treatment, although instances are very rare (21, 22).

It has also been reported that KRAS mutations are associated with a lack of sensitivity to gefitinib and erlotinib (23, 24). Therefore, it is possible that acquired KRAS mutations are also associated with acquired resistance.

In this study, we looked for the T790M mutation and other secondary mutations of the EGFR gene in tumors from patients who showed disease progression after presenting with an initial response to EGFR-specific tyrosine kinase inhibitor treatment and in tumors before gefitinib treatment. We also looked for KRAS mutations in the same tumors.

Materials and Methods

Patients. Patients with non-small cell lung cancer who initially responded but subsequently experienced disease progression while on gefitinib treatment were defined as having "acquired resistance." A detailed definition of the effectiveness of gefitinib treatment was described in our previous study (25). Briefly, gefitinib treatment is judged to be effective when tumors show a decrease of at least a 30% in tumor diameter in imaging studies or when elevated carcinoembryonic antigen levels decrease to a level less than half the baseline level.

Fourteen tumor samples and 10 corresponding pretreatment tumor samples from eligible patients were obtained according to this definition at the time of diagnosis or treatment. The selection of patients depended only on whether a second tumor sample collected at the time of progression could be obtained. Appropriate approval from the institutional review board and the patients' written informed consent were obtained. Patient characteristics and details of the samples are shown in Table 1. All patients had adenocarcinomas, and the median duration of gefitinib treatment was 367 days (range, 69–921 days). We also analyzed the samples of 52 patients who had been treated with gefitinib for recurrent disease after they had undergone pulmonary resection. This cohort was part of our previous study, and their clinical details are described elsewhere (25).

Subcloning mutational analysis of the EGFR gene. Genomic DNA and total RNA (if possible) were extracted from each sample (Table 1). Exons 18 to 21 of the EGFR tyrosine kinase domain were amplified using PCR or reverse transcription-PCR (RT-PCR) methods. PCR for genomic DNA was done using AmpliTaq Gold (Applied Biosystems, Foster City, CA) and the following primers: exon 18, 5'-GAGGTGACCC-TTGTCTCTGTGT-3' (forward) and 5'-CCCAACACTCAGTGAACA-3' (reverse); exon 19, 5'-TGCCAGTTAACGCTTCTCTCT-3' (forward) and 5'-ATGTGGAGATGAGCAGGGTCTA-3' (reverse); exon 20, 5'-TGAAATC-AAGATCGCATT-3' (forward) and 5'-CATGGCAACTCTTGTCTAC-3' (reverse).

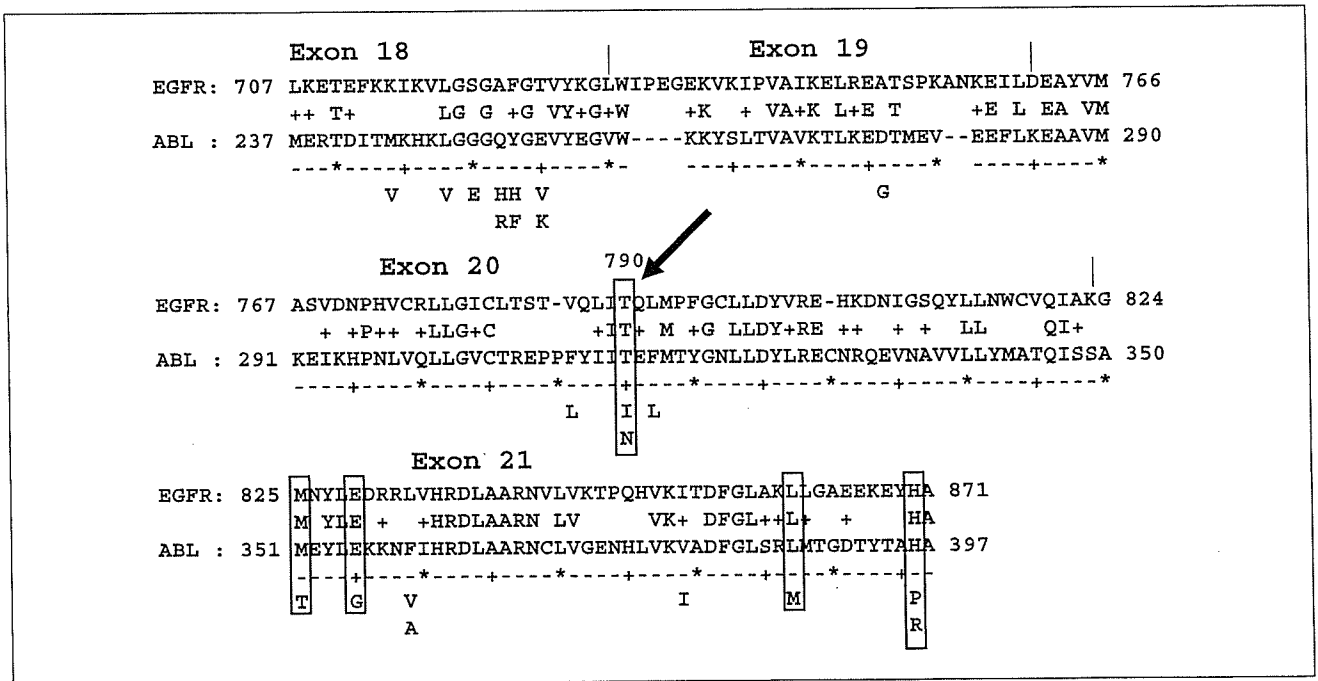


Fig. 1. Structural similarity between EGFR tyrosine kinase and ABL. This amino acid alignment was obtained using basic local alignment search tool, and both sequences were obtained from Genbank (accession nos.: EGFR, NM 005228; ABL, NM 005157). Top line, EGFR; bottom line, ABL. Vertical lines, boundaries between exons. Numbers at each end, codon numbers. Capital letters under the alignment, amino acid changes in ABL that have been reported as acquired imatinib resistance mutations. Square frames, qualifying codons as common codons in EGFR and ABL and as acquired resistance mutant codons in ABL. Arrow, location of codon 790 of EGFR and codon 315 of ABL.

Table 1. Patient characteristics and results of sequencing analysis

Patient no.	Sex	Smoking status	Prior treatment	Gefitinib response	Gefitinib treatment days	Analyzed specimen (state)	Nucleic acid	Activating mutation	T790M mutation	T790M (pre-gefitinib samples)
1	F	NS	S	E	642	LN (Fr)	RNA	Δ2	+	—
2	M	FS	S	E	368	PE (Al)	RNA	Δ3	—	—
3	M	NS	S	E	116	PE (Al)	RNA	Δ1	—	—
4	F	FS	CT	E	599	PE (CL)	RNA	Δ1	—	NA
5	F	NS	CRT	E	921	LU (Al)	RNA	Δ1	+	NA
6	F	NS	None	E	181	PE (Al)	RNA	Δ1	+	—
7	F	FS	CT	E	346	BO (Al)	RNA	Δ1	+	—
8	F	NS	S→CRT	E	623	LN (Al)	RNA	L858R	—	NA
9	M	FS	S	E	915	BR (Fr)	DNA	L858R*	—	—
10	M	FS	S→CRT	NE	69	PE (Al)	DNA	L858R	—	—
11	F	FS	None	E	560	LU (Fr)	RNA	L858R*	+	NA
12	F	NS	CT	E	239	PE (Al)	RNA	Δ1	+	—
13	F	NS	S	E	367	PE (Al)	RNA	L858R	—	—
14	F	NS	CRT	E	235	LN (Al)	RNA	Δ1	+	—

NOTE: Patients 1, 4, and 13 received gefitinib therapy twice. Pretreatment samples from patients 4, 5, 8, and 11 were not available. Patient 10 was defined as not evaluable according to our definition. However, this patient showed a 46% decrease in carcinoembryonic antigen and a marked reduction in pleural effusion on initial treatment before subsequent progression. Therefore, we regarded this case as eligible for this study.

Abbreviations: Al, alcohol fixed; BO, bone metastasis; BR, brain metastasis; CL, cell line; CRT, chemoradiotherapy; CT, chemotherapy; del, deletion; E, effective; F, female; Fr, frozen; FS, former smoker; ins, insertion; LN, lymph node; LU, lung tumor; M, male; NA, not available; NE, not evaluable; NS, never smoker; PE, pleural effusion; RT, radiotherapy; S, surgery; Δ1, del E746-A750; Δ2, del L747-P753 insS; Δ3, del L747-A750 insP.

*Patients 9 and 11 had another point mutation (L833V in patient 9 and R776H in patient 11).

(reverse); and exon 21, 5'-GAGCTTCCTCCCATGATGATCT-3' (forward) and 5'-GAAAATGCTGGCTGACCTAAAG-3' (reverse). The PCR conditions were as follows: 1 cycle of 95°C for 11 minutes, 45 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 40 seconds followed by 1 cycle of 72°C for 4 minutes.

RT-PCR for RNA was done with primers 5'-AGCTTGTGGAGCCTCT-TACACC-3' (forward 1) and 5'-TAAAATTGATTCCAATGCCATCC-3' (reverse 1) in a one-step RT-PCR setup using Qiagen OneStep RT-PCR kits (Qiagen, Valencia, CA) as described previously (26). RT-PCR conditions were as follows: 1 cycle of 50°C for 30 minutes and 95°C for 15 minutes, 40 cycles of 94°C for 50 seconds, 62°C for 50 seconds, and 72°C for 1 minute followed by 1 cycle of 72°C for 10 minutes.

The PCR products were subcloned using TOPO TA Cloning kits (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Each clone was then directly amplified with the same primers using AmpliTaq Gold and cycle sequenced using BigDye Terminator v3.1/1.1 cycle sequencing kits (Applied Biosystems). Subcloning PCR conditions were as follows: 1 cycle of 95°C for 11 minutes, 45 cycles of 95°C for 50 seconds, 62°C for 50 seconds, and 72°C for 70 seconds followed by 1 cycle of 72°C for 4 minutes.

The sequencing reaction products were electrophoresed using an ABI PRISM 3100 system (Applied Biosystems). Both forward and reverse sequences were analyzed with basic local alignment search tool, and the chromatograms were analyzed by manual review.

Cycleave real-time PCR assay. Details of the cycleave real-time PCR assay have been described previously (27). Briefly, genomic DNA was extracted, and exon 20 of the *EGFR* gene was amplified by real-time quantitative PCR assay on a SmartCycler (TaKaRa, Gifu, Japan) using Cycleave PCR Core kits (TaKaRa) with a T790M-specific cycling probe and a wild-type cycling probe. As few as ~5% of tumor cell molecules could be detected in this assay.

Mutational analysis of the *KRAS* gene. A RT-PCR direct sequence assay was done for RNA, and a cycleave real-time PCR assay was done for DNA. *KRAS* primers for PCR were 5'-GGCCTGCTGAAAATGACTGA-3' (forward 1) and 5'-TCTTGCTAAGTCTGAGCCTGTT-3' (reverse 3).

Codon 12 cycling probes and a wild-type cycling probe were used in cycleave real-time PCR assays. Direct sequencing was used to identify codon 12, 13, and 61 mutations.

Results

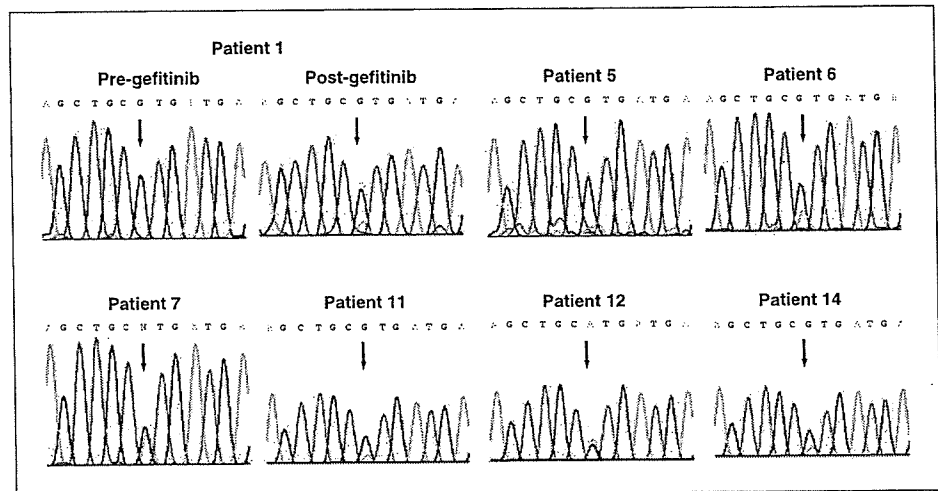
Detection of secondary mutations in the *EGFR* gene or the *KRAS* gene. For the analysis of secondary mutations, we first amplified exons 18 to 21 of the *EGFR* gene, which include the region homologous to the region of the *ABL* gene that contains all the secondary mutations thus far reported to be responsible for imatinib resistance in CML. All 14 tumors with acquired resistance had activating mutations of the *EGFR* gene, either deletion mutations, including codons 746 to 750 (nine patients), or L858R (five patients). Seven tumors had a secondary T790M mutation (Table 1; Fig. 2).

When we sequenced corresponding tumor samples that had been obtained before gefitinib treatment, the same activating mutations were always present, whereas T790M was not detected in any of the available pretreatment samples (samples for patients 4, 5, 8, and 11 were not available).

Mutant bands for T790M in the sample from patient 7 were as strong as the wild-type bands, and the mutant bands were stronger than the wild-type bands in patient 12 (Fig. 2). However, in most cases, the T790M mutant bands were weaker than the wild-type bands.

Two tumors had another point mutation as well as L858R (L833V in patient 9 and R776H in patient 11). L833 corresponds to F359 of *ABL*, where a secondary mutation to valine or alanine has been reported in CML (Fig. 1; ref. 12). However, the pretreatment sample of patient 9 revealed that L833V existed before treatment in the same ratio as the L858R band. The ratios of L833V and L858R bands were unchanged

Fig. 2. Sequencing chromatograms for *EGFR* exon 20. Secondary T790M mutations were observed in seven patients. Antisense strands of each chromatogram. Arrows, small peaks of the C→T substitution at nucleotide 2,369 (G→A on the antisense strand), which results in the T790M mutation. This substitution was observed only in posttreatment samples. T790M mutant bands were clearly detected on sequencing chromatograms, except in that of patient 5; in this patient, it was unclear because of artifacts.



before and after gefitinib treatment. Although the T790M mutant band was weaker than the L858R mutant band in patient 11, the intensity of the R776H mutant band was the same as that of the L858R mutant band and both mutations were heterozygous. We considered these point mutations to be primary mutations and not associated with "acquired" resistance.

To increase the sensitivity for the detection of T790M and other possible secondary mutations in the tyrosine kinase domain, each PCR product was subcloned and multiple subclones were amplified and sequenced directly. All the T790M mutations found by sequencing the noncloned PCR products were confirmed by this subcloning method, but no new T790M mutations were detected even when >50 clones were analyzed in samples from patients 2 and 3 (Table 2). Furthermore, we detected no secondary mutations in exons 18 to 21 other than T790M.

The T790M mutations were either present in clones with activating (or sensitizing) mutations or in other clones without activating mutations (Table 2). In three tumors (of patients 1, 5, and 14), T790M was present only in clones with activating mutations, whereas in the remaining four tumors (patients 6,

7, 11, and 12), T790M was present in both clones with and without activating mutations. No tumor carried the T790M mutation only in the wild-type clones. However, four of five T790M mutations were in clones without activating mutations in the tumor of patient 6.

We also looked for mutations in codon 12 (and codons 13 and 61 in RNA samples) in the *KRAS* gene. However, none of the samples from the tumors studied had *KRAS* mutations.

Relationship between T790M mutation and clinical and genetic features. T790M mutations were more frequent in women (women, 7 of 10; men, 0 of 4), who had never smoked (never smoker, 5 of 8; previous smoker, 2 of 6), and with deletion mutations (deletion, 6 of 9; L858R, 1 of 5). There was no difference in the incidence of T790M in the presence or absence of prior chemotherapy (with, 4 of 8; without, 3 of 6; Table 1).

We also compared the duration of gefitinib treatment, which is considered to correlate roughly with the time to progression, with the presence or absence of T790M. However, the median treatment times were almost identical (tumors with T790M, 346 days; tumors without T790M, 368 days; Fig. 3).

Analysis of corresponding tumor tissues before gefitinib treatment in patient 1. To determine whether rare T790M

Table 2. Analysis of acquired mutation using the subcloning method

Patient no.	Activating mutation	Total clones	Activating mutant clones		Wild-type clones	
			With T790M	Without T790M	With T790M	Without T790M
1	Δ2	21	8	10	0	3
2	Δ3	54	0	52	0	2
3	Δ1	51	0	50	0	1
4	Δ1	21	0	13	0	8
5	Δ1	51	3	39	0	9
6	Δ1	47	1	17	4	25
7	Δ1	20	4	5	1	10
8	L858R	18	0	14	0	4
9	L858R	20	0	14	0	6
10	L858R	20	0	5	0	15
11	L858R	21	5	10	1	5
12	Δ1	23	11	9	1	2
13	L858R	21	0	8	0	13
14	Δ1	19	7	8	0	4

mutant clones existed before gefitinib treatment, we analyzed the corresponding tumor tissues of patient 1, whose tissue after gefitinib treatment had a secondary T790M mutation. Tumor tissue was obtained at the time of operation. PCR products from the tumor before gefitinib treatment were subcloned, and 103 subclones were amplified and sequenced directly. However, at this sensitivity, we detected no clone carrying the T790M mutation. Among 103 clones, 92 (89%) had activating deletion mutations, suggesting that the mutant allele was amplified before gefitinib treatment. The incidence of clones with deletional mutations was similar (18 of 21, 85%) in a cervical lymph node taken after gefitinib resistance had developed.

To further explore of possible association of T790M with metastatic spread, we looked for the T790M mutation in hilar and mediastinal lymph nodes with metastases dissected at the time of surgery. Genomic DNA was extracted from lymph nodes from four stations (aortopulmonary, ascending aorta, main bronchus, and intrapulmonary) and analyzed using cycleave real-time PCR. However, we detected no T790M mutations.

Analysis of tumors for T790M before gefitinib treatment in 52 patients who were treated with gefitinib. The possible presence of T790M at a low frequency in tumors before gefitinib treatment might affect the tumor response or the time to progression after gefitinib treatment. In a previous study, we sequenced exons 18 to 23 of the *EGFR* genes of 52 patients who had been treated with gefitinib for recurrent disease after they had undergone pulmonary resection. None of them had the T790M mutation. Here, we used a cycleave real-time PCR assay, which is more accurate analysis than normal sequence, to investigate whether rare T790M mutant cells were present. However, we detected no T790M mutations in these 52 tumors.

Discussion

We studied 14 tumors with acquired resistance to gefitinib for secondary mutations occurring in the *EGFR* tyrosine kinase domain. Seven of the 14 tumors had a secondary T790M mutation, an incidence consistent with those of previous studies (9, 10). Whereas clones with activating mutations (deletion or L858R) might well have been eliminated by selection pressure during gefitinib treatment, those clones were always present in tumors that developed acquired resistance. In most cases, clones with the T790M mutation were not predominant.

The T790M mutations occur more frequently in women who had never smoked and who had a deletion-type mutation. Time to progression did not differ between tumors that acquired secondary T790M mutations and those that did not. However, these tendencies require careful interpretation because of the number of samples was small.

In a previous report, Kobayashi et al. (9) showed that the T790M mutation was observed with either wild-type or deletion mutation sequences, whereas Pao et al. (10) showed that both the T790M and L858R mutations were in the same allele. Our data showed that three samples had the T790M mutation only in the clones with activating mutation and four samples had the T790M mutation in the clones with and without activating mutation, whereas the most of T790M mutation was in the clones with activating mutation, except for

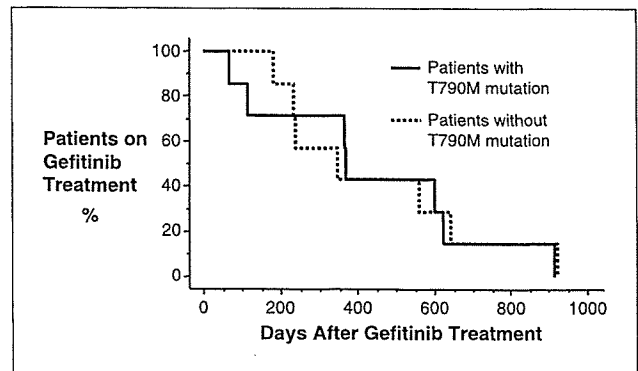


Fig. 3. Effect of the T790M mutation on the length of gefitinib treatment. The length of gefitinib treatment was considered to be roughly related to time to progression. Median treatment times were almost identical in both the presence and absence of the T790M mutation.

the samples of patient 6. It is possible that this could result from a PCR error or DNA repair error at the subcloning step. Bell et al. (28) have reported that artifactual PCR-generated allelic separation occurred with probability of ~30% in their analysis. However, it is also possible that the T790M mutation occurs in both alleles or that tumor heterogeneity exists.

In CML, 20 to 30 mutations in the *ABL* gene are responsible for acquired resistance to imatinib. Many types of mutations have been detected, and there are four distinguishable clusters (P-loop, T315, M351, and A-loop; ref. 29). Furthermore, secondary mutations in the *ABL* kinase domain are found in 50% to 90% of patients (29), many more than in patients with non-small cell lung cancer. We detected no novel mutations in the *EGFR* gene other than T790M. Two tumors had another point mutations together with L858R, L833V, or R776H. We considered these point mutations to be primary mutations and not associated with acquired resistance. However, these conclusions were based only on sequencing and subcloning methods, and we have no evidence of the functional effects of these mutations. There may be differences in the mechanisms of acquired resistance between non-small cell lung cancer and CML.

We previously reported that, in a series of 397 unselected patients with non-small cell lung cancer who had undergone surgery, 2 female patients with no history of smoking had L858R plus T790M mutations (21). Because these patients were not treated with gefitinib, T790M might well have conferred a growth advantage. These tumors were aggressive and later developed recurrent disease. One was treated with gefitinib but was refractory to treatment. A similar case was reported by another group (22). Inspired by this observation and because the secondary mutations related to imatinib resistance in CML were detected at low frequencies (0.01-0.9%) in pretreatment samples (16, 20), we attempted to detect minor clones with the T790M mutation in samples before gefitinib treatment. However, we could not detect the T790M mutation by assays that can detect mutant cells if there is about 1% to 5% at least. It remains unclear whether a more sensitive method would have detected rare clones with the T790M mutation in our samples.

Why tumors with T790M mutant cells acquire resistance to gefitinib despite the fact that mutant band for the T790M

mutation was almost always weaker than wild-type band remains unclear. It is possible that cells with the T790M mutation preexist at a very low frequency and gradually increase during gefitinib treatment by clonal selection as in cases of CML (16). It is also possible that amplification of the activating mutant allele occurs in resistant tumors and parts of them have the T790M mutation. Another possibility is that multiple coexisting mechanisms, including the T790M mutation, cause acquired resistance cooperatively or independently. A recent study suggested that increased internalization of ligand-bound EGFR is one of the mechanisms underlying acquired gefitinib resistance (30). It is also likely that *EGFR* gene amplification (31) by alteration of downstream molecules, such as AKT (32), might play a role in the acquisition of resistance to gefitinib.

Mutations in *KRAS* are associated with a lack of sensitivity to gefitinib and erlotinib (23). We looked for *KRAS* mutations because of the possibility that acquired *KRAS* mutations are associated with acquired resistance. There were no *KRAS* mutations in any tumor. The same finding has been reported in a previous study (10), suggesting that *KRAS* mutations are not associated with acquired resistance.

In conclusion, half of tumors with acquired resistance to gefitinib had secondary T790M mutations. No novel mutations in the *EGFR* gene were present in contrast to CML.

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Establishment and characterization of four malignant pleural mesothelioma cell lines from Japanese patients

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Malignant pleural mesothelioma (MPM) is an asbestos-related malignancy that is highly resistant to current therapeutic modalities. We established four MPM cell lines (ACC-MESO-1, ACC-MESO-4, Y-MESO-8A and Y-MESO-8D) from Japanese patients, with the latter two from the same patient with biphasic-like characteristics of MPM, showing epithelial and sarcomatous phenotypes, respectively, in cell culture. These cells grew well in RPMI-1640 medium supplemented with 10% fetal bovine serum under 5% CO₂. Mutation and expression analyses demonstrated that the tumor suppressor gene *NF2*, which is known to be one of the most frequently mutated in MPM, is mutated in ACC-MESO-1. We detected homozygous deletion of *p16^{INK4A}/p14^{ARF}* in all four MPM cell lines. However, mutations of other tumor suppressor genes, including *TP53*, and protooncogenes, including *KRAS*, *NRAS*, *BRAF*, *EGFR* and *HER2*, were not found in these cell lines. Polymerase chain reaction amplification of the simian virus 40 sequence did not detect any products. We also analyzed genetic alterations of six other MPM cell lines and confirmed frequent mutations of *NF2* and *p16^{INK4A}/p14^{ARF}*. To characterize the biological differences between Y-MESO-8A and Y-MESO-8D, we carried out cDNA microarray analysis and detected genes that were differentially expressed in these two cell lines. Thus, our new MPM cell lines seem to be useful as new models for studying various aspects of the biology of human MPM as well as materials for the development of future therapies. (*Cancer Sci* 2006; 97: 387–394)

Malignant mesothelioma is an aggressive neoplasm arising from mesothelial cells that most often occurs in the pleural cavity as MPM. MPM is considered to be associated with previous exposure to asbestos fibers. Owing to the long latency period after exposure and the widespread use of asbestos fibers for many years, the incidence of MPM is projected to rise sharply worldwide in the next two decades.⁽¹⁾ In Japan, 500 patients with MM died in 1995, and that number increased to approximately 900 patients in 2003.⁽²⁾

Several clinical problems regarding the diagnosis, pathophysiology and treatment of MM remain unsolved. In particular, MM has been demonstrated to be resistant to all conventional therapy regimens, including chemotherapy, radiotherapy and surgery, and the prognosis of patients remains very poor.⁽³⁾

The discrepancy between the rising incidence of MM and the lack of success of new more effective therapeutic strategies may be related at least in part to inadequate knowledge of the biological properties of this tumor. It is hoped that a better understanding of MM biology may provide the rationale for new therapeutic strategies. In this regard, the development of tumor cell lines has been an important tool in setting up suitable *in vitro* models for studying the biological properties of many tumors and to assess tumor sensitivity to various drugs or biological response modifiers. However, as opposed to lung cancer, for example, where several hundred cell lines have been established, a relatively small number of MPM cell lines have been established,^(4–8) and only a few cell lines are available in tissue culture banks such as the ATCC. Furthermore, according to previous reports, only a few cell lines were established from Japanese patients with MPM.⁽⁹⁾

In the present study, four MPM cell lines, designated ACC-MESO-1, ACC-MESO-4, Y-MESO-8A and Y-MESO-8D (the latter two being from the same patient with biphasic-like characteristics of MPM), were established from Japanese patients, and their genetic alterations were analyzed. The TSG and protooncogenes analyzed were *NF2*, *p16^{INK4A}/p14^{ARF}*, *TP53*, *KRAS*, *NRAS*, *BRAF*, *EGFR* and *HER2*, the first three of which were reported to be inactivated in MPM.⁽¹⁰⁾ We found a point mutation of *NF2* in ACC-MESO-1 and homozygous deletion of *p16^{INK4A}* in all four cell lines. As there has been no prior report of two distinct morphologically different MPM cell lines being established from the same patient, we characterized the biological and genetic properties of Y-MESO-8A and Y-MESO-8D in detail, including tumorigenicity in nude mice, and found different gene expression profiles between these cell lines, with some genes encoding molecules involved in cell structural activity or cell adhesion being preferentially expressed in one cell line rather than the other.

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Abbreviations: ATCC, American Type Tissue Culture Collection; IL8, interleukin 8; MM, malignant mesothelioma; MPM, malignant pleural mesothelioma; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SDS, sodium dodecylsulfate; SSC, saline-sodium citrate; SSCP, single-strand conformation polymorphism; STR, short tandem repeat; SV40, simian virus 40; TSG, tumor suppressor gene.

Materials and Methods

Patient and establishment of cell lines

Three Japanese patients with pleural thickening or pleuritis were diagnosed with malignant mesothelioma through routine histopathological examination of haematoxylin–eosin staining and/or immunohistochemical studies (including carcinoembryonic antigen [CEA], vimentin and carletinin). ACC-MESO-1 was established from a 61-year-old Japanese woman, ACC-MESO-4 from a 59-year-old Japanese man, and Y-MESO-8 A and Y-MESO-8D from a 60-year-old Japanese man. The patient with ACC-MESO-4 had a history of asbestos exposure, but the remaining two patients did not have any obvious history. Cell cultures were established using a method similar to that described previously,⁽¹¹⁾ with approval by local ethical committees. Briefly, after collection of the materials, the pleural effusion of 20 mL or dissected tumor samples were transferred into a 75-cm² culture flask. They were incubated at 37°C in a humidified incubator containing 5% CO₂ with replacement of fresh RPMI-1640 medium (Sigma Aldrich, Irvine, UK) supplemented with 10% fetal bovine serum (Equitech-Bio, Ingram, TX, USA) and 1 × antibiotic-antimycotic (Gibco BRL Life Technologies, Rockville, MD, USA). Thereafter, the medium was replaced twice a week. Significant contaminant cells, such as fibroblast cells, were removed by scraping.

During the subsequent period of continuous propagation by culture, the cells were sampled at intervals, resuspended in the Cell Banker freezing medium (Juji Field, Tokyo, Japan), and stored in liquid nitrogen. After thawing, the stored cells could be propagated in culture without noticeable change in growth and morphology. Tumor cells grown in the flasks were examined directly with an inverted microscope, and phase-contrast photographs were taken periodically.

Source of other cell lines

Three MPM cell lines (NCI-H28 [CRL5820], NCI-H2373 [CRL5943] and MSTO-211H) were purchased from ATCC (Rockville, MD, USA). The other three MPM cell lines (NCI-H290, NCI-H513 and NCI-H2052) and a lung cancer cell line of an adenocarcinoma (NCI-H358) were gifts from Dr Adi F. Gazdar. All cells were grown in RPMI-1640 supplemented with 10% fetal bovine serum and 1 × antibiotic-antimycotic at 37°C in a humidified incubator with 5% CO₂.

Preparation of DNA and RNA

DNA and RNA were prepared from cell lines by standard techniques.⁽¹²⁾ Random-primed, first-strand cDNAs were synthesized from 2 µg of total RNA using Superscript II according to the manufacturer's instructions (Invitrogen, New York, NY, USA).

Mutation analysis

Mutation analyses were carried out either by direct sequencing after genomic PCR amplification and/or SSCP analysis followed by sequencing using aberrant bands. Sequencing analysis was carried out using an Applied Biosystems Model 3100 DNA sequencer (Perkin-Elmer Cetus, Norwalk, CT, USA) with a PCR primer and a BigDye terminator Cycle sequencing FS Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). SSCP analysis was carried out on mutation detection

enhancement (MDE) gels (FMC Bioproducts, Rockland, ME, USA) containing 10% glycerol, as described previously.⁽¹³⁾

For *TP53* mutation, PCR-SSCP analysis was carried out for exons 2–11 using genomic DNA to cover the entire coding frame of *TP53*. Primers used were as described previously.⁽¹⁴⁾

For *NF2* mutation, direct sequencing was carried out, and primers used were as follows: NF2exon1S, 5'-AGGCCTGTGC-AGCAACTC-3'; NF2exon1AS, 5'-GAGAACCTCTCGAGCT-TCCAC-3'; NF2exon2S, 5'-GAGAGTTGAGAGTGTCAGAG-3'; NF2exon2AS, 5'-TCAGCCCCACCAGTTTCATC-3'; NF2exon3S, 5'-GCTTCTTTGAAGGTAGCAC-3'; NF2exon3AS, 5'-GGTCAACTCTGAGGCCAACT-3'; NF2exon4S, 5'-CCTCACTTCCCCTCACAGAG-3'; NF2exon4AS, 5'-CCCATGACCCAAATTAACGC-3'; NF2exon5S, 5'-ATCTT-TAGAATCTCAATCGC-3'; NF2exon5AS, 5'-AGCTTTCTT-TTAGACCACAT-3'; NF2exon6S, 5'-CATGTGTAGGTTTT-TTATTTTGC-3'; NF2exon6AS, 5'-GCCCATAAAGGAATG-TAAACC-3'; NF2exon7S, 5'-CAGTGTCTCCGTTCTCC-3'; NF2exon7AS, 5'-AGCTCAGAGAGGTTTCAA-3'; NF2exon8S, 5'-CCACAGAATAAAAAGGGCAC-3'; NF2exon8AS, 5'-GATCTGCTGGACCCATCTGC-3'; NF2exon9S, 5'-GTTCTGCTTCTTCTCC-3'; NF2exon9AS, 5'-GTAATG-AAAACCAGATC-3'; NF2exon10S, 5'-CCTTTTAGTCTG-CTTCTG-3'; NF2exon10AS, 5'-TCAGTTAAAACAAGGTTG-3'; NF2exon11S, 5'-TCGAGCCCTGTGATTCAATG-3'; NF2exon11AS, 5'-AAGTCCCCAAGTAGCCTCCT-3'; NF2exon12S, 5'-CCCCTTCAGCTAAGAGCAC-3'; NF2exon12AS, 5'-CTCCTCGCCAGTCTGGTG-3'; NF2exon13S, 5'-GGTGTCTTTTCTGCTACCT-3'; NF2exon13AS, 5'-GGGAGGAAAGAGAACATCAC-3'; NF2exon14S, 5'-TGTGCCATTGCCTCTGTG-3'; NF2exon14AS, 5'-AGGG-CACAGGGGGCTACA-3'; NF2exon15S, 5'-TCTCACTGT-CTGCCAAAG-3'; NF2exon15AS, 5'-GATCAGCAAATA-CAAGAAA-3'; NF2exon16S, 5'-CTCTCAGCTTCTTCTC-TGCT-3'; NF2exon16AS, 5'-CCAGCCAGCTCCTATGGATG-3'; NF2exon17S, 5'-GGCATTGTTGATATCACAGGG-3'; and NF2exon17AS, 5'-GGCAGCACCATCACCACATA-3'. Numbers after exon indicate exon number, and S and AS indicate sense and antisense, respectively.

Polymerase chain reaction of *p16^{INK4A}* was carried out using the primer sets: p16ex1S, 5'-TGCCACATTCGCTAAGTGCT-3'; p16ex1AS, 5'-GCTGGCGGAAGAGCCC-3'; p16ex2S, 5'-GTGGACCTGGCTGAGGAGC-3'; p16ex2AS, 5'-TCTCAGGGTACAAATTCTCAGATCAT-3'; p16ex3S, 5'-AAGAAAAACACCGCTTCTGCC-3'; and p16ex3AS, 5'-TCCCTAGTTCACAAATGCTTGTC-3'.

For *KRAS*, *NRAS* and *BRAF* mutations, direct sequencing was carried out, and the primers for *KRAS* and *NRAS* were as described previously.⁽¹⁵⁾ PCR of *BRAF* was carried out using the following primer sets: BRAF11S, 5'-TTCTGTTTGGC-TTGACTTGAC-3' and BRAF11AS, 5'-CTATTATGACTT-GTCACAATGTCACC-3' for exon 11; and BRAF15S, 5'-TCATAATGCTTGCTCTGATAGGA-3' and BRAF15AS, 5'-GGCCAAAATTTAATCAGTGGA-3' for exon 15.

Tumorigenicity in nude mice

The cultured cells (4×10^6) were washed, resuspended in 0.2 mL of PBS, and injected subcutaneously into the left flank of 10 6-week-old BALB/c (nu/nu) female nude mice. As a control, 0.2 mL of PBS alone was similarly injected into the right

flank of the nude mice. The animals were examined every week for the development of tumors. Tumor volume was calculated as length \times height \times width \times 0.5. All animal care was in accordance with institutional guidelines. After the single tumor-bearing mouse was killed, the tumor tissue was excised, fixed in 10% formalin and processed for routine histopathological examination.

Genetic analysis

To confirm that there was no cross-contamination of cell lines, the uniqueness of the established cell line was evaluated by analysis of STR polymorphisms using the AmpFISTR Identifier Kit (Applied Biosystems). This kit includes 16 STR loci, which are D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, Amelogenin, D5S818 and FGA.

Western blot analyses

Preparation of total cell lysates and western blotting were carried out as described previously.⁽¹⁶⁾ In brief, cells growing subconfluently were rinsed twice with PBS, lysed in SDS sample buffer (62.5 mM Tris pH 6.8, 2% SDS, 2% 2-mercaptoethanol, 10% glycerol) and homogenized. Total cell lysate protein (15 μ g) was subjected to SDS-polyacrylamide gel electrophoresis and transferred to poly(vinylidene fluoride) (PVDF) membranes (Millipore, Bedford, MA, USA). Following blocking with 5% non-fat dry milk, the filters were incubated with the primary antibody, washed with PBS, reacted with the secondary antibody, and then detected with ECL (Amersham Biosciences, Buckinghamshire, UK). The primary antibodies used were anti-E-cadherin antibody (C20820; Pharmingen/Transduction Laboratories, San Diego, CA, USA), anti-N-cadherin antibody (C70320; Pharmingen/Transduction Laboratories), and anti-ERC/mesothelin antibody (IBL, Gunma, Japan).

Microarray analysis

The fluorescently labeled cRNA targets were prepared by incorporation of CyDye-NTP through *in vitro* transcription reaction. Aliquots of total RNA (4 μ g) from Y-MESO-8A and Y-MESO-8D were labeled using RNA Transcript SureLABEL Core Kit (TAKARA BIO, Otsu, Japan) with Cy5-UTP and Cy3-UTP (Amersham Biosciences), respectively, in each paired case. We used the commercially available IntelliGene HS Human Expression chip (TAKARA BIO) to carry out microarray analysis.

Labeled probes were mixed with hybridization solution (6 \times SSC, 0.2% SDS, 5 \times Denhardt's solution, 0.1 mg/mL denatured salmon sperm DNA, 50% formamide). After hybridization for 16 h at 65°C, the slides were washed twice in 2 \times SSC and 0.1% SDS for 5 min at 65°C, once in 2 \times SSC and 0.1% SDS for 5 min at 65°C, and once in 0.05 \times SSC for 5 min at room temperature. The slides were scanned using the Affymetrix 428 scanner (Affymetrix, Santa Clara, CA, USA). The signal intensity of hybridization was evaluated photometrically by the ImaGene computer program (BioDiscovery, El Segundo, CA, USA) and normalized to the averaged signals of housekeeping genes (or global normalization). A cut-off value for each expression level was calculated according to the background fluctuation.

Results

Establishment of MPM cell lines

The new MPM cell lines ACC-MESO-1, ACC-MESO-4, Y-MESO-8A and Y-MESO-8D were established successfully from a 61-year-old Japanese woman, a 59-year-old Japanese man, and a 60-year-old Japanese male (Fig. 1). Microscopically, the original tumors of ACC-MESO-1 and ACC-MESO-4 mainly consisted of epithelioid cells, and that of Y-MESO-8A

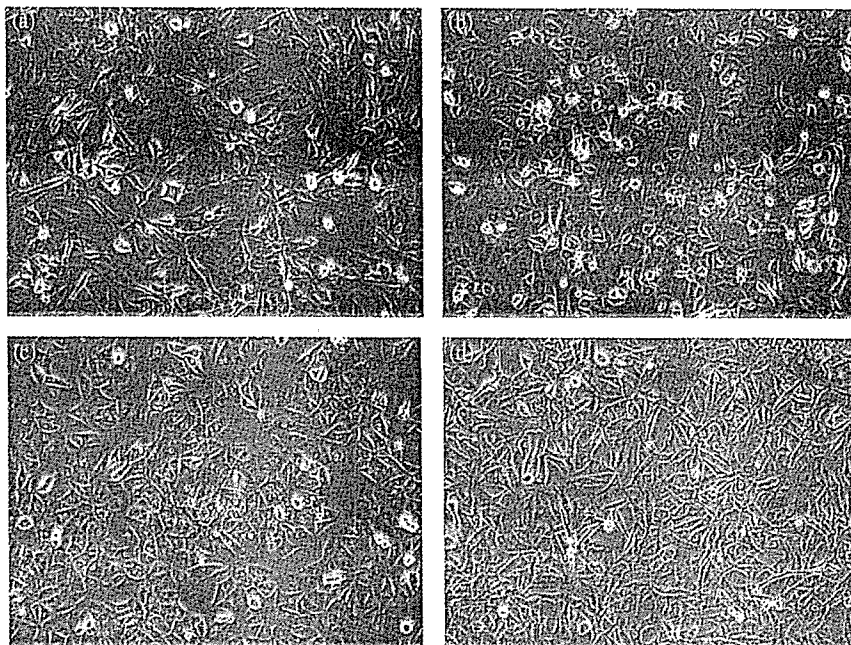


Fig. 1. Morphology of the four malignant pleural mesothelioma (MPM) cell lines. Micrographs of cultured (a) ACC-MESO-1 cells, (b) ACC-MESO-4 cells, (c) Y-MESO-8A cells, and (d) Y-MESO-8D cells (original magnifications \times 100).

Table 1. Genetic alterations in 10 mesothelioma cell lines

Cell line	ACC-MESO-1	ACC-MESO-4	Y-MESO-8A	Y-MESO-8D	H28	H290	H513	H2052	H2373	MSTO-211H
<i>TP53</i>	+	+	+	+	+	+	Nt717insA	+	+	+
<i>NF2</i>	Q389X	+	+	+	+	HD [†]	ND	R341X [†]	HD [†]	+
<i>p16^{INK4A}/p14^{ARF}</i>	HD	HD	HD	HD	HD	HD	HD	HD	HD	HD
<i>KRAS</i>	+	+	+	+	+	+	+	+	+	+
<i>NRAS</i>	+	+	+	+	+	+	+	+	+	+
<i>BRAF</i>	+	+	+	+	+	+	+	+	+	+
<i>EGFR</i>	+	+	+	+	+	+	+	+	ND	+
<i>HER2</i>	+	+	+	+	+	+	+	+	ND	+

[†]Refer to Sekido *et al.* 1995. +, Undetectable mutation for target regions; HD, homozygous deletion; ND, not determined; Nt, nucleotide.

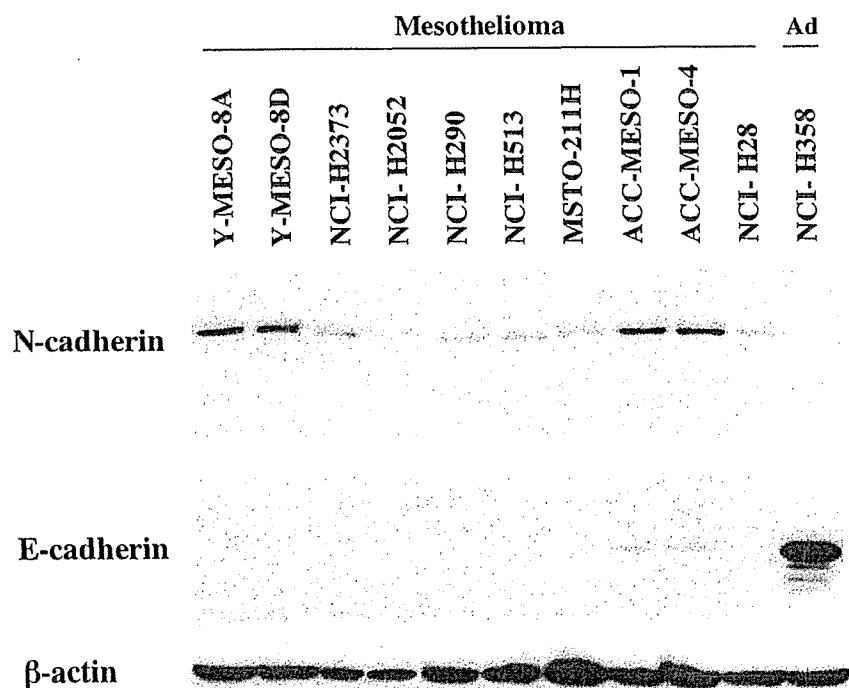


Fig. 2. Western blot analysis of E-cadherin and N-cadherin. Each lane was loaded with 15 µg of total cell lysate from Y-MESO-8A (lane 1), Y-MESO-8D (lane 2), NCI-H2373 (lane 3), NCI-H2052 (lane 4), NCI-H290 (lane 5), NCI-H513 (lane 6), MSTO-211H (lane 7), ACC-MESO-1 (lane 8), ACC-MESO-4 (lane 9), NCI-H28 (lane 10) and NCI-H358 (lane 11). β-Actin is shown as an internal control at the bottom.

and Y-MESO-8D mainly consisted of spindle cells. Because the primary cultures of Y-MESO-8 showed several colonies with different morphological types, they were subcloned. Y-MESO-8A showed polygonal and epithelial-like morphology, whereas Y-MESO-8D showed spindle-like morphology (Fig. 1c,d). These cell lines grew as adherent monolayers and maintained a consistent morphology from the primary culture to the following passages. After thawing, the cryopreserved cells were able to propagate in culture without noticeable change in growth and morphology.

Mutation and expression analyses

To determine whether the four new cell lines harbored genetic changes of tumor suppressor genes, reported as frequently detected in MPM, we carried out mutation and expression analyses along with six other MPM cell lines. The tumor suppressor genes studied were *TP53*, *NF2* and *p16^{INK4A}/p14^{ARF}*. We also analyzed protooncogenes, including *KRAS*, *NRAS*, *BRAF*, *EGFR* and *HER2*. The results are summarized in Table 1. Among the four new cell lines, homozygous deletions of *p16^{INK4A}/p14^{ARF}* were detected in all four, whereas *NF2* was

shown to be inactivated by a nonsense mutation (Q389X) only in ACC-MESO-1. Neither mutation nor homozygous deletion of *TP53* was found.

To determine whether the SV40 large T antigen was involved in the pathogenesis of our new MPM cell lines, we carried out PCR analysis to detect the DNA of large T antigen. However, we found no evidence to indicate implication of the SV40 (data not shown).

Furthermore, we also tested expression of cell adhesion molecules, E-cadherin and N-cadherin, with western blot analysis, as these have been reported to be expressed aberrantly in MPM as well as being useful for differential diagnosis from poorly differentiated adenocarcinoma. E-cadherin expression was detected in an adenocarcinoma cell line, NCI-H358, and two mesothelioma cell lines, ACC-MESO-1 and ACC-MESO-4, but not in the other mesothelioma cell lines, Y-MESO-8A, Y-MESO-8D, NCI-H2373, NCI-H2052, NCI-H290, NCI-H513, MSTO-211H and NCI-H28. However, N-cadherin expression was detected in Y-MESO-8A, Y-MESO-8D, NCI-H2373, NCI-H2052, NCI-H290, NCI-H513, MSTO-211H and NCI-H28 (Fig. 2). Finally, we tested the expression of ERC/

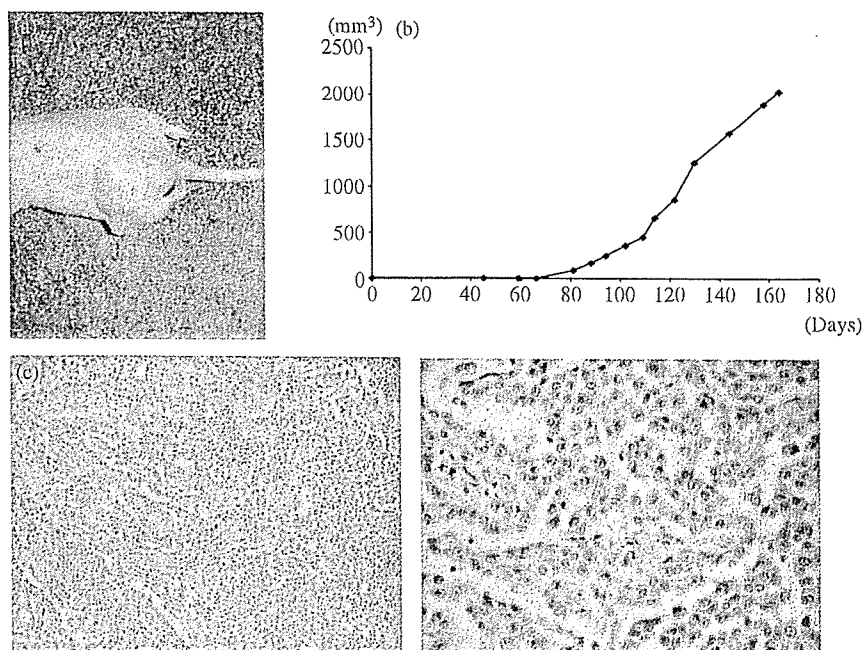


Fig. 3. (a) Xenografted tumor of Y-MESO-8A. (b) Growth curve of the xenografted tumor in nude mice. (c) Histology of xenografted Y-MESO-8A tumor (haematoxylin and eosin, original magnification $\times 100$ and $\times 400$). As in epithelioid mesothelioma, the tumor shows papillary proliferation consisting of atypical epithelioid cells.

mesothelin, which has been reported as being expressed in MPM, using western blot analysis. An expected strong 41-kDa band was observed for ACC-MESO-1, ACC-MESO-4, Y-MESO-8A, NCI-H2052 and NCI-H2373 (data not shown).

Genetic and biological differences between Y-MESO-8A and Y-MESO-8D

Y-MESO-8A and Y-MESO-8D were established from pleural effusion at the same time and showed distinct morphological patterns. As MPM are frequently composed from two separate subpopulations of cell groups (epithelial and sarcomatous types, which are diagnosed as biphasic MPM), we speculated that these two cell lines may represent both phenotypes, respectively.

First, we confirmed that Y-MESO-8A and Y-MESO-8D were from the same patient using a multiplex amplification and typing system for 16 STR with DNA derived from the white blood cells of the patient (data not shown). To determine the differences in tumorigenicity between Y-MESO-8A and Y-MESO-8D cells, we inoculated the cells into athymic nude mice. After subcutaneous injection of the Y-MESO-8A cells, a visible subcutaneous tumor developed in only one of the 10 nude mice at the site of inoculation, with histological examination of the xenotransplanted nodules showing papillary proliferation consisting of atypical epithelioid cells (Fig. 3). Meanwhile, the Y-MESO-8D cells showed no visible subcutaneous tumors in any of the 10 inoculated nude mice. Lastly, we performed cDNA microarray analysis to determine the differences in expression profiles. The 43 genes showing over a 5-fold difference of expression between the two cell lines are listed in Table 2. With selected cDNA probes synthesized, we carried out northern blot analyses for genes including *CRIP1* and *VCAM1* and confirmed the differences of expression between Y-MESO-8A and Y-MESO-8D (data not shown).

In addition, we also carried out cytogenetic analysis of the Y-MESO-8A cells at passages 11 (data not shown). The

modal chromosome number from the 12-karyotype analysis ranged from 41 to 91 with a median of 57. Extra copies of chromosomes 1, 3, 8, 11, 12, 15, 16, 17, 20 and X were noted in some metaphases, whereas loss of chromosomes 9, 13, 14 and 22 was noted in others. Homogeneous staining regions or double minutes were not detected.

Discussion

The development of tumor cell lines has been an important tool in establishing suitable *in vitro* models for studying the biological properties of many tumors. Various types of human tumors have been selected for establishment of cell cultures, and in lung cancer, for example, there are several hundred cell lines that have been developed in many laboratories worldwide during recent decades.⁽¹⁷⁻²⁰⁾ In contrast, the number of other specific tumor cell lines is still small because of the rare incidence of diseases, infrequent availability of fresh specimens, and technical difficulties in cell culture development, all of which result in a large impediment for studying tumors aimed at basic and preclinical research. MPM is one such example, and MPM cell lines, which are available from public bioresource banks including ATCC, are very few.⁽⁴⁻⁸⁾ Furthermore, only a few cell lines have been established from Japanese patients with MPM,⁽⁹⁾ and there are only several abstracts from the Japan Medical Abstracts Society reporting the establishment of a single MPM cell line. In the present study, we established four MPM cell lines (ACC-MESO-1, ACC-MESO-4, Y-MESO-8A and Y-MESO-8D), derived from Japanese patients, characterized their genetic abnormalities and detected genes differentially expressed between Y-MESO-8A and Y-MESO-8D, which were derived from the same patient.

Traditional cytogenetic and loss of heterozygosity analyses, followed by recent comparative genomic hybridization techniques, identified common chromosomal abnormalities

Table 2. Genes showing over a five-fold difference in expression between Y-MESO-8A and Y-MESO-8D

Gene name	Accession no.	Molecular function	Ratio
Y-MESO-8A/Y-MESO-8D ≥ 5			
Potassium large conductance calcium-activated channel, subfamily M, α member 1 (KCNMA1)	NM_002247.2	Unknown	19.2
Microfibril-associated glycoprotein-2 (MAGP2)	NM_003480.1	Structural molecule activity	15.4
Filaggrin (FLG)	XM_048104.1	Structural molecule activity	14.5
Cysteine-rich protein 1 (intestinal) (CRIP1)	NM_001311.2	Binding	13.9
UDP-N-acetyl- α -D-galactosamine : polypeptide N-acetylgalactosaminyltransferase 5 (GALNT5)	XM_050509.6	Unknown	11.8
Hypothetical protein FLJ14834 (FLJ14834)	NM_032849.2	Unknown	11.1
Glutathione peroxidase 6 (GPX6)	NM_015696.2	Unknown	10.7
Decorin (DCN), transcript variant A1	NM_001920.2	Unknown	10.5
KIAA0193 gene product (KIAA0193)	NM_014766.2	Catalytic activity	10.4
Lumican	NM_002345.2	Morphogenesis	9.24
Selenium binding protein 1	NM_003944.2	Unknown	8.99
ATP-binding cassette, subfamily B (MDR/TAP)	NM_000927.2	Cell growth	8.63
S100 calcium binding protein A4	NM_002961.2	Cell growth	7.06
Plasminogen activator	NM_000930.2	Unknown	7.01
Adenylate cyclase activating polypeptide 1	NM_001117.2	Cell communication	6.88
Serine protease inhibitor	NM_021102.1	Cell motility	6.77
Rec8p, a meiotic recombination and sister chromatid cohesion phosphoprotein of the rad21p family	NM_005132.1	Unknown	6.58
Adipose specific 2	NM_006829.1	Unknown	6.52
Tissue inhibitor of metalloproteinase 3	NM_000362.3	Unknown	6.38
EphA3	NM_005233.2	Cell communication	6.34
Sodium channel, non-voltage-gated 1 α	NM_001038.1	Cell growth	6.31
Podocalyxin-like	NM_005397.1	Unknown	6.21
Cut-like 1, CCAAT displacement protein	NM_001913.1	Unknown	5.98
Ocular albinism 1	NM_000273.1	Cell communication	5.90
Paternally expressed 10	NM_015068.1	Unknown	5.89
Cytochrome P450, family 26, subfamily A, polypeptide 1 (CYP26A1)	NM_057157.1	Unknown	5.84
Desmoplakin	NM_004415.1	Morphogenesis	5.64
Complement component 4B	NM_000592.3	Unknown	5.60
Y-MESO-8D/Y-MESO-8A ≥ 5			
Kynureninase (L-kynurenine hydrolase) (KYNU)	NM_003937.1	Unknown	69.4
Aminopeptidase (LOC64167)	NM_022350.1	Unknown	32.8
Aldo-keto reductase family 1, member B10 (aldose reductase) (AKR1B10)	NM_020299.3	Unknown	17.8
Annexin A10 (ANXA10)	NM_007193.2	Unknown	15.3
Vascular cell adhesion molecule 1 (VCAM1), transcript variant 1	NM_001078.2	Cell communication	14.2
Hypothetical protein FLJ30834 (FLJ30834)	NM_152399.1	Unknown	10.9
Hypothetical protein FLJ33957 (FLJ33957)	NM_152322.1	Unknown	9.47
Protease inhibitor 3, skin-derived (SKALP) (PI3)	NM_002638.1	Unknown	9.16
Interleukin 8 (IL8)	NM_000584.2	Cell growth	9.00
Interleukin 1, α (IL1A)	NM_000575.3	Cell growth	7.50
Aldo-keto reductase family 1, member C3 (AKR1C3)	NM_003739.4	Cell growth	6.35
Transmembrane 4 superfamily member 2 (TM4SF2)	NM_004615.2	Unknown	6.14
Glutathione S-transferase theta 2 (GSTT2)	NM_000854.2	Unknown	5.84
Hypothetical protein FLJ22761 (FLJ22761)	NM_025130.1	Unknown	5.74
Solute carrier family 21 (organic anion transporter), member 9 (SLC21A9)	NM_007256.1	Cell growth	5.04

in MPM cells, including deletions on chromosomes 1, 3, 4, 9, 11, 14 and 22, some of which have already been shown to harbor target TSG for MPM.⁽²¹⁻²⁶⁾ *NF2*, which is located on chromosome 22q12 and is known to be one of the most frequently mutated TSG in MPM,⁽¹⁰⁾ was mutated in ACC-MESO-1, although we found no *NF2* mutation in the other three new MPM cell lines. *p16^{INK4A}*, which is located on chromosome 9p21 and is involved in the development of many other types of cancers, has also been shown to be a target gene for MPM with frequent homozygous deletions being identified.⁽²⁷⁾ In the present study, we found homozygous

deletions of *p16^{INK4A}* in all four cell lines using primers of exons 1, 2 and 3 (Table 1), indicating that in the *p14^{ARF}* gene, a second coding frame using another exon 1 (exon1 β), along with exons 2 and 3, is also completely inactivated. However, a detailed analysis of 9p21 homozygous deletions in lung cancer have also identified that the deletion regions extend beyond the *p16^{INK4A}/p14^{ARF}* gene locus and affect other genes in the vicinity, including *p15*.⁽²⁸⁾ Thus, further analyses concerning these homozygous deletions in 9p21 should determine whether genes other than *p16^{INK4A}/p14^{ARF}* are also targeted in MPM.

In contrast, although *TP53* is known to be one of the most frequently mutated TSG, previous analyses have shown that only a small subset of MPM have a *TP53* mutation.⁽²¹⁾ Consistent with this, we also did not find any activating mutation of the *TP53* gene in any of the four new cell lines. Taken together, although the number of cell lines analyzed was small, the frequencies of genetic alterations in our new MPM cell lines, including mutation of *NF2* and *TP53* and homozygous deletion of *p16^{INK4A}/p14^{ARF}*, seem to be similar to previous reports, which may suggest that MPM from Japanese patients share common genetic abnormalities with Caucasians.

Meanwhile, SV40, which encodes two tumor antigens (large T and small t), has a well-characterized ability to trigger transformation of cells in culture. Recently, several studies suggested that SV40 is involved in the development of human mesothelioma, which was shown by detection of DNA sequences encoding the SV40 large T antigen and/or its protein expression.^(29–32) However, some reported findings are strongly against a role for SV40 in the development of human MPM, and thus the implication of SV40 remains controversial.⁽³³⁾ To determine whether the SV40 large T antigen is involved, we also carried out PCR analysis to detect the DNA of large T antigen in our new MPM cell lines, as well as in six other MPM cell lines. However, we did not find any evidence for implication of the SV40 large T antigen.

Differential diagnosis of MPM from other thoracic malignancies, including poorly differentiated lung adenocarcinoma, is often difficult. Several molecular markers, including carletinin, Wilms' tumor 1, cytokeratin 5/6 and mesothelin, have been suggested to be useful in distinguishing them.⁽³⁴⁾ E-cadherin and N-cadherin expression has also been used to distinguish MPM from adenocarcinoma, which is related to tumor invasion or progression.⁽³⁵⁾ We also confirmed the expression of these cadherins in our newly established cell lines. E-cadherin is expressed in epithelial cells, and reduction in the expression of E-cadherin has been associated with higher malignancy potential and invasiveness in epithelial neoplasms of the colon, ovary, stomach, pancreas, lung, breast, and head and neck, due to lack of cell–cell adhesion. Meanwhile, N-cadherin is restricted to tissues of nerve cells, developing skeletal muscle, embryonic and mature cardiac muscle cells, and mesothelial cells. Because the mesoderm-derived mesothelial cells that form the pleura express N-cadherin during embryonic development, it is suggested that N-cadherin plays an important role in the development and differentiation of mesothelial cells. Thus, the expression of N-cadherin in malignant mesothelioma has been thought to reflect its cell lineage and phenotype. Although it is not clearly understood how N-cadherin expression affects MPM cells pathologically, cell adhesion molecules including N-cadherin should be reconsidered in terms of the uniqueness

of MPM progression, such as highly aggressive invasion of the thoracic region but only rare metastasis to distant organs.

Malignant pleural mesothelioma is usually classified into three pathological subtypes, epithelioid, sarcomatous and biphasic, but it is not clearly understood why MPM shows these variations in morphology, even in a single tumor. To our knowledge, this is the first report of two morphologically distinct MPM cell lines being established from the same patient. During many passages of Y-MESO-8A and Y-MESO-8D, both cell lines maintained consistent phenotypes, even under tissue culture condition, which is thought to be useful for *in vitro* models of MPM biphasic type. In the present study, we tried to determine the underlying mechanisms that affect the morphological differences in Y-MESO-8A and Y-MESO-8D. As described above, because we did not find any differences in the genetic abnormalities of major cancer-associated genes between the two cell lines, we suspect other genetic alterations or epigenetic alterations may account for the differences. To study this in more detail, we are currently carrying out further analyses, including comparative genomic hybridization, and preliminary data suggest that there are some differences in chromosomal gains and losses among most shared genotypes (data not shown). However, we also carried out expression profiling and found 15 genes with over a 10-fold difference in mRNA expression between the two cell lines. Among these genes, vascular cell adhesion molecule 1, microfibril-associated glycoprotein-2 and filaggrin, which are related to morphology, were found to be expressed in one cell line but not the other, which also needs to be analyzed for genetic or epigenetic changes to discover underlying mechanisms. It is also interesting to note that *IL8* expression was different between the two cell lines. Whereas *IL8* has been suggested to be involved in cell growth of MPM,⁽³⁶⁾ the expression might also be related to morphological differences between these cell lines.

In conclusion, we have established and characterized new human malignant mesothelioma cell lines, designated ACC-MESO-1, ACC-MESO-4, Y-MESO-8A and Y-MESO-8D, from Japanese patients. These cell lines will provide us with a new experimental system to study pathogenesis and biological behavior, as well as to test new therapeutic reagents of MPM.

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Prospective Validation for Prediction of Gefitinib Sensitivity by Epidermal Growth Factor Receptor Gene Mutation in Patients with Non-Small Cell Lung Cancer

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Introduction: We evaluated the efficacy of gefitinib monotherapy prospectively in patients with advanced or pretreated non-small cell lung cancer (NSCLC) harboring epidermal growth factor receptor (EGFR) mutations.

Methods: Patients with NSCLC were examined for EGFR exon 19 deletion mutations by fragment analysis and for EGFR L858R point mutations by the Cycleave polymerase chain reaction technique. EGFR mutation-positive patients with locally advanced, metastatic, or recurrent/refractory NSCLC that was not curable with surgery or thoracic radiotherapy were candidates for gefitinib treatment administered at 250 mg/day until disease progression.

Results: Mutations of the EGFR gene were detected in 27 (41%) of 66 patients. Ten had exon 19 deletion, and 17 had L858R. Twenty-one patients harboring EGFR mutations were treated with gefitinib and were considered assessable for responses and adverse events. Nineteen patients with EGFR mutations achieved objective responses (three complete responses and 16 partial responses), resulting in an overall response rate of 90.5% (95% confidence interval, 69.6%–98.8%). The median progression-free survival was 7.7 months (95% confidence interval, 6.0 mo to not reached). The median overall survival has not been reached. Common adverse events were skin toxicity, diarrhea, and elevated aminotransferases, but no pulmonary toxicity was observed.

Conclusions: Detection of common EGFR mutations seems to be useful for selecting patients with NSCLC who would likely benefit from gefitinib monotherapy.

Key Words: EGFR, Gefitinib, Lung cancer, Mutations, Drug sensitivity.

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Lung cancer remains the most common cause of cancer death in both men and women worldwide. Lung cancer frequently presents at an advanced and biologically aggressive stage, resulting in poor prognosis. Non-small cell lung cancer (NSCLC) accounts for more than 80% of all lung cancers. Currently, platinum-based combination chemotherapy regimens, including several active new chemotherapeutic agents, comprise the standard option for patients with advanced NSCLC. However, various combinations of drugs have similar efficacy, producing objective response rates of 30 to 40%, median survival time of eight to 10 months, and 1-year survival rates of 30 to 40%.^{1,2} These results remain unsatisfactory, and new modalities of treatment are urgently awaited. Recently, novel molecular targeted strategies that block cancer progression pathways have been suggested as the ideal treatment to control cancer and are considered an exciting therapeutic approach for treating NSCLC.³

The epidermal growth factor receptor (EGFR) is a 170 kDa receptor tyrosine kinase and a member of the erbB receptor family that plays a pivotal role in the signaling processes of tumor progression.^{4–6} EGFR is overexpressed in several solid tumors, including NSCLC, and it is one of the leading therapeutic molecular targets.⁷ Gefitinib is an orally bioavailable, selective EGFR tyrosine kinase inhibitor (TKI) and was the first targeted drug for NSCLC. Phase II and III monotherapy trials for patients pretreated for NSCLC demonstrated objective response rates of only 8 to 18%.^{8–10} However, subset analyses of these trials and a retrospective study¹¹ showed a small group of clinical responders comprising women, patients with adenocarcinomas, nonsmokers, and Japanese or Asian patients. These results suggest that identifying predictive molecular or genetic biomarkers for gefitinib sensitivity may be useful for selecting patients who are most likely to benefit from treatment.

In 2004, three independent groups reported that somatic EGFR mutations correlated with sensitivity of NSCLC to gefitinib or erlotinib, another EGFR TKI.^{12–14} Subsequently, several groups confirmed this striking correlation between EGFR mutations and gefitinib sensitivity, yielding a response rate of about 60 to 94% in retrospective analyses.^{15–22} EGFR mutations are likely to be significantly associated with survival benefit attributed to gefitinib treatment.^{17,18,21} In con-

trast to these results, recent reports concerning molecular analyses of large-scale phase II and III trials showed lower response rates than previously reported and no survival benefit in patients with mutations treated with TKIs.^{23–26} Around the same time, the EGFR gene amplification/copy number was demonstrated as another useful predictive molecular marker of TKI efficacy.^{23,26–28} However, these contradictory results were obtained through the retrospective collection of tumor samples, and prospective validation studies that predict TKI efficacy by EGFR mutations are needed.

Data from previous reports show that in-frame deletions in exon 19 and specific missense mutation of codon 858 in exon 21 (L858R) account for about 90% of all EGFR mutations, and about 80% of responders to gefitinib or erlotinib harbor either of these two hotspot mutations. Therefore, we developed a rapid, sensitive screening assay of two hotspot mutations²⁹ and conducted a prospective cohort study to explore the prediction of gefitinib sensitivity in EGFR mutation-positive patients.

MATERIALS AND METHODS

Study Design

This prospective cohort study was conducted to identify patients with NSCLC who would most likely benefit from gefitinib treatment according to their EGFR mutation. Patients with EGFR mutation were treated with oral administration of gefitinib at a dose of 250 mg once a day until disease progression or intolerable toxicity occurred, or until the patient refused to continue treatment. The primary endpoint was objective tumor response rate. Secondary endpoints included adverse effects, disease control rate (response + stable disease), progression-free survival (PFS), and overall survival (OS). This study was approved by the institutional review board of Aichi Cancer Center Hospital.

Patient Eligibility

Eligibility criteria for gefitinib treatment were adult (age ≥ 20 yr) with cytologic or histologic confirmation; locally advanced, metastatic, or recurrent/refractory NSCLC that was not curable by surgery or radiotherapy; harboring EGFR mutation; and one or more measurable or assessable lesions. All patients were admitted to the study regardless of prior treatment, extent of performance status (PS), or main organ functions. The exclusion criteria were pulmonary fibrosis, interstitial pneumonia, or prior treatment with an EGFR TKI or antibody. All patients gave written informed consent in accordance with institutional regulations before entering the study.

Efficacy and Toxicity Evaluation

Tumor responses were evaluated according to the Response Evaluation Criteria in Solid Tumors³⁰ and were confirmed by repeated imaging studies after 4 to 8 weeks of gefitinib treatment. During the treatment and for 30 days after the last dose of gefitinib, patients were monitored for adverse events, which were graded using Common Terminology Criteria for Adverse Events, version 3.0. PFS was assessed from the date of gefitinib treatment until the date of objective

disease progression, death from any cause, or the last follow-up. OS was assessed from the date of gefitinib treatment until the date of death from any cause, or the last follow-up.

Detection of EGFR Mutations

Genomic DNA was extracted from tumors embedded in paraffin blocks or from aspirated tumors obtained in pleural effusions, superficial lymph nodes, or subcutaneous metastasis. All specimens were reviewed by a single reference pathologist (Y.Y.) and marked grossly near the tumor-rich lesion on an unstained slide to enrich the tumor cell population as much as possible.

We performed mutational analyses of exon 19 deletion and the L858R point mutation of the EGFR gene, as previously described.²⁹ Briefly, exon 19 deletion was determined by common fragment analysis using polymerase chain reaction (PCR) with an FAM-labeled primer set, and the PCR products were electrophoresed on an ABI PRISM 310 (Applied Biosystems, Foster City, CA). The shorter segment of DNA amplified by PCR showed a deletion mutation in a new peak in an electropherogram. The L858R mutation was detected by the Cycleave real-time quantitative PCR technique using the Cycleave PCR core kit (Takara Co. Ltd., Ohtsu, Japan) with an L858R-specific cycling probe and a wild-type probe. Fluorescence intensity was measured with a Smart Cycler system (SC-100, Cepheid, Sunnyvale, CA).

Statistical Analysis

Data were analyzed using the chi-square test; $p < 0.05$ was regarded as significant. Confidence intervals (CI) were calculated using binomial CIs. PFS and OS were calculated using the Kaplan–Meier method and compared between two EGFR mutation groups using log-rank test. All the analyses were performed with Stata 8.2 for Macintosh (Stata Corp, College Station, TX).

RESULTS

Sampling Procedure for Detecting EGFR Mutations

Sixty-six consecutive patients with NSCLC were examined to detect the EGFR mutations from November 2004 through August 2005 at Aichi Cancer Center Hospital. Of these patients' samples, 23 specimens were obtained from bronchoscopic biopsy, 22 from computed tomography/ultrasound-guided needle biopsy, 13 from percutaneous aspiration (seven from pleural effusion, four from lymph nodes, and two from skin metastases), two from biopsy (one from tonsil metastasis and one from skin metastasis), and six from surgery with general anesthesia (three from thoracotomy, two from thoracoscopy, and one from mediastinoscopy (Table 1). Sixty samples (91%) were obtained from the biopsy or aspiration method. Tumor tissues or aspirates were procured at the time of initial diagnosis in 52 patients and at the time of tumor progression in 14 patients.

Patient Characteristics and EGFR Mutations

Mutations of the EGFR gene were detected in 27 (41%) of 66 patients. Ten of these had the deletion in exon 19, and

TABLE 1. Patient Characteristics and Sample Procurement According to EGFR Mutation Status

	EGFR Mutation Status			p
	All	Mutation	Wild type	
All cases	66	27 (21)	39	
Sex				0.175
Male	36	10 (8)	26	
Female	30	17 (13)	13	
Age (yr)				0.5084
≤64	31	14 (11)	17	
>64	35	13 (10)	22	
Histology				0.0199
Adenocarcinoma ^a	59	27 (21)	32	p (^a vs. ^b)
Squamous cell ^b	2	0	2	
Large cell ^b	2	0	2	
Pleomorphic ^b	1	0	1	
NSCLC NOS ^b	2	0	2	
Smoking status				0.0002
Never smoker ^c	24	17 (13)	7	p (^c vs. ^d)
Former smoker ^d	17	9 (7)	8	
Current smoker ^d	25	1 (1)	24	
Stage at initial diagnosis				0.6348
IA ^e	2	1	1	p (^e vs. ^f)
IIB ^e	4	2 (2)	2	
IIIA ^f	3	0	3	
IIIB ^f	16	3 (2)	13	
IV ^f	41	21 (17)	20	
Performance status				0.6059
0/1	51	20 (14)	31	p (0/1 vs. ≥2)
2	7	3 (3)	4	
3	3	1 (1)	2	
4	5	3 (3)	2	
Prior first treatment				ND
No	8	5 (5)	3	
Surgery	3	3 (1)	0	
Thoracic irradiation	4	2 (2)	2	
Chemoradiotherapy	10	2 (1)	8	
Bone irradiation	6	3 (3)	3	
Brain irradiation	6	3 (2)	3	
Sclerotherapy for effusion	1	1 (1)	0	
Chemotherapy	28	8 (6)	20	
Prior chemotherapy				0.4337
0	28	13 (12)	15	p (0 vs. ≥1)
One regimen	28	10 (6)	18	
Two regimens	8	4 (3)	4	
Three regimens	2	0	2	
Method for sample procurement				ND
Bronchoscopic biopsy	23	11	12	
CT/US-guided needle biopsy	22	6	16	
Pleural effusion aspiration	7	4	3	
LN/skin aspiration	6	2	4	
Tonsil/skin biopsy	2	0	2	
Thoracotomy	3	2	1	
VATS	2	1	1	
Mediastinoscopy	1	1	0	

EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer; NOS, not otherwise specified; ND, not done; CT/US, computed tomography/ultrasound; LN, lymph node; VATS, video-assisted thoracoscopy. Superscript letters indicate groups compared in the statistical analysis. Numbers in parentheses represent the numbers of patients receiving gefitinib treatment.

17 were the point mutation at codon 858. As previously reported,^{12-14,17} the EGFR mutations were significantly associated with adenocarcinoma histology and never-smoking status (Table 1). However, the EGFR mutation status was not significantly correlated with sex, age, PS, stage at initial diagnosis, or prior chemotherapy. Twelve patients received gefitinib treatment as the first-line chemotherapy; five patients desired first-line gefitinib therapy, and the other seven were unfit for conventional chemotherapy because of age (one patient, age 84 yr), cardiac disease (one patient), widespread bone metastases (two patients), and poor PS (3-4 in three patients).

Clinical Response and Survival

Of 27 patients harboring EGFR mutation, 21 were treated with gefitinib and were assessable for objective responses (Table 2) and adverse events (Table 3). The median interval of gefitinib treatment was 5.9 months (range, 0.67 to 11.4 mo). Of the assessable 21 patients, 19 patients achieved objective responses (three complete response and 16 partial response), for an overall response rate of 90.5% (95% CI, 69.6-98.8%). One patient had stable disease, giving an overall disease control rate of 95.2% (95% CI, 76.2-99.9%). According to EGFR mutation classes and PS, the objective responses were seven of eight for the exon 19 deletion, 12 of 13 for the L858R point mutation, 13 of 14 in PS 0 to PS 1 patients, and 6 of seven in PS 2 to PS 4 patients. The response to gefitinib did not differ significantly according to the mutation class or PS.

The median PFS was 7.7 months (95% CI, 6.0 mo to not reached) (Figure 1A). The median OS has not been reached at present (Figure 1B). Subset analyses showed that PFS was greater in patients with the exon 19 deletion than in those with the L858R point mutation (log rank test, $p = 0.04$; Fig 2A). The median PFS for the exon 19 deletion group was 7.8 months (95% CI, 7.6 mo to not reached); for the L858R mutation group, median PFS was 6.0 months (95% CI, 2.6 to 7.7 mo). OS did not differ significantly between the two types of mutations (Figure 2B). No difference was observed in PFS

TABLE 2. Response of EGFR Mutation-Positive Patients to Gefitinib Treatment

	EGFR Mutation Status		
	Exon 19 Deletion ($n = 8$)	L858R Mutation ($n = 13$)	Total ($n = 21$)
CR	1 (12.5%)	2 (15.4%)	3 (14.3%)
PR	6 (75%)	10 (76.9%)	16 (76.2%)
Overall response rate (CR + PR)	7 (87.5%)	12 (92.3%)	19 (90.5%)
SD	1 (12.5%)	0	1 (4.8%)
Disease control (CR + PR + SD)	8 (100%)	12 (92.3%)	20 (95.2%)
Progressive disease	0	1 (7.7%)	1 (4.8%)

EGFR, epidermal growth factor receptor; CR, complete response; PR, partial response; SD, stable disease.

TABLE 3. Number (%) of Patients with Treatment-Related Adverse Events ($n = 21$)

	Grade				
	0	1	2	3	4
Skin toxicity	15 (71)	4 (19)	2 (10)	0	0
Diarrhea	13 (62)	3 (14)	3 (14)	2 (10)	0
Elevated aspartate aminotransferase/ alanine aminotransferase	15 (71)	1 (5)	2 (10)	3 (14)	0
Nail changes	17 (81)	3 (14)	1 (5)	0	0
Mucositis	20 (95)	1 (5)	0	0	0
Joint pain	20 (95)	1 (5)	0	0	0

and OS between never-smokers and current/former smokers (data not shown).

Adverse Events

All 21 patients were evaluated for drug-related adverse events. The most common adverse events were skin toxicity, diarrhea, and elevated aspartate aminotransferase/alanine aminotransferase (AST/ALT) (Table 3). The grade 3 adverse events of diarrhea and elevated AST/ALT occurred in two (10%) and three (14%) patients, respectively. These events occurred slightly more frequently than in previous studies.^{8,9} No grade 4 adverse events or pulmonary toxicity were observed. Seven patients required an interruption of treatment, lasting 2 to 4 weeks, because of grade 2/3 diarrhea or grade 3 elevated transaminases. Two patients withdrew: one after 3 weeks of gefitinib treatment because of grade 3 diarrhea, and the other after 9 weeks of gefitinib treatment because of grade 2 nail changes.

DISCUSSION

In the present study, we have observed that the objective response rate in our patients was similar to that in previous reports. We also found that PFS and OS seem promising in identifying gefitinib-sensitive patients regardless of whether the study includes patients unsuited for conventional cytotoxic chemotherapy because of age, cardiac disease, widespread bone metastases, or poor PS (3 to 4). Our favorable data might have resulted because we selected patients harboring one of two hotspot mutations (exon 19 deletion and exon 21 L858R mutation). Greulich et al.³¹ examined NIH-3T3 cells transformed with various EGFR mutants and showed that a distinct EGFR mutation confers differential sensitivity to TKIs. They demonstrated greater sensitivity to TKIs in cell lines with the two hotspot mutations than with the G719S mutation, and insensitivity to TKIs in cell lines with exon 20 insertion (D770-N771 ins) mutation. These in vitro data may explain, at least partially, our promising results for detecting these two sensitive mutations.

We previously reported that patients with the EGFR exon 19 deletion respond significantly better to gefitinib than those with the L858R mutation ($p = 0.0108$).¹⁷ Our current data show no difference in gefitinib sensitivity and OS after

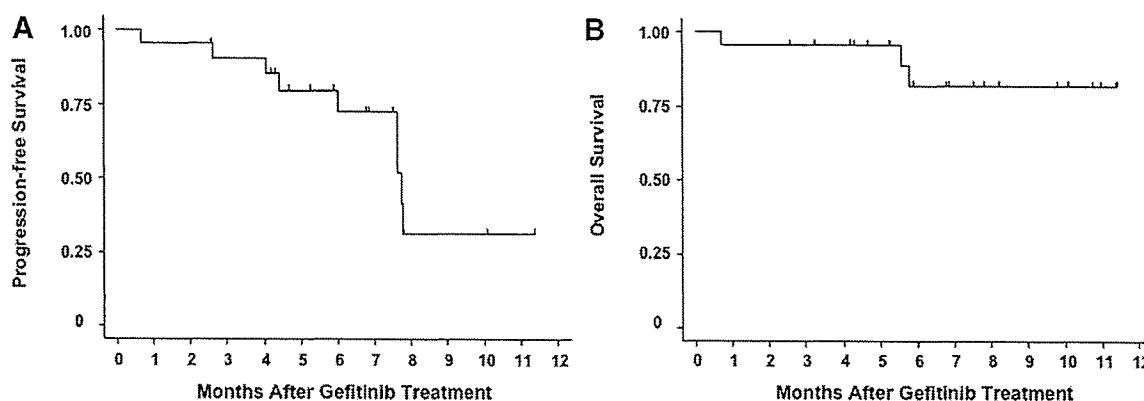


FIGURE 1. Kaplan-Meier estimates of (A) progression-free survival and (B) overall survival for patients with EGFR mutations ($n = 21$). The median progression-free survival was 7.7 months (95% CI, 6.0 mo to not reached). The median survival was not reached.

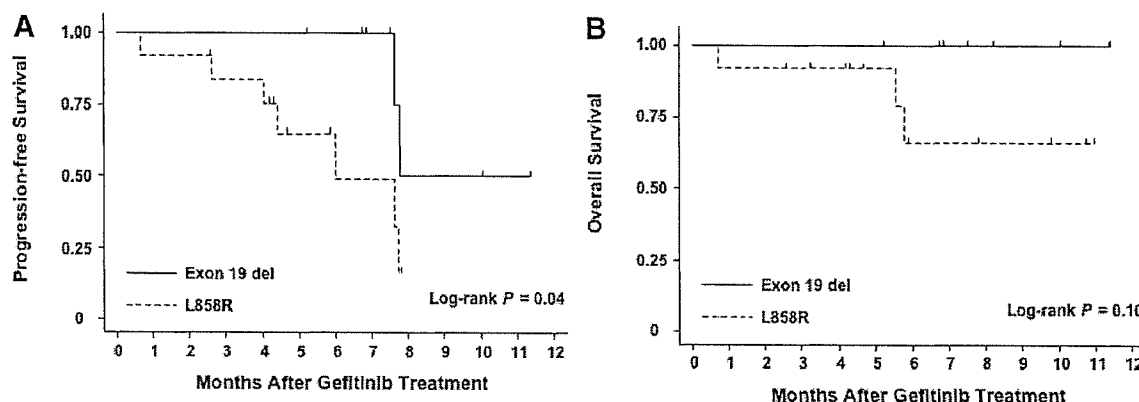


FIGURE 2. Kaplan-Meier estimates of (A) progression-free survival and (B) overall survival for patients with EGFR mutations according to the exon 19 deletion ($n = 8$) and L858R mutation ($n = 13$). The median PFS for the exon 19 deletion group was 7.8 months (95% CI, 7.6 mo to not reached); for the L858R mutation group, median PFS was 6.0 months (95% CI, 2.6 to 7.7 mo).

gefitinib treatment between these two groups of patients, although we observed a greater PFS in the EGFR exon 19 deletion group than in the L858R group. It is possible that the number of patients (eight with exon 19 deletion and 13 with L858R) was too small to detect a statistically significant difference in OS. Riely et al.³² reported recently that patients with exon 19 deletion have a significantly longer survival after TKI treatment than those with the L858R mutation ($p = 0.01$). These findings suggest that the EGFR exon 19 deletion might be a better predictor of the efficacy of TKIs than the L858R mutation.

EGFR mutations are significantly associated with patients with adenocarcinomas, patients of Asian origin, females, and patients who had never smoked—clinical factors also associated with patients who respond to gefitinib.^{13,14,24,33} A phase II trial using gefitinib monotherapy as the first-line therapy for patients with adenocarcinoma histology and never-smoking status was recently completed in South Korea and reported promising data (e.g., an objective response rate of 69% and estimated 1-year survival rate of 73%).³⁴ However, this trial did not select patients using

biomarkers, and we believe the benefit of gefitinib therapy could be enhanced by selecting individual patients according to appropriate biomarkers. Very recently, two prospective phase II studies that had selected patients based on molecular biomarkers demonstrated that EGFR mutations³⁵ and gene copy number assessed by fluorescence in situ hybridization (FISH)³⁶ can predict clinical outcomes in TKI-treated NSCLC patients.

The grade 3 adverse events of diarrhea and elevated AST/ALT were observed in five patients (24%); this is a higher rate than that reported in two previous phase II studies that reported rates of adverse events of 1.5%⁸ and 7%⁹ at a gefitinib dose of 250 mg per day. The reasons for our higher rate of adverse events are unknown. Although adverse events related to gefitinib treatment are generally thought to be mild and tolerable, they should not be discounted.

Most studies have detected EGFR mutations using direct sequencing or single-strand conformation polymorphism analysis for exons 18 to 21.³⁷ These techniques are less sensitive when applied to a small amount of tumor cells from the biopsy or aspiration samples.³⁸ We were able to detect