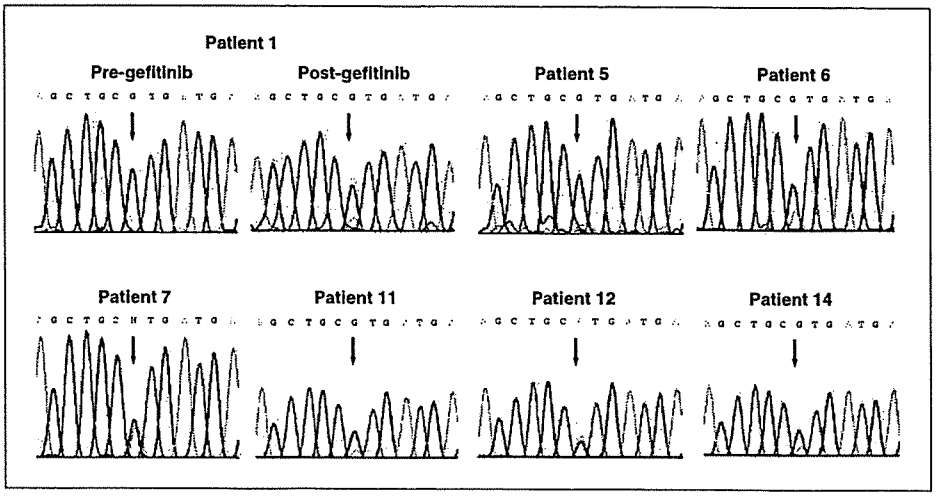

Fig. 2. Sequencing chromatograms for *EGFR* exon 20. SecondaryT790M mutations were observed in seven patients. Antisense strands of each chromatogram. Arrows, small peaks of the C→T substitution at nucleotide 2,369 (G→A on the antisense strand), which results in the T790M mutation. This substitution 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 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.

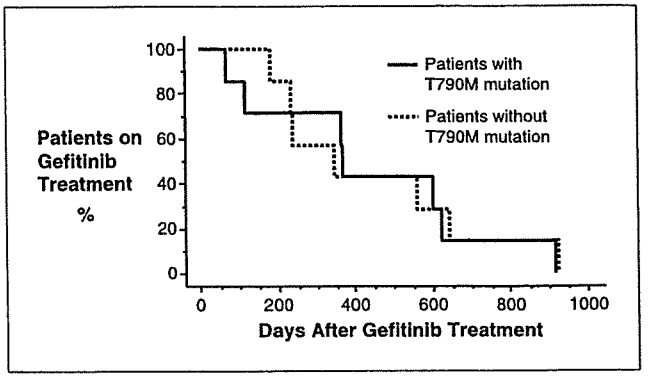

Fig. 3. Effect of theT790M mutation on the length of gefitinib treatment. The length of gefitinib treatment was considered to be roughly related to time to progression. Median treatment times were almost identical in both the presence and absence of theT790M mutation.

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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A Rapid, Sensitive Assay to Detect EGFR Mutation in Small Biopsy Specimens from Lung Cancer

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It has been demonstrated that lung cancers, specifically a subset of pulmonary adenocarcinomas, with epidermal growth factor receptor (EGFR) mutation are highly sensitive to EGFR-targeted drugs. Therefore, a rapid, sensitive assay for mutation detection using routine pathological specimens is demanded in clinical practice to predict the response. We therefore developed a new assay for detecting EGFR mutation using only a paraffin section of a small biopsy specimen. The method was very sensitive, detecting as few as 5% cancer cells in a background of normal cells, the results usually being obtained within 4 hours. Furthermore, it was accurate, as shown by the high concordance with reverse transcriptase-polymerase chain reaction-coupled direct sequencing (186 of 195, 95%). The practical application of this assay to 29 cases treated with gefitinib resulted in a high prediction rate: 10 of the 11 responders were shown to be positive for the mutation, and all patients with progressive disease were negative. In addition, a mutation at codon 790, conferring gefitinib resistance, was successfully analyzed in a similar manner. In conclusion, the assay is a rapid, sensitive method using paraffin sections of biopsy specimens without a tumor cell-enrichment procedure and is quite useful to select a treatment of choice in clinical practice. *(J Mol Diagn 2006, 8:335–341; DOI: 10.2353/jmoldx.2006.050104)*

During the last decade, small molecules that inhibit receptor protein kinase activity have been developed.[1] Gefitinib is one such drug that targets epidermal growth factor receptor (EGFR) kinase. The EGFR, also known as HER1 or ErbB, is a 170-kd receptor tyrosine kinase (TK) that dimerizes and phosphorylates several tyrosine residues on the binding of several specific ligands.[2,3] These phosphorylated tyrosines serve as binding sites for several signal transducers that initiate multiple signaling pathways, resulting in cell proliferation, migration and metastasis, evasion of apoptosis, or angiogenesis, through Ras-Raf-MEK-ERK, phosphatidylinositol-3 kinase-AKT, and PAK-JNKK-JNK pathways. EGFR is expressed in more than 80% of non-small-cell lung cancers (NSCLCs), in addition to a wide range of epithelial cancers. However, clinical trials have shown significant variability in response to gefitinib: 10 to 20% of patients respond to gefitinib treatment, and in some patients, the response is dramatic, whereas the remaining patients show no response. Although further analysis has revealed some prevalence in responders, no definite determinant of the response has been established.

Recently, it has been reported that EGFR somatic mutation can be identified in a subset of pulmonary adenocarcinomas and that tumors with EGFR mutations are highly sensitive to gefitinib.[4,5] This correlation has subsequently been confirmed by our group and others,[6–9] and thus the development of a rapid and sensitive assay to predict gefitinib response by means of the presence or absence of the mutation is demanded clinically. Paraffin sections are a convenient source for such an assay in practice, but most studies using immunohistochemistry failed to predict the response.[10–12]

In this study, we introduce a practical approach using a rapid screening assay of EGFR mutation to predict gefitinib response. This method uses only a single paraffin section of a small biopsy specimen and does not require a tumor cell-enrichment procedure. The result is usually obtained within 4 hours and can be applied to a large number of samples.

Materials and Methods

Patients and Tissues

A series of 195 NSCLCs, in which the mutational status of the EGFR-TK domain with both reverse transcriptase-polymerase chain reaction (RT-PCR)-coupled direct sequencing and the new assay presented here was accessible, was used for this study. Some of the mutational results by RT-PCR-coupled direct sequencing have been

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reported previously.[13] DNA for the new assay was prepared from a section of tissue microarray blotted with 0.6-mm tissue cores of the 195 cases. To examine a correlation with the clinical response evaluated according to the guidelines of Response Evaluation Criteria in Solid Tumors (RECIST), a paraffin section of each biopsy specimen was examined for EGFR mutation in 29 patients treated with gefitinib because of the failure of first or second line therapy. To analyze the codon 790 mutation, which has been reported in association with acquired resistance to gefitinib treatment, four tissues were examined. One, reported as a rare case, was shown to have T790M, independent of gefitinib treatment.[13,14] The other three presented with a recurrent tumor after gefitinib treatment, and the recurrent tumor and corresponding initial tumor tissue were examined. Appropriate approval was obtained from the institutional review committee in addition to written informed consent from the patients.

Mutation Assay by RT-PCR-Coupled Direct Sequencing

Frozen tissue from the tumor specimens was grossly dissected to pass as many tumor cells as possible into the extraction solution (at least 25% of tumor cell content), followed by the extraction of total RNA with an RNeasy kit (Qiagen, Valencia, CA). For RT-PCR-coupled direct sequencing, the EGFR tyrosine kinase domain (exon 18 to 24) was amplified, and then the products were directly sequenced with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). The primer set used was described previously.[13]

DNA Extraction from Paraffin-Embedded Tissues

Tumor cell-rich area in a hematoxylin and eosin-stained section was 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 with 95°C for 3 minutes, the solution was directly used for template DNA for the assay.

EGFR Mutation Detection

To detect the point mutations at codons 858 and 790 of the EGFR gene, we used the cycleave PCR technique. This technique is based on a chimeric DNA-RNA-DNA probe labeled with a fluorescent dye and quencher at each end. The RNA sequence of the probes corresponds to that of the wild type and point mutation labeled with FMA and ROX, respectively. When mutant molecules are present in the sample and PCR-amplified DNA generates a complete hybrid with the RNA portion of the mutant probe, RNase-H digests the probe at the RNA-DNA heteroduplex into two pieces, leading to a significant increase in fluorescence intensity by separation of the fluorescent dye from the quencher. The intensity of the wild-type probe served as an internal control for the assay. This assay was performed using a cycleave PCR core kit (TAKARA, Co., Ltd., Ohtsu, Japan), and sequences of the primer set and the probes were as follows: PCR forward primer for L858R, 5′-AGGAACGTACTGGTGAAAAC-3′; PCR reverse primer for L858R, 5′-TCCCTGGTGTCAGGAAAATG-3′; wild-type probe for L858R, 5′ FAM-CCA *U* CCCAAAAT-Eclipse 3′; probe for L858R mutation, 5′ FAM-CCC*G*CCCAAAAT-Eclipse 3′; PCR forward primer for T790M, 5′-ATCTGCCTCACCTCCAC-3′; PCR reverse primer for T790M, 5′-CAATATTGTCTTTGTGTTC-3′; wild-type probe for T790M, 5′ FAM-TGC*G*TGATGAG-Eclipse 3′; probe for T790M mutation, 5′ FAM-TGC*A*TGATGAG-Eclipse 3′ (italics represent RNA). Fluorescent signals were quantified with a Smart Cycler system (SC-100; Cepheid, Sunnyvale, CA).

To detect the deletion in exon 19 of the EGFR gene, common fragment analysis was used. Sample DNA was amplified with an FAM-labeled primer set as follows: forward, 5′ FAM-TCACAATTGCCAGTTAACGTCT-3′, and reverse, 5′-CAGCAAAGCAGAAACTCACATC-3′. PCR products were electrophoresed on an ABI PRISM 310. When a deletion mutation was present, PCR amplified the shorter segment of DNA, creating a new peak in an electropherogram.

Sensitivity Assay

In the preliminary examination, we prepared a mutation-positive control DNA, which contained exactly one-half each of wild-type and mutant molecules. According to the mixture ratio, the mutation-positive control DNA was mixed up with normal DNA, the concentration of which was equal to that of the mutation-positive control DNA. Therefore, 5% of tumor cells corresponded to 2.5% of mutant molecules in background of wild-type molecule. Using these mixtures of DNA, we examined the sensitivity of the assays (deletion of exon 19 and point mutation of L858R and T790M).

Statistical Analysis

The χ^2 test and Fisher's exact test for independence compared incidences of EGFR mutation, using SYSTAT software (SYSTAT Software Inc., Richmond, CA). A P value below 0.05 was considered statistically significant.

Results

Sensitivity of the New Assay

It is known that mutations in the EGFR tyrosine kinase domain are restricted to four exons, and the results of previous reports[4,5,8,9,13,15] revealed that the deletion in exon 19 and the point mutation of codon 858 in exon 21 covers about 90% of cases with EGFR-TK mutation. We therefore established assays using fragment analysis for

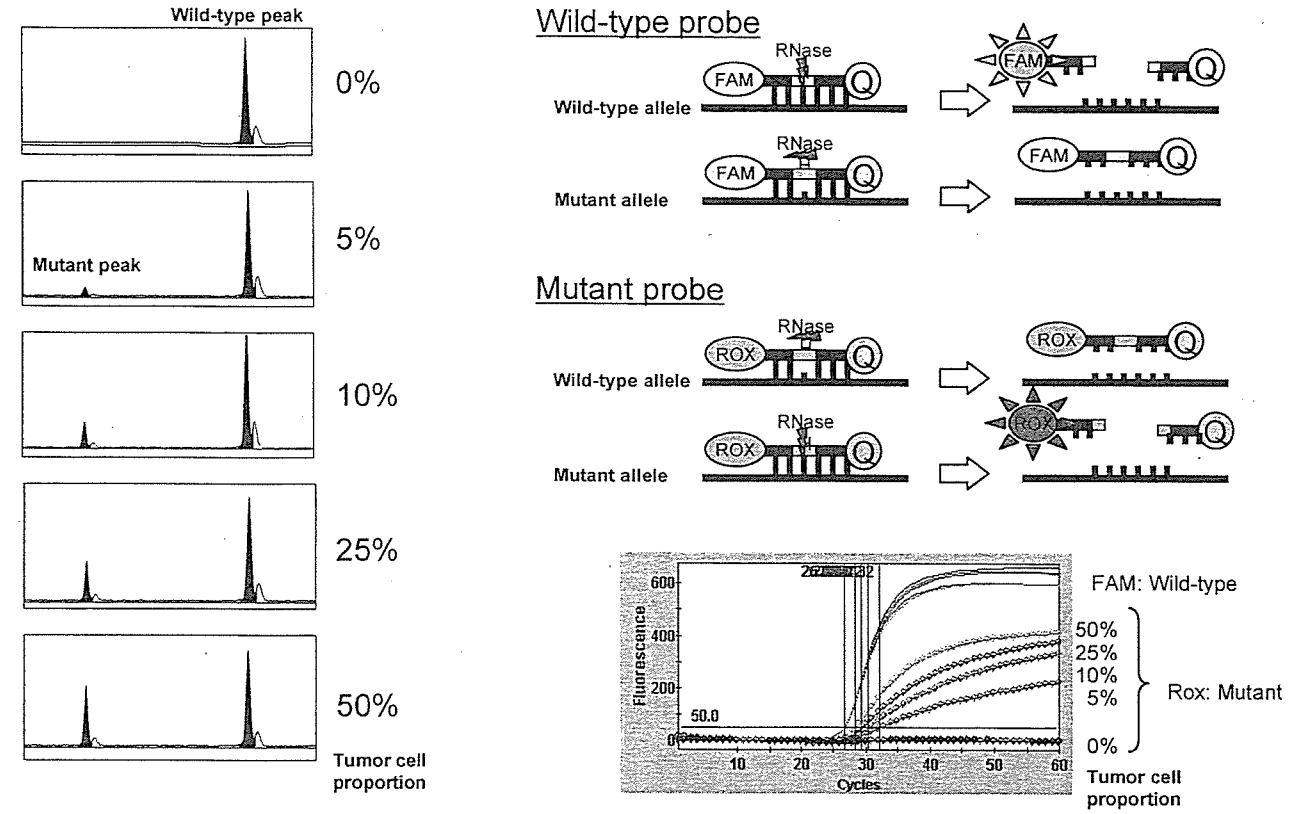


Figure 1. Sensitivity of the new assay. **A:** The sensitivity of the fragment analysis in the new assay. As few as 5% of tumor cells with the deletion could be detected. In the **top** of **B**, a brief explanation of the cycleave technology is displayed. Using this technique, as few as 5% of tumor cells with point mutation at codon 858 could be detected (**bottom** of **B**).

the deletion and cycleave real-time PCR for the point mutation of codon 858. The positive detection of mutated molecules makes this assay very sensitive, as shown in Figure 1. As few as ~5% of tumor cells could be detected in this assay.

Specificity of the New Assay and Concordance with Direct Sequencing

We evaluated the concordance of results between the new assay and conventional direct sequencing using 195 NSCLCs. The results are summarized in Table 1. Overall concordance was 186 of 195 (95%). When we excluded the seven evaluation cases, which were mutated in regions other than the targets of this assay, 99% of cases were concordant. In one case, mutation was only detected with the new assay, whereas one case was negative for mutation with the new assay but positive with direct sequencing. This disagreement resulted from the different tumor cell population in the samples examined. In the preliminary examination, at least 25% of tumor cells were required for detection of the gene mutation by direct sequencing (data not shown). Although tumor tissues in this analysis were dissected to contain more than 25% of tumor cells from most frozen sections, this case contained around 25% tumor cells, on the threshold of that detectable by the sequencing approach. In contrast, the paraffin section used for the new assay was rich in tumor cells. This difference in tumor cell content between frozen and paraffin sections may be the cause of the discrepancy. We confirmed this result by direct sequencing of the frozen section, using DNA microdissected with a laser capture microdissection system.

Practical Application for the Prediction of Gefitinib Response

To confirm whether the new assay is useful for the prediction of gefitinib response in clinical practice, we applied the assay to 29 gefitinib-treated cases whose response had been evaluated according to RECIST. A paraffin section of the large tumor tissue, which had been surgically resected a few years before relapse, was used in seven cases, whereas DNA was extracted from a paraffin section of transbronchial biopsy or computer tomography-assisted fine needle biopsy in 20 cases (Figure 2). Partial response was achieved in 11 cases; all but one were positive for the mutation, whereas five cases with progressive disease were negative with this assay (Table 2). EGFR mutation was detected in only 2 of 13 cases evaluated as stable disease. The correlation be-

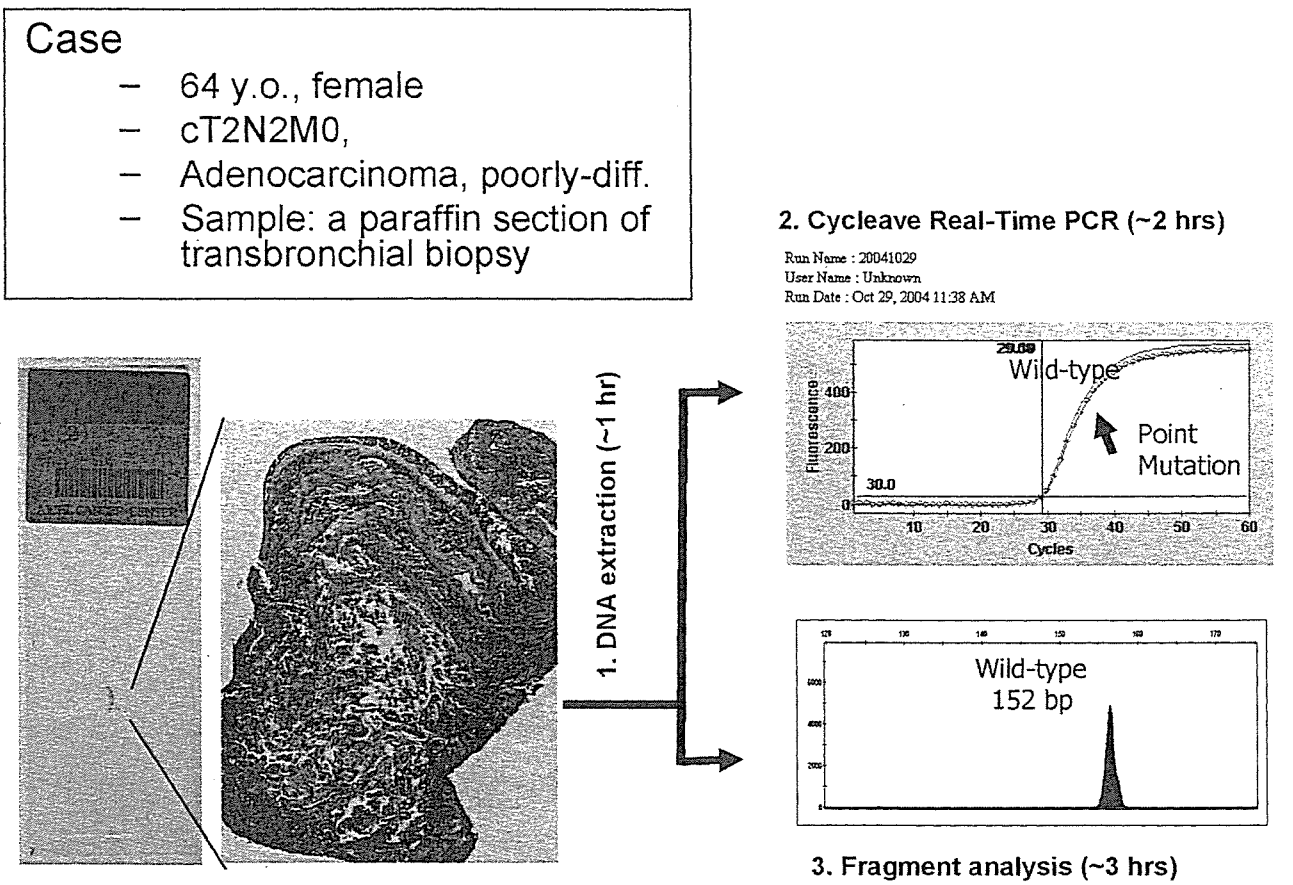


Figure 2. A representative result of the new assay. DNA was extracted from a paraffin section of the biopsy followed by simultaneous analysis using cycleave real-time PCR and fragment analysis. The entire procedure was completed within 4 hours. In this case, point mutation at codon 858 was detected, and the patient responded to gefitinib therapy.

tween EGFR mutation and gefitinib response was highly significant ($P = 0.0001$).

All of the 12 EGFR-mutated specimens were also examined by direct sequencing. In seven cases, identical results were obtained with both methods, whereas background noise prevented us from evaluating the results in the other five cases, all of which were small biopsy specimens. This may not indicate a lack of confirmation but rather suggests the superiority of this new assay, considering the good correlation of this result with clinical response and with the results obtained with direct sequencing using sufficient amounts of surgical tissue.

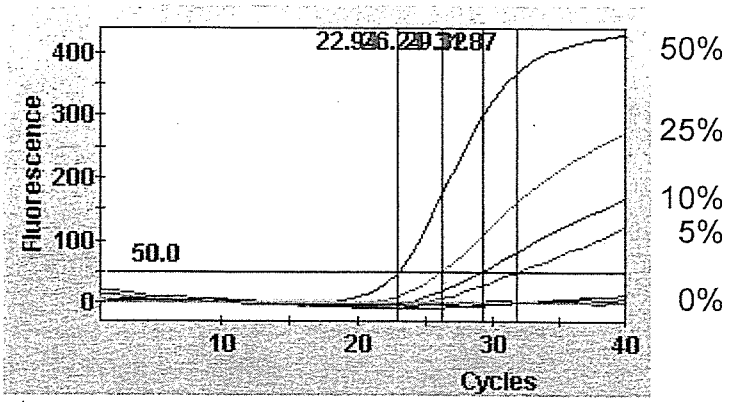
Detection of Mutation at Codon 790 Conferring Acquired Resistance to Gefitinib

Recently, it has been reported that a second mutation, at codon 790, was associated with acquired resistance to gefitinib.[16,17] On very rare occasions, the mutation was also detected independently of gefitinib treatment.[13,14] An assay for this mutation, using cycleave PCR, was similarly established (Table 3). In this assay, as few as 5% of tumor cells could be detected, as shown in Figure 3. A rare case, whose tumor was known to have T790M

Table 1. Comparison of Results between the Conventional and New Assays

	New assay		
Direct sequencing	Wild type	Mutation at codon 858	Deletion at exon 19
Wild type	116	1	0
Point mutation at codon 858	0	32	0
Deletion at exon 19	1	0	38
Point mutation at codon 719	3	0	0
Insertion at exon 20	3	0	0
Point mutation at codon 742	1	0	0

A. Sensitivity analysis

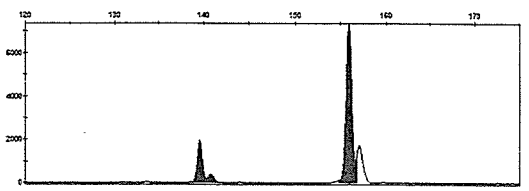


B. Representative case of acquired second mutation

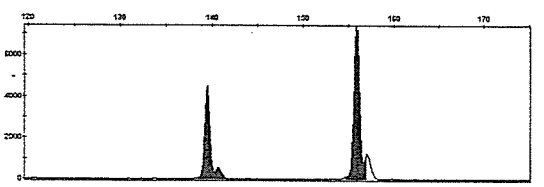
Primary tumor before treatment

Recurrent tumor in pleural effusion

Exon 19, fragment analysis

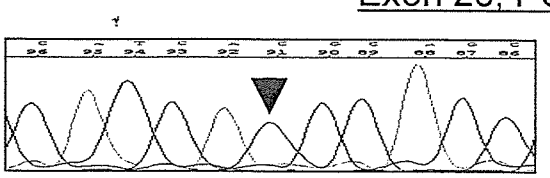


15bp-deleted

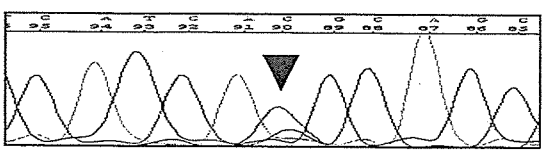


15bp-deleted

Exon 20, PCR-direct sequencing

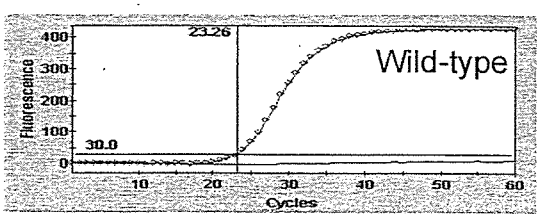


Wild-type



T790M

T790M cycleave PCR



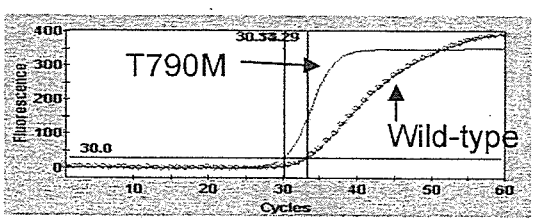


Figure 3. Detection of acquired mutation at codon 790. **A:** The sensitivity of this cycleave assay for T790M mutation. As few as 5% of tumor cells with T790M mutation could be detected. A representative result of acquired mutation at codon 790 after gefitinib treatment is displayed in **B** (Table 3, case 2). In contrast to the 15-bp deletion in exon 19 of the EGFR gene in both primary and recurrent tumors, T790M was detected only in the recurrent tumor, suggesting acquired mutation after gefitinib treatment. The result of the cycleave method was more obvious than that with direct sequencing.

Table 2. Practical Application of the New Assay

	Clinical response		
EGFR status	PD	NC	PR
Wild type	5	11	1
Mutated	0	2	10
Deletion in exon 19	0	2	5
Point mutation of codon 858	0	0	5

PD, progressive disease; NC, no change; PR, partial response.

mutation independently of gefitinib treatment,[16,17] was also positive in this assay. In one of the other three recurrent tumors, this assay clearly demonstrated the mutation (Figure 3), although it was often difficult to detect the mutated signal with direct sequencing of the PCR product.

Discussion

Paez et al[5] and Lynch et al[4] simultaneously published the result that somatic mutation of EGFR in lung adenocarcinoma predicts a clinical response to gefitinib. Erlotinib is another targeted small-molecule inhibitor of EGFR, and lung adenocarcinoma sensitive to erlotinib also harbored EGFR mutations. In addition, *in vitro* studies support the observation that EGFR mutations make tumor cells significantly sensitive to gefitinib[18] and erlotinib. This increased sensitivity may be explained by the "addiction to oncogene" hypothesis proposed by Weinstein.[19] Tumor cells with EGFR mutation are highly dependent on the activated EGFR pathway and are thus very susceptible to inhibition of this dependence. We have reported that patients with EGFR mutations survived longer than those without mutations after the initiation of gefitinib treatment.[7] Recently, failure to show a survival benefit in the IRESSA Survival Evaluation in Lung Cancer was announced. Gefitinib may not be effective enough to kill tumor cells that are not under a state of "addiction to EGFR mutation." Conversely, these findings suggest that selection of patients with EGFR-mutated tumors has the advantage of increasing the response rate of EGFR-targeted therapy. Furthermore, selection may also be efficient at preventing serious interstitial pneumonia occurring as a side effect.[20]

Although an assay using paraffin sections is very practical, immunohistochemical analysis of the tumors failed to predict the response. Currently, the microdissection of tumor cells and direct sequencing of PCR products is commonly used as a standard method. Regarding practical applications, the new assay reported here provides two benefits compared with the conventional method. First, microdissection is not necessary for the assay because a positive mutated signal makes this assay very sensitive. Second, this assay is rapid, does not require a purification step, and is usually completed within 4 hours: digestion with proteinase K for 1 hour, real-time PCR or regular PCR for 3 hours, and electrophoresis for 1 hour. In addition to paraffin sections, pleural effusion and specimens for fine needle aspiration cytology can be used. All three specimens of pleural effusion for the T790M cycleave assay were successfully analyzed, whereas direct sequencing occasionally resulted in an ambiguous result (Figure 3). The main targets for gefitinib or erlotinib therapy are recurrent and refractory tumors, and an assay using such specimens is therefore quite useful. However, the examination of limited regions of the EGFR gene appears to be a disadvantage of this study. Recent studies suggested that an insertion of exon 20 was shown to be resistant to EGFR inhibitors[25], whereas the gefitinib sensitivity of cells expressing the G719S mutant was significantly less than that of cells expressing the L858R mutant form[26]. Therefore, these results suggest that examination of the L858R mutation and deletion in exon 19 is reasonable, because these two mutations are likely to be a major target of the EGFR inhibitors.

A few approaches for the detection of EGFR mutation have been reported recently.[21–23] Comparing the assays, the advantage of the method presented here is its practical clinical use. Biopsy specimens frequently result in small, fragmented tissues containing only a few cancer cells. Using such biopsy specimens, the assay successfully demonstrated the EGFR mutations that correlate with gefitinib response, in contrast to failure of the direct sequencing of some biopsy specimens. Furthermore, the cycleave technique can be simultaneously applied for the detection of the K-ras mutation, which has been proposed to be an adverse prognostic marker for chemotherapy with erlotinib.[24]

In summary, we have introduced a new practical approach for the detection of EGFR mutations. This assay is very sensitive and useful for predicting gefitinib response. This rapid screening assay uses paraffin sections from biopsy without the need for a microdissection

Table 3. Detection of T790M Mutation Associated with Acquired Resistance to Gefitinib

Patient	Gefitinib treatment	Tissue examined	T790M mutation	Comments
Case 1*	No	Primary tumor	Yes	A rare case, harboring T790M mutation independent of gefitinib treatment
Case 2	Yes	Pleural effusion	Yes	15-bp deletion of exon 19 in the primary and recurrent cancers (Figure 3)
Case 3	Yes	Pleural effusion	No	9-bp deletion of exon 19 in the primary and recurrent cancers
Case 4	Yes	Pleural effusion	No	15-bp deletion of exon 19 in the primary and recurrent cancers

*The mutation of codon 790 in a primary cancer, which was demonstrated with RT-PCR direct sequencing, has been reported previously.

procedure and has significant advantages over other methods.

Acknowledgments

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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% CO_2. 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.

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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-cm^2 culture flask. They were incubated at 37°C in a humidified incubator containing 5% CO_2 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% CO_2.

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′-GAGAGTTGAGAGTGCAGAG-3′; NF2exon2AS, 5′-TCAGCCCCACCAGTTTCATC-3′; NF2exon3S, 5′-GCTTCTTTGAAGGTAGCACA-3′; NF2-exon3AS, 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′-CAGTGTCTTCCGTTCTCC-3′; NF2exon7AS, 5′-AGCTCAGAGAGGTTTCAA-3′; NF2exon8S, 5′-CCACAGAATAAAAAGGGCAC-3′; NF2exon8AS, 5′-GATCTGCTGGACCCATCTGC-3′; NF2exon9S, 5′-GTTCTGCTTCATTCTTCC-3′; NF2exon9AS, 5′-GTAATG-AAAACCAGGATC-3′; NF2exon10S, 5′-CCTTTTAGTCTG-CTTCTG-3′; NF2exon10AS, 5′-TCAGTTAAAACAAGGTTG-3′; NF2exon11S, 5′-TCGAGCCCTGTGATTCAATG-3′; NF2exon11AS, 5′-AAGTCCCCAAGTAGCCTCCT-3′; NF2exon12S, 5′-CCCACTTCAGCTAAGAGCAC-3′; NF2-exon12AS, 5′-CTCCTCGCCAGTCTGGTG-3′; NF2exon13S, 5′-GGTGTCTTTTCCTGCTACCT-3′; NF2exon13AS, 5′-GGGAGGAAAGAGAACATCAC-3′; NF2exon14S, 5′-TGTGCCATTGCCTCTGTG-3′; NF2exon14AS, 5′-AGGG-CACAGGGGGCTACA-3′; NF2exon15S, 5′-TCTCACTGT-CTGCCCAAG-3′; NF2exon15AS, 5′-GATCAGCAAAATA-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′-TCCCTAGTTCACAAAATGCTTGTC-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′-GGCCAAAAATTTAATCAGTGGA-3′ for exon 15.

Tumorigenecity 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

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flank of the nude mice. The animals were examined every week for the development of tumors. Tumor volume was calculated as length × height × width × 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 Identifiler 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 μg) 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 μg) 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 × SSC, 0.2% SDS, 5 × 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 × SSC and 0.1% SDS for 5 min at 65°C, once in 2 × SSC and 0.1% SDS for 5 min at 65°C, and once in 0.05 × 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

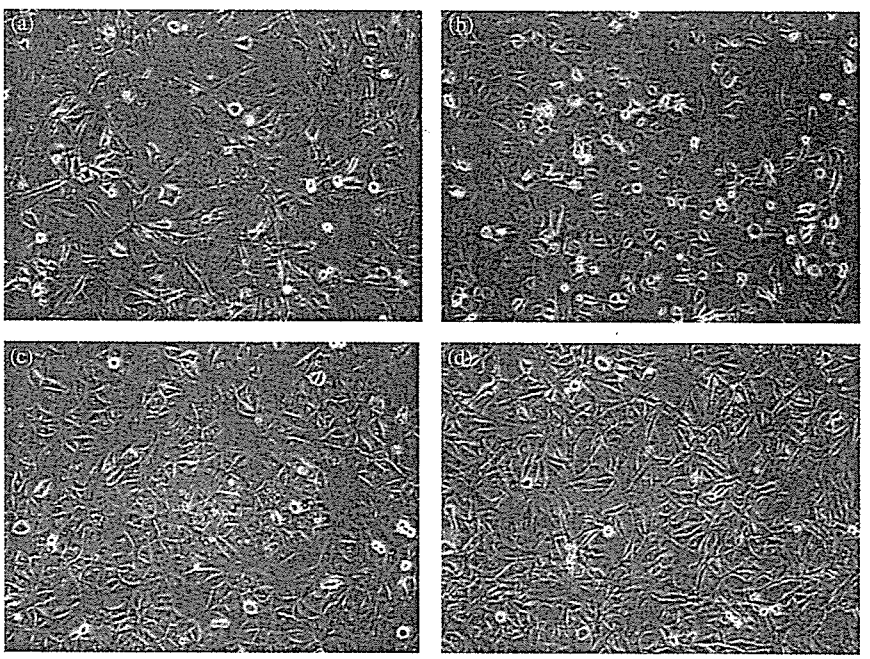

Fig. 1. Morphology of the four malignant pleural mesothelioma (MPM) cell lines. Micrographs of cultured (a) ACC-MESO-1 cells, (b) ACC-MESO-4 cells, (c) Y-MESO-8A cells, and (d) Y-MESO-8D cells (original magnifications ×100).

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
TP53	+	+	+	+	+	+	Nt717insA	+	+	+
NF2	Q389X	+	+	+	+†	HD†	ND	R341X†	HD†	+†
p16^{INK4A}/p14^{ARF}	HD	HD	HD	HD	HD	HD	HD	HD	HD	HD
KRAS	+	+	+	+	+	+	+	+	+	+
NRAS	+	+	+	+	+	+	+	+	+	+
BRAF	+	+	+	+	+	+	+	+	+	+
EGFR	+	+	+	+	+	+	+	+	ND	+
HER2	+	+	+	+	+	+	+	+	ND	+

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

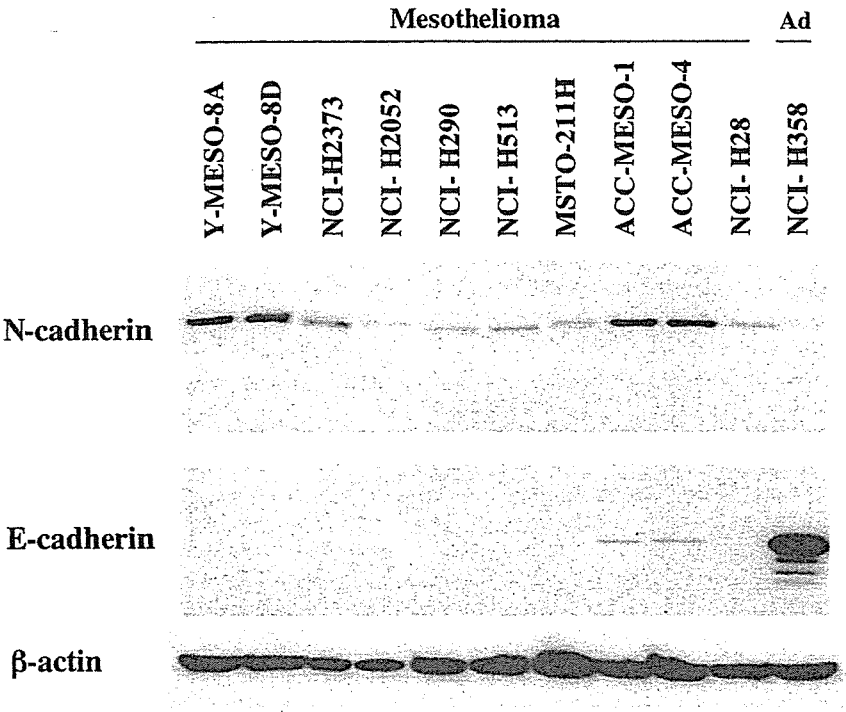

Fig. 2. Western blot analysis of E-cadherin and N-cadherin. Each lane was loaded with 15 μg of total cell lysate from Y-MESO-8A (lane 1), Y-MESO-8D (lane 2), NCI-H2373 (lane 3), NCI-H2052 (lane 4), NCI-H290 (lane 5), NCI-H513 (lane 6), MSTO-211H (lane 7), ACC-MESO-1 (lane 8), ACC-MESO-4 (lane 9), NCI-H28 (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 suppresser 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/

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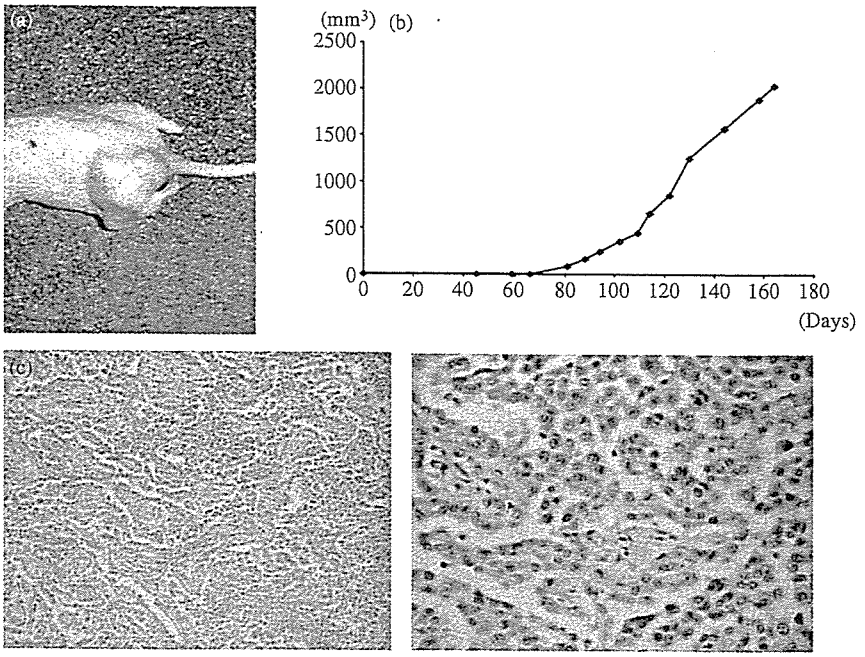

Fig. 3. (a) Xenografted tumor of Y-MESO-8A. (b) Growth curve of the xenografted tumor in nude mice. (c) Histology of xenografted Y-MESO-8A tumor (haematoxylin and eosin, original magnification ×100 and ×400). As in epithelioid mesothelioma, the tumor shows papillary proliferation consisting of atypical 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 1α	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 (exon1β), 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.

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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 study[11] 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 mutations[29] 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 Tumors[30] 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