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.

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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

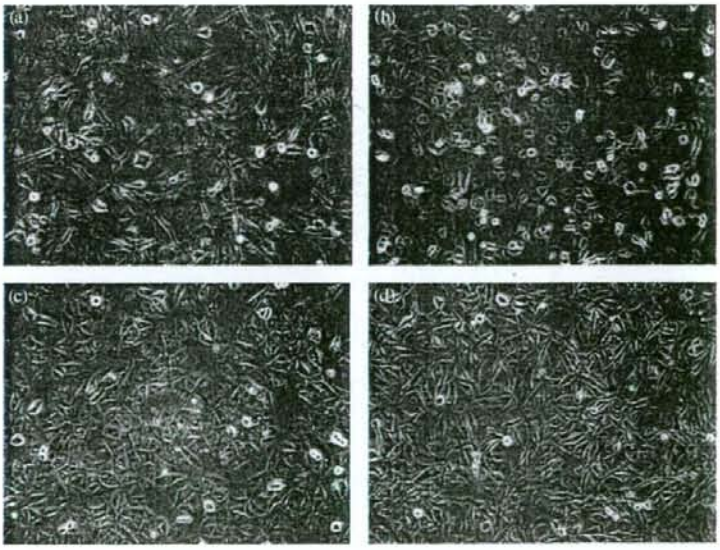

Fig. 1. Morphology of the four malignant pleural mesothelioma (MPM) cell lines. Micrographs of cultured (a) ACC-MESO-1 cells, (b) ACC-MESO-4 cells, (c) Y-MESO-8A cells, and (d) Y-MESO-8D cells (original magnifications ×100).

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.

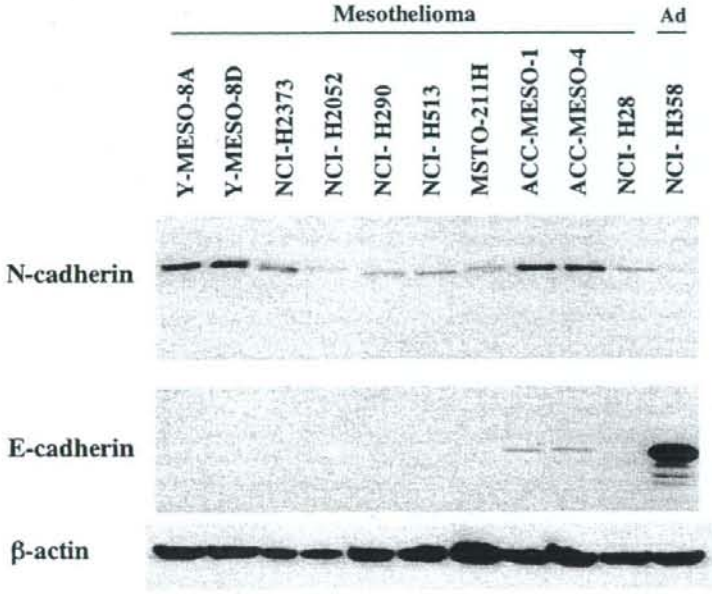

Fig. 2. Western blot analysis of E-cadherin and N-cadherin. Each lane was loaded with 15 μg of total cell lysate from Y-MESO-8A (lane 1), Y-MESO-8D (lane 2), NCI-H2373 (lane 3), NCI-H2052 (lane 4), NCI-H290 (lane 5), NCI-H513 (lane 6), MSTO-211H (lane 7), ACC-MESO-1 (lane 8), ACC-MESO-4 (lane 9), NCI-H28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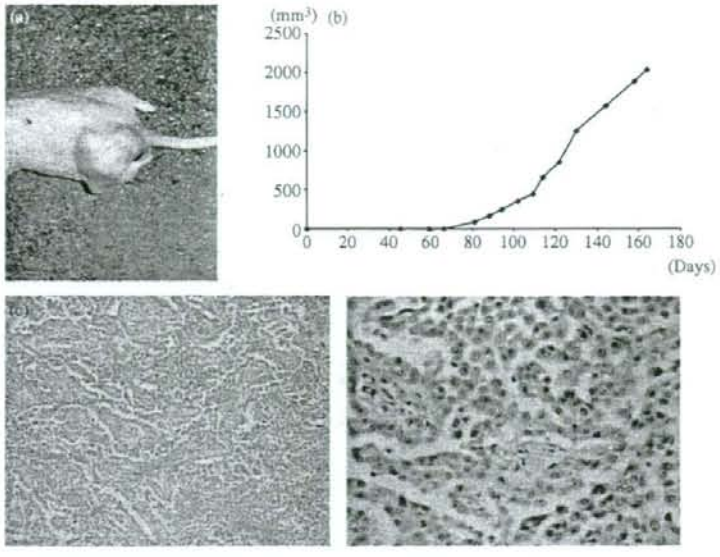

Fig. 3. (a) Xenografted tumor of Y-MESO-8A. (b) Growth curve of the xenografted tumor in nude mice. (c) Histology of xenografted Y-MESO-8A tumor (haematoxylin and eosin, original magnification ×100 and ×400). As in epithelioid mesothelioma, the tumor shows papillary proliferation consisting of atypical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

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

	EGFR Mutation Status			
	All	**Mutation**	**Wild type**	***p***
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 2*A*). 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 and OS between never-smokers and current/former smokers (data not shown).

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

Adverse Events

All 21 patients were evaluated for drug-related adverse events. The most common adverse events were skin toxicity, diarrhea, and elevated asparatate 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

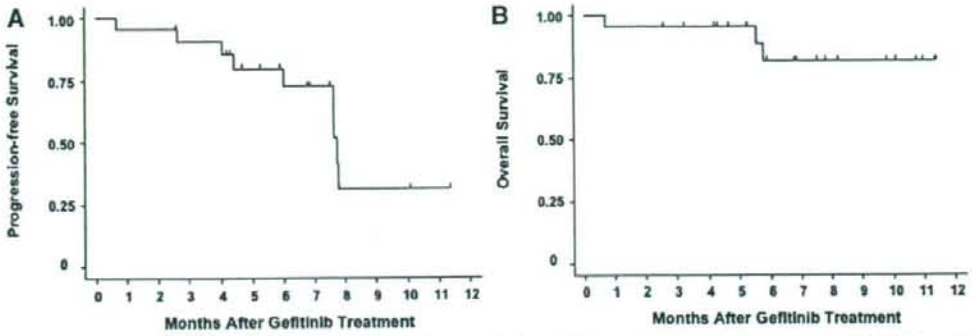

FIGURE 1. Kaplan–Meier estimates of (*A*) progression-free survival and (*B*) overall survival for patients with EGFR mutations ($n = 21$). The median progression-free survival was 7.7 months (95% CI, 6.0 mo to not reached). The median survival was not reached.

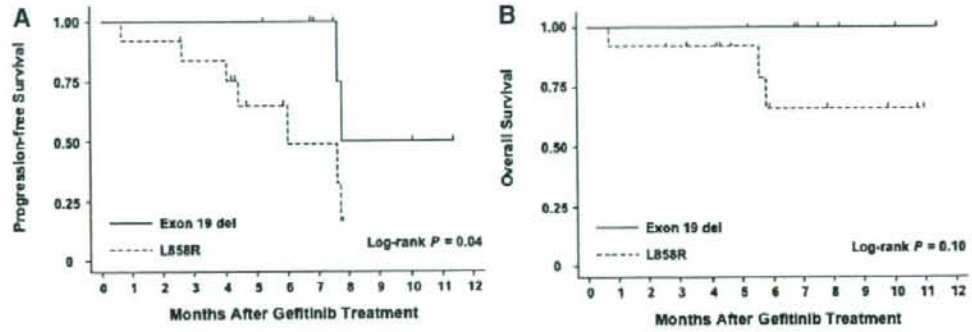

FIGURE 2. Kaplan–Meier estimates of (*A*) progression-free survival and (*B*) overall survival for patients with EGFR mutations according to the exon 19 deletion ($n = 8$) and L858R mutation ($n = 13$). The median PFS for the exon 19 deletion group was 7.8 months (95% CI, 7.6 mo to not reached); for 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 mutations[35] 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

two hotspot mutations with our sensitive rapid screening assay in most biopsy or aspiration samples in the routine clinical setting. Although this assay needs precise assessment of tumor samples by a pathologist to enrich the tumor cells, it is very sensitive and accurate for detection, and it can be completed within 4 hours without need for microdissection or nested PCR process.[29]

The key genetic event for TKI sensitivity has not been perfectly identified and is the subject of a growing debate about the role of EGFR mutations versus EGFR gene amplification/copy number in NSCLC. EGFR mutant NSCLC cell lines are strongly associated with increased EGFR gene copy number.[39,40] Cappuzzo et al.[27] and Takano et al.[22] found that EGFR mutations in NSCLC patients correlate significantly with gene copy number assessed by FISH and quantitative real-time PCR, respectively. However, Cappuzzo et al.[27] demonstrated that in patients treated with gefitinib, a high EGFR gene copy number is a better predictor of survival than EGFR mutations.[27] In contrast, Takano et al.[22] reported that the status of the EGFR mutations, rather than gene copy number, is the major determinant of gefitinib efficacy. Recent reports of the molecular analyses from the largest phase III TKI monotherapy trials failed to show that the EGFR mutation is superior to gene copy number in predicting the efficacy of TKIs.[23,26] These conflicting results on EGFR mutations and gene amplification/copy number could be explained by (i) differences in the detection methodologies and assessment of mutation and gene amplification/copy number (e.g., direct sequence versus PCR-based DNA testing for detecting EGFR mutations, or FISH versus PCR-based amplification for detecting EGFR gene amplification/copy number), (ii) failure to reconfirm these results in other institutions, and (iii) other unknown factors underlying drug sensitivity, especially those related to ethnicity. Further prospective studies are needed to investigate the crucial molecular markers involved in the EGFR network, using adequate tissue samples and assays to more precisely detect molecular events.

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Original contribution

Gemcitabine/Carboplatin in a Modified 21-Day Administration Schedule for Advanced-Stage Non–Small-Cell Lung Cancer

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Abstract

PURPOSE: Gemcitabine/carboplatin is active for advanced-stage non–small-cell lung cancer. Although it has a better toxicity profile than gemcitabine/cisplatin, severe thrombocytopenia can be a problem. We conducted a phase II study of gemcitabine/carboplatin on a 21-day schedule with administration of carboplatin delayed until day 8, intending to decrease the severity of thrombocytopenia and evaluate the feasibility and efficacy of this schedule. PATIENTS AND METHODS: Thirty-one patients with stage IIIB or stage IV non–small-cell lung cancer received gemcitabine 1000 mg/m^2 on days 1 and 8 and carboplatin at an area under the curve of 5 mg × minute/mL on day 8, every 21 days. RESULTS: The response rate was 22.6%, including 1 complete response. The median time to progression was 161 days, and the median survival was 454 days. Grade 3/4 thrombocytopenia, according to the National Cancer Institute Common Toxicity Criteria, version 3.0, was observed in 2 patients (6.5%) in the first 2 cycles. Nonhematologic toxicity included rash, depression, fever, nausea/vomiting and increased hepatic transaminase. The median courses of delivery were 3, and 13 patients (42%) received the first 3 courses without treatment delay. Dose intensity for each drug was 638 mg/m^2 per week for gemcitabine and 1.56 mg × minute/mL per week for carboplatin area under the curve, respectively. CONCLUSION: This study suggests that gemcitabine/carboplatin with a day-8 administration of carboplatin in a 21-day schedule reduces the severity of thrombocytopenia without having a detrimental effect on efficacy.

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Key words: Dose intensity, Feasibility, Phase II studies, Thrombocytopenia

Introduction

Non–small-cell lung cancer (NSCLC) constitutes 75%-80% of lung cancer cases and currently represents a leading cause of cancer-related death throughout the world.[1] Significant proportions of the patients present with locally advanced or metastatic disease at the time of diagnosis.[2] Although a recent overview suggested that platinum agent–based chemotherapy improves survival and quality of life,[3] the long-term prognosis of these patients is still generally poor. In the past 2 decades, several new chemotherapeutic agents have been developed and have proven to be active in advanced-stage NSCLC. Gemcitabine, a pyrimidine antimetabolite, is one of the most promising among these agents, showing definite efficacy and mild toxicity profiles. Initial phase I studies using a schedule of weekly administrations of 3 weeks for every 4 weeks established 790 mg/m^2 weekly as the maximum tolerated dose. Dose-limiting toxicity was myelosuppression, with thrombocytopenia more significant than granulocytopenia.[4] Later phase I/II studies have established 1250 mg/m^2 weekly as an optimal tolerated dose.[5-7] Several phase II studies of single-agent gemcitabine in advanced-stage NSCLC have demonstrated response rates of 20%-26% and a median survival of 7-9.4 months.[8-13] In these studies, 800-1250 mg/m^2 gemcitabine was administered weekly for 3 weeks every 4 weeks. Toxicities reported in these studies were myelosuppression, such as granulocytopenia and thrombocytopenia, transient increase of hepatic transaminases, rash, flu-like symptoms, and lethargy.

The combination of gemcitabine and a platinum compound has demonstrated a synergistic effect in preclinical settings, and a number of phase II/III studies of gemcitabine/cisplatin have been performed.[14-22] This combination chemotherapy has proved to be very promising, showing

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an objective response rate (ORR) of 28%-54% and a median survival of 8.4-15.4 months. Gemcitabine/cisplatin is now one of the standard chemotherapy combinations for advanced-stage NSCLC. However, the toxicity profile of cisplatin, such as nausea/vomiting, nephrotoxicity, and neurotoxicity, can be troublesome for patients with advanced-stage NSCLC, who generally have poor prognosis. Moreover, cisplatin is often intolerable for certain patients, especially the elderly and/or those with concomitant severe diseases. Carboplatin is a cisplatin analogue, and its nonhematologic toxicity is milder compared with cisplatin. Carboplatin is also expected to exert a synergistic effect with gemcitabine. Several phase II studies of gemcitabine/carboplatin have been reported. The early studies adopted a schedule of weekly administration of gemcitabine for 3 weeks (day 1, 8, and 15 administrations) and day-1 administration of carboplatin every 4 weeks.[23-29] However, those studies reported high incidences of thrombocytopenia, prompting the investigation of other schedules that are less myelosuppressive. Iaffaiolli et al recommended a 28-day schedule that decreased myelotoxicity around day 15 by administrating carboplatin on day 8 and eliminating the administration of gemcitabine on day 15.[30] Edelman et al recommended a 21-day schedule that decreased myelotoxicity around day 15 by simply eliminating the administration of gemcitabine on day 15.[31] Several large phase II studies have been performed using these schedules. Among them, Mott et al reported a phase II study with a 28-day schedule described by Iaffaiolli et al, with an ORR of 10% and a median survival of 8.3 months.[32] On the other hand, Yamamoto et al reported the results of a comparative phase II study in which a 21-day schedule described by Edelman et al was compared with gemcitabine/vinorelbine as a control arm.[33] The ORR of gemcitabine/carboplatin was 20%, and the median survival of 432 days was favorable. However, a high incidence of dose reduction as a result of myelosuppression and early withdrawal from the study were reported. These studies suggest that the schedule for gemcitabine/carboplatin still needs improvement. In the present article, we report another 21-day schedule, with the intent to be more dose intense than Mott et al and less myelosuppresive than Yamamoto et al.

Patients and Methods

Eligibility Criteria

Eligibility criteria of patients were as follows: age 20-80 years, a histologic or cytologic diagnosis of clinical stage IIIB NSCLC with malignant pleural effusion or clinical stage IV NSCLC, and Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0-2. Patients were required to have adequate bone marrow reserve (leukocyte count > 4000/μL, platelet count > 100,000/μL, and hemoglobin > 10 g/dL), normal hepatic function (serum bilirubin < 1.5 mg/dL, transaminases < 2 times the upper limit of normal), normal renal function (serum creatinine < 1.2 mg/dL), and a life expectancy of > 3 months. Patients who did not have measurable disease based on Response Evaluation Criteria in Solid Tumors[34] were excluded from the study. Neither previous chemotherapy nor thoracic irradiation was allowed. Patients were excluded from the study when they met one of the following conditions: active uncontrolled infection, unstable concomitant disease (ischemic heart disease, hypertension, or diabetes mellitus), active concomitant malignant disease, pregnancy, or breastfeeding. Written informed consent was obtained from all patients.

Study Design

This was a single-arm phase II study. Because the response rate of gemcitabine/carboplatin has been reported by a variety of authors, we determined the primary endpoint of our study as the rate of treatment completion without treatment delay. It has been reported that the median courses of delivery of platinum-doublet chemotherapy was approximately three[35] and that there was no statistical significance in survival of patients between 3 and 6 courses of platinum agent–containing chemotherapy.[36] Therefore, we analyzed drug delivery in the first 3 courses to evaluate the feasibility of the schedule and defined the treatment completion rate to be the percentage of patients who received the first 3 courses with no delay from the intended schedule. The expected and threshold value of the treatment completion rates were 90% and 70%, respectively. The number of patients required was determined with an α risk of 0.05 and a β risk of 0.2. Simon's optimal design was applied to recruit the patients[37]: if completion of treatment was observed in < 5 patients among the first 6 patients, the study was to be terminated; if it was observed in ≥ 5 patients, recruitment of as many as 27 patients was allowed. This schedule was judged to be feasible when, in an analysis of 27 patients, treatment completion was observed in > 22 patients. The secondary endpoints included the evaluation of response rate, toxicities, median time to progression (TTP), and overall survival. This study was approved by the Institutional Review Board of Osaka Medical Center for Cancer and Cardiovascular Diseases.

Treatment Plan

Patients received carboplatin at an area under the curve (AUC) of 5 mg × minute/mL, calculated using the Calvert formula[38] with creatinine clearance evaluation by the Cockcroft formula.[39] Carboplatin was administrated in a 60-minute infusion on day 8 of a 21-day cycle. Gemcitabine was administrated at 1000 mg/m^2 in a 30-minute infusion on days 1 and 8. The planned dose intensity for each drug was 667 mg/m^2 per week for gemcitabine and 1.67 mg × minute/mL every week for carboplatin AUC. Four cycles of treatment were intended. On day 1 and day 8 of each cycle, complete blood count was evaluated. Drug administration was delayed until recovery in cases with leukocyte count < 3000/μL or platelet count < 100,000/μL on day 8.

The hematologic criteria to start the next cycles were loosened to increase dose intensity (leukocyte count > 2500/μl.

Table 1 Patient Characteristics (N = 31)

Characteristic	Number of Patients
Median Age, Years (Range)	63 (42-76)
Sex	
Male	12
Female	19
Stage	
IIIB	8
IV	23
Histology	
Adenocarcinoma	25
Squamous cell carcinoma	6
ECOG PS	
0	22
1	9

and platelet count > 75,000/μL). The start of the new cycles was postponed until blood count met these criteria. Doses of gemcitabine were adjusted according to leukocyte, neutrophil, and platelet counts. If grade 4 leukopenia or neutropenia continued > 3 days despite the use of granulocyte colony-stimulating factor or if platelet count decreased to < 25,000/μL, the gemcitabine dose was reduced by 200 mg/m^2 intervals until 600 mg/m^2. Patients were withdrawn from the study in cases of disease progression, development of grade > 3 nonhematologic toxicities, unacceptable treatment delay as a result of hematologic toxicities, or necessity of gemcitabine dose reduction to < 600 mg/m^2. After withdrawal from the study, subsequent treatment was to be decided by the investigator.

Evaluation

Response was evaluated by chest and abdominal computed tomography (CT) scans after the second and fourth cycles of chemotherapy according to Response Evaluation Criteria in Solid Tumors. Brain magnetic resonance imaging, chest CT scan, and abdominal CT scan were performed at any time if assessment for the disease progression was necessary. Confirmation was necessary to determine partial and complete response. During the study, all enrolled patients were evaluated weekly by physical examination, complete blood count, and blood chemistries. Toxic effects were graded according to National Cancer Institute Common Toxicity Criteria, version 3.0.

Statistical Analysis

Time to progression was calculated from the date of enrollment to the date of progression using the Kaplan-Meier method.[40] Overall survival was calculated from the date of enrollment until the date of death or last known contact using the Kaplan-Meier method. Statistical analysis in the study was carried out using the SPSS program.

Table 2 Hematologic Toxicities

Adverse Event	Grade 3	Grade 4	N (%)
Leukopenia	10	0	10 (32.2)
Neutropenia	16	5	21 (67.7)
Anemia	3	0	3 (9.7)

Results

From June 2003 to April 2005, 31 eligible patients were enrolled in the study. There were 12 men and 19 women; 6 patients with squamous cell carcinoma and 25 with adenocarcinoma; 8 patients with clinical stage IIIB and 23 with clinical stage IV; 22 patients with an ECOG PS of 0 and 9 with a PS of 1. Sixteen patients had a smoking history. Patient characteristics are summarized in Table 1. Tumor response was assessable in all 31 patients. One complete response and 6 partial responses were observed, resulting in a response rate of 22.6%. Median TTP was 161 days (95% confidence interval, 109-213 days). At the time of analysis, when the median follow-up time was 356 days (range, 40-946 days), 12 patients were alive, 16 patients were dead, and 3 patients were lost to follow-up. Median survival time was 454 days (95% confidence interval, 230-678 days).

Toxicity profiles are summarized in Tables 2, 3, and 4. Table 2 shows hematologic toxicities except thrombocytopenia in the first 2 cycles. Neutropenia was frequently observed, with grade 3/4 neutropenia occurring in 51.6% (16 of 31 patients) and 16.1% (5 of 31 patients) of the patients, respectively. However, febrile neutropenia was not observed. Grade 3 anemia was observed in 9.7% of patients (3 of 31 patients), and grade 4 anemia was not observed. The incidence of red blood cell and platelet transfusions was 3.2% (1 of 31 patients) and 3.2% (1 of 31 patients), respectively. Because the grading of thrombocytopenia is substantially different among versions of the National Cancer Institute Common Toxicity Criteria, we show detailed results of platelet numbers in Table 3. Thrombocytopenia was relatively mild; grade 3/4 thrombocytopenia occurred in 3.2% (1 of 31 patients) and 3.2% (1 of 31 patients) of patients in the first 2 cycles, without serious hemorrhagic events. The lowest platelet count was 15,000/μL and was observed in the first cycle in a 74-year-old man. Grade 2/3 nausea/vomiting occurred in 3.2% (1 of 31 patients) and 3.2% (1 of 31 patients) of patients, respectively, grade 2 and 3 rash in 6.5% (2 of 31 patients) and 12.9% (4 of 31 patients), grade 3 depression in 3.2% (1 of 31 patients), grade 1 fever (in the absence of neutropenia) in 3.2% (1 of 31 of patients), and grade 1 hepatic transaminase increase in 9.7% (3 of 31 patients). A total of 94 cycles with a median of 3 cycles for each patient were administered. Treatment was delayed in 42.6% of cycles and required dose reduction in 6.4% of cycles. The median number of days per cycle was 24 days (22, 29, and 26 days for the first, second, and third cycles, respectively). The dose intensity was 638 mg/m^2 per week for gemcitabine and 1.56 mg × minute/mL per week for carboplatin AUC.