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′-AGGCCTGTGCAGCAACTC-3′; NF2exon1AS, 5′-GAGAACCTCTCGAGCTTCCAC-3′; NF2exon2S, 5′-GAGAGTTGAGAGTGCAGAG-3′; NF2exon2AS, 5′-TCAGCCCCACCAGTTTCATC-3′; NF2exon3S, 5′-GCTTCTTTGAAGGTAGCACA-3′; NF2exon3AS, 5′-GGTCAACTCTGAGGCCAACT-3′; NF2exon4S, 5′-CCTCACTTCCCCTCACAGAG-3′; NF2exon4AS, 5′-CCCATGACCCAAATTAACGC-3′; NF2exon5S, 5′-ATCTTTAGAATCTCAATCGC-3′; NF2exon5AS, 5′-AGCTTTCTTTTAGACCACAT-3′; NF2exon6S, 5′-CATGTGTAGGTTTTTTATTTTGC-3′; NF2exon6AS, 5′-GCCCATAAAGGAATGTAAACC-3′; NF2exon7S, 5′-CAGTGTCTTCCGTTCTCC-3′; NF2exon7AS, 5′-AGCTCAGAGAGGTTTCAA-3′; NF2exon8S, 5′-CCACAGAATAAAAAGGGCAC-3′; NF2exon8AS, 5′-GATCTGCTGGACCCATCTGC-3′; NF2exon9S, 5′-GTTCTGCTTCATTCTTCC-3′; NF2exon9AS, 5′-GTAATGAAAACCAGGATC-3′; NF2exon10S, 5′-CCTTTTAGTCTGCTTCTG-3′; NF2exon10AS, 5′-TCAGTTAAAACAAGGTTG-3′; NF2exon11S, 5′-TCGAGCCCTGTGATTCAATG-3′; NF2exon11AS, 5′-AAGTCCCCAAGTAGCCTCCT-3′; NF2exon12S, 5′-CCCACTTCAGCTAAGAGCAC-3′; NF2exon12AS, 5′-CTCCTCGCCAGTCTGGTG-3′; NF2exon13S, 5′-GGTGTCTTTTCCTGCTACCT-3′; NF2exon13AS, 5′-GGGAGGAAAGAGAACATCAC-3′; NF2exon14S, 5′-TGTGCCATTGCCTCTGTG-3′; NF2exon14AS, 5′-AGGGCACAGGGGGCTACA-3′; NF2exon15S, 5′-TCTCACTGTCTGCCCAAG-3′; NF2exon15AS, 5′-GATCAGCAAAATACAAGAAA-3′; NF2exon16S, 5′-CTCTCAGCTTCTTCTCTGCT-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′-TTCTGTTTGGCTTGACTTGAC-3′ and BRAF11AS, 5′-CTATTATGACTTGTCACAATGTCACC-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

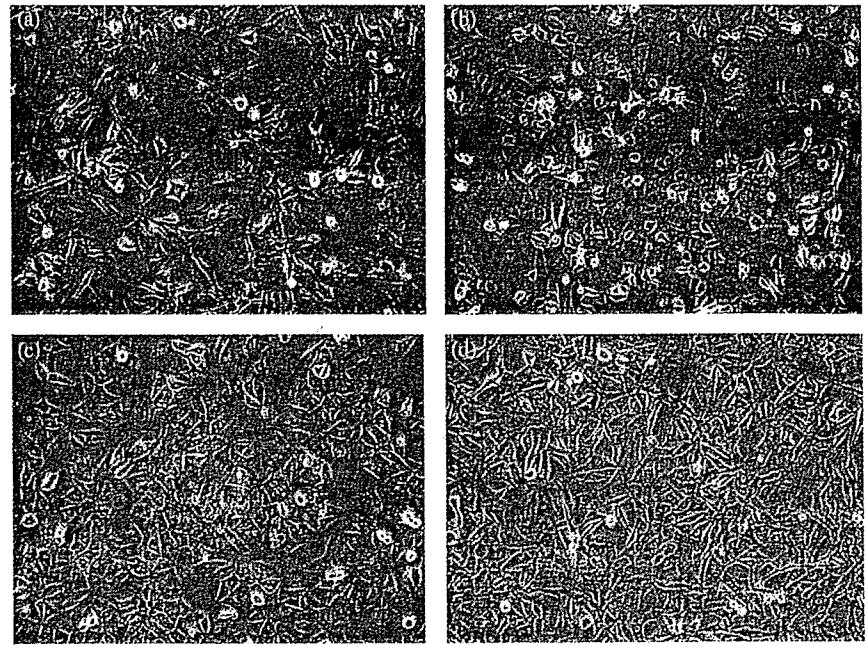

Fig. 1. Morphology of the four malignant pleural mesothelioma (MPM) cell lines. Micrographs of cultured (a) ACC-MESO-1 cells, (b) ACC-MESO-4 cells, (c) Y-MESO-8A cells, and (d) Y-MESO-8D cells (original magnifications ×100).

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.

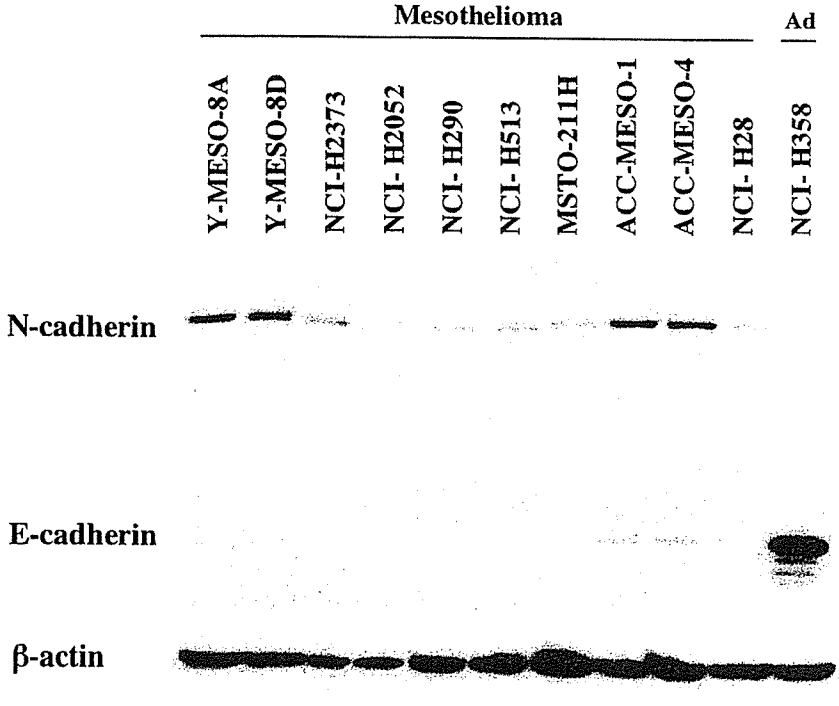

Fig. 2. Western blot analysis of E-cadherin and N-cadherin. Each lane was loaded with 15 μg of total cell lysate from Y-MESO-8A (lane 1), Y-MESO-8D (lane 2), NCI-H2373 (lane 3), NCI-H2052 (lane 4), NCI-H290 (lane 5), NCI-H513 (lane 6), MSTO-211H (lane 7), ACC-MESO-1 (lane 8), ACC-MESO-4 (lane 9), NCI-H28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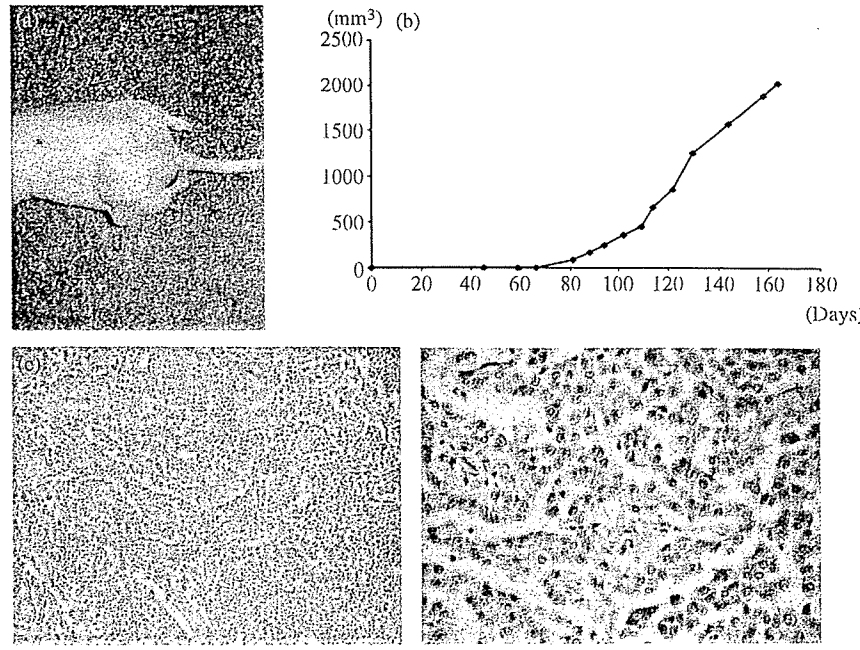

Fig. 3. (a) Xenografted tumor of Y-MESO-8A. (b) Growth curve of the xenografted tumor in nude mice. (c) Histology of xenografted Y-MESO-8A tumor (haematoxylin and eosin, original magnification ×100 and ×400). As in epithelioid mesothelioma, the tumor shows papillary proliferation consisting of atypical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

Acknowledgments

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References

1 Peto J, Hodgson JT, Matthews FE *et al.* Continuing increase in mesothelioma mortality in Britain. *Lancet* 1995; **345**: 535–9.
2 Statistics and Information Department. *Labour and Welfare of Japan. Vital Statistics of Japan 2003*. Tokyo: Health and Welfare Statistics Association, 2003.
3 Sugarbaker DJ, Norberto JJ. Multimodality management of malignant pleural mesothelioma. *Chest* 1998; **113**: 61S–5S.
4 Orengo AM, Spoletini L, Procopio A *et al.* Establishment of four new mesothelioma cell lines: characterization by ultrastructural and immunophenotypic analysis. *Eur Respir J* 1999; **13**: 527–34.
5 Pass HI, Stevens EJ, Oie H *et al.* Characteristics of nine newly derived mesothelioma cell lines. *Ann Thorac Surg* 1995; **59**: 835–44.
6 Tange T, Hasegawa Y, Oka T *et al.* Establishment and characterization of a new human mesothelioma cell line (T-85) from malignant peritoneal mesothelioma with remarkable thrombocytosis. *Pathol Int* 1995; **45**: 791–800.
7 Versnel MA, Bouts MJ, Hoogsteden HC *et al.* Establishment of human malignant mesothelioma cell lines. *Int J Cancer* 1989; **44**: 256–60.

8 Manning LS, Whitaker D, Murch AR *et al.* Establishment and characterization of five human malignant mesothelioma cell lines derived from pleural effusions. *Int J Cancer* 1991; **47**: 285–90.
9 Ishiwata I, Ishiwata C, Anzo M *et al.* Establishment and characterization of a human malignant mesothelioma cell line (HMMME). *Hum Cell* 2003; **16**: 231–9.
10 Sekido Y, Pass HI, Bader S *et al.* Neurofibromatosis type 2 (NF2) gene is somatically mutated in mesothelioma but not in lung cancer. *Cancer Res* 1995; **55**: 1227–31.
11 Sekido Y, Sato M, Usami N *et al.* Establishment of a large cell lung cancer cell line (Y-ML-1B) producing granulocyte colony-stimulating factor. *Cancer Genet Cytogenet* 2002; **137**: 33–42.
12 Sambrook J, Fritsch E, Maniatis T. *Molecular Cloning: A Laboratory Manual*, 2nd edn. New York: Cold Spring Harbor Laboratory Press, 1989.
13 Sato M, Sekido Y, Horio Y *et al.* Infrequent mutation of the *hBUB1* and *hBUBR1* genes in human lung cancer. *Jpn J Cancer Res* 2000; **91**: 504–9.
14 Mori S, Ito G, Usami N *et al.* p53 apoptotic pathway molecules are frequently and simultaneously altered in nonsmall cell lung carcinoma. *Cancer* 2004; **100**: 1673–82.
15 Uchiyama M, Usami N, Kondo M *et al.* Loss of heterozygosity of chromosome 12p does not correlate with KRAS mutation in non-small cell lung cancer. *Int J Cancer* 2003; **107**: 962–9.
16 Sekido Y, Bader SA, Carbone DP *et al.* Molecular analysis of the *HuD* gene encoding a paraneoplastic encephalomyelitis antigen in human lung cancer cell lines. *Cancer Res* 1994; **54**: 4988–92.
17 Carney DN, Gazdar AF, Bepler G *et al.* Establishment and identification of small cell lung cancer cell lines having classic and variant features. *Cancer Res* 1985; **45**: 2913–23.
18 Masuda N, Fukuoka M, Takada M *et al.* Establishment and characterization of 20 human non-small cell lung cancer cell lines in a serum-free defined medium (ACL-4). *Chest* 1991; **100**: 429–38.
19 Liu C, Tsao MS. Proto-oncogene and growth factor/receptor expression in the establishment of primary human non-small cell lung carcinoma cell lines. *Am J Pathol* 1993; **142**: 413–23.
20 Phelps RM, Johnson BE, Ihde DC *et al.* NCI-Navy Medical Oncology Branch cell line data base. *J Cell Biochem Suppl* 1996; **24**: 32–91.
21 Pass HI, Vogelzang N, Hahn S *et al.* Malignant pleural mesothelioma. *Curr Probl Cancer* 2004; **28**: 93–174.
22 Robinson BWS, Musk AW, Lake RA. Malignant mesothelioma. *Lancet* 2005; **366**: 397–408.
23 Popescu NC, Chahinian AP, DiPaolo JA. Nonrandom chromosome alterations in human malignant mesothelioma. *Cancer Res* 1988; **48**: 142–7.
24 Hagemeijer A, Versnel MA, Van Drunen E *et al.* Cytogenetic analysis of malignant mesothelioma. *Cancer Genet Cytogenet* 1990; **47**: 1–28.
25 Taguchi T, Jhanwar SC, Siegfried JM *et al.* Recurrent deletions of specific chromosomal sites in 1p, 3p, 6q, and 9p in human malignant mesothelioma. *Cancer Res* 1993; **53**: 4349–55.
26 Lee WC, Balsara B, Liu Z *et al.* Loss of heterozygosity analysis defines a critical region in chromosome 1p22 commonly deleted in human malignant mesothelioma. *Cancer Res* 1996; **56**: 4297–301.
27 Cheng JQ, Jhanwar SC, Klein WM *et al.* p16 alterations and deletion mapping of 9p21–p22 in malignant mesothelioma. *Cancer Res* 1994; **54**: 5547–51.
28 Hamada K, Kohno T, Takahashi M *et al.* Two regions of homozygous deletion clusters at chromosome band 9p21 in human lung cancer. *Genes Chromosomes Cancer* 2000; **27**: 308–18.
29 Carbone M, Pass HI, Rizzo P *et al.* Simian virus 40-like DNA sequences in human pleural mesothelioma. *Oncogene* 1994; **9**: 1781–90.
30 Gazdar AF, Butel JS, Carbone M. SV40 and human tumours: myth, association or causality? *Nat Rev Cancer* 2002; **2**: 957–64.
31 Carbone M, Pass HI, Miele L *et al.* New developments about the association of SV40 with human mesothelioma. *Oncogene* 2003; **22**: 5173–80.
32 Pass HI, Bocchetta M, Carbone M. Evidence of an important role for SV40 in mesothelioma. *Thorac Surg Clin* 2004; **14**: 489–95.
33 Manfredi JJ, Dong J, Liu WJ *et al.* Evidence against a role for SV40 in human mesothelioma. *Cancer Res* 2005; **65**: 2602–9.
34 Robinson BWS, Lake RA. Advances in malignant mesothelioma. *N Engl J Med* 2005; **353**: 1591–603.
35 Peralta Soler A, Knudsen KA, Jaurand MC *et al.* The differential expression of N-cadherin and E-cadherin distinguishes pleural mesotheliomas from lung adenocarcinomas. *Hum Pathol* 1995; **26**: 1363–9.
36 Galffy G, Mohammed KA, Dowling PA *et al.* Interleukin 8: an autocrine growth factor for malignant mesothelioma. *Cancer Res* 1999; **59**: 367–71.

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平成１６年～１８年度　総合研究報告書

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Mutations of the Epidermal Growth Factor Receptor Gene Predict Prolonged Survival After Gefitinib Treatment in Patients With Non–Small-Cell Lung Cancer With Postoperative Recurrence

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Authors' disclosures of potential conflicts of interest are found at the end of this article.

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ABSTRACT

Purpose
To evaluate the relationship between mutations of the epidermal growth factor receptor (*EGFR*) gene and the effectiveness of gefitinib treatment in patients with recurrent lung cancer after pulmonary resection.

Patients and Methods
We sequenced exons 18-21 of the *EGFR* gene using total RNA extracted from 59 patients with lung cancer who were treated with gefitinib for recurrent lung cancer. Gefitinib effectiveness was evaluated by both imaging studies and change in serum carcinoembryonic antigen (CEA) levels.

Results
EGFR mutations were found in 33 patients (56%). Of these mutations, 17 were deletions around codons 746-750 and 15 were point mutations (12 at codon 858, three at other codons), and one was an insertion. *EGFR* mutations were significantly more prevalent in females, adenocarcinoma, and never-smokers. Gefitinib treatment resulted in tumor shrinkage and/or CEA decrease to less than half of the baseline level in 26 patients, tumor growth and/or CEA elevation in 24 patients, and gefitinib effect was not assessable in nine patients. Female, never-smoking patients with adenocarcinoma tended to respond better to gefitinib treatment. Gefitinib was effective in 24 of 29 patients with *EGFR* mutations, compared with two of 21 patients without mutations ($P < .0001$). Of note, del746-750 might be superior to L858R mutations for prediction of gefitinib response. Patients with *EGFR* mutations survived for a longer period than those without the mutations after initiation of gefitinib treatment ($P = .0053$).

Conclusion
EGFR mutations were a good predictor of clinical benefit of gefitinib in this setting.

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INTRODUCTION

Lung cancer has long been the leading cause of cancer death in North America. In 1998, it became the leading cause of cancer death in Japan, and now claims more than 55,000 lives annually.[1] Lung cancer is divided into two morphologic types: small-cell lung cancer and non–small-cell lung cancer (NSCLC). NSCLCs are further subdivided into adenocarcinoma, squamous cell carcinoma, and large-cell carcinoma. Adenocarcinoma is the predominant histologic subtype, and is increasing among patients with lung cancer who are candidates for surgical treatment in Japan. In our institution, adenocarcinoma accounted for 76% of 407 patients who were operated on from 2001 through 2003. Adenocarcinomas are characterized by a high degree of morphologic heterogeneity. Analyses of various cancer-associated genes, including *K-ras*,[2] *p53*,[3,4] cyclin D1,[5] $p27^{Kip1}$,[6] and cyclooxygenase-2,[7]

suggests a different molecular pathway for carcinogenesis in lung adenocarcinomas at least partly accounts for this heterogeneity. In addition, the NSCLC frequently overexpresses receptors of the ErbB family, including the epidermal growth factor receptor (EGFR) encoded by ErbB1 (HER-1).[8,9]

EGFR is a 170 kd receptor tyrosine kinases (TK) that dimerizes and phosphorylates several tyrosine residues upon binding of several specific ligands including epidermal growth factor and transforming growth factor alpha.[8] These phosphorylated tyrosines serve as the binding sites for several signal transducers that initiate multiple signaling pathways resulting in cell proliferation, migration and metastasis, evasion from apoptosis, or angiogenesis, all of which are associated with cancer phenotypes.[8] Downstream pathways include ras-raf-MEK-ERK, phosphatidylinositol-3 kinase-Akt, and PAK-JNKK-JNK.[8]

Gefitinib is an orally bioavailable small molecule that specifically inhibits EGFR tyrosine phosphorylation.[10] Clinical trials revealed that there is significant variability in response to gefitinib. Good clinical responses have been observed most frequently in women, in nonsmokers, in patients with adenocarcinomas, and in Japanese patients.[11,12] However, it was not possible to predict gefitinib sensitivity by levels of EGFR overexpression as determined by immunohistochemistry[13] or immunoblotting.[14] The factors that determine gefitinib sensitivity have long been an enigma. Recently, it has been reported that activating mutations of *EGFR* are present in a subset of pulmonary adenocarcinomas and that tumors with *EGFR* mutations are highly sensitive to gefitinib[15-17] or erlotinib, another EGFR TK inhibitor. Furthermore, the incidence of *EGFR* mutations is significantly higher in female, never-smoking, Japanese patients with adenocarcinoma.[15] These features coincide with those of good responders to gefitinib.

In this study, we studied patients who had recurrent disease after pulmonary resection for NSCLC and who were subsequently treated with gefitinib. We searched for mutations of the *EGFR* gene in tumor specimens taken at the time of surgery and we correlated *EGFR* mutations with gefitinib effectiveness, including tumor response and patient survival.

PATIENTS AND METHODS

Patients

Seventy-five patients were treated with gefitinib for their recurrent diseases after they had undergone surgery between 1999 and 2003. We studied 59 patients whose tumors were available for RNA extraction, which was a sole determinant of inclusion into the present study. There were 32 men and 27 women with ages ranging from 48 to 79 years. Fifty patients had adenocarcinomas, five had squamous cell carcinomas, three had large-cell carcinomas, and one had adenosquamous carcinoma. Eight patients had stage IA disease; seven stage IB; three stage IIA; five stage IIB; 24 stage IIIA; eight stage IIIB; and three stage IV at the time of surgery. Lobectomy had been performed in 57, and pneumonectomy and partial resection in one patient each. Four patients received postoperative adjuvant chemotherapy (two with oral uracil/tegafur and two with gemcitabine monotherapy). Forty patients had had chemotherapy before gefitinib treatment (23 patients, platinum doublet; 16 patients, monotherapy with vinorelbine or gemcitabine, one patient, oral uracil/tegafur). Gefitinib treatment with a daily dose of 250 mg was initiated between July 2002 and May 2004, with the median interval between operation and gefitinib treatment being 778 days (range, 107 to 1,931 days). Fifty patients had distant metastatic tumors, eight patients had pleural dissemination and malignant effusion, and one patient had hilar lymph-node metastasis at initiation of gefitinib treatment.

Molecular Analysis of Lung Cancer Specimens

After we obtained appropriate approval from the institution and written informed consent for comprehensive use of molecular and pathologic analysis from the patients, tumor samples were collected during surgery, rapidly frozen in liquid nitrogen and stored at −80°C. A surgical pathologist (Y.Y.) grossly dissected the frozen tumor specimens to enrich the tumor cell population as much as possible. Total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA).

The first four exons (exons 18-21) of the seven exons (exons 18-24) that code for TK domain of the *EGFR* gene (which includes all the mutations reported so far[15-17]) was amplified with primers F1 (5′-AGCTTGTGGAGCCTCTTACACC-3′) and R1 (5′-TAAAATTGATTCCAATGCCATCC-3′) in a one-step reverse transcription polymerase chain reaction (RT-PCR) using the QIAGEN OneStep RT-PCR Kit (Qiagen). The cDNA sequence of the *EGFR* gene was obtained from GenBank (accession number NM 005228). The RT-PCR conditions were: one cycle of 50°C for 30 minutes, 95°C for 15 minutes, 40 cycles of 94°C for 50 seconds, 62°C for 50 seconds, and 72°C for 60 seconds, followed by one cycle of 72°C for 10 minutes.

RT-PCR products were diluted and cycle-sequenced using the Big Dye Terminator v3.1/1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Sequencing products were electrophoresed on an ABI PRISM 3100 (Applied Biosystems). Both the forward and reverse sequences obtained were analyzed by BLAST (basic local alignment search tool) and chromatograms by manual review. High-quality sequence variations found in both directions were scored as candidate mutations.

Definition of Effectiveness of Gefitinib

Because this study was a retrospective analysis of the daily clinical practice of oncology, the evaluation of tumor response could not be performed strictly according to predefined criteria, such as Response Evaluation Criteria in Solid Tumors (RECIST).[18] RECIST are not necessarily applicable or complete in such a context and the evaluation may instead be based on a subjective medical judgment that results from clinical and laboratory data.[18] Therefore, gefitinib treatment was judged as effective when the tumors showed at least a 30% decrease in tumor diameter in imaging studies. However, because of the nature of the study, confirmation of tumor response no less than 4 weeks apart, as in RECIST,[18] was not necessarily required.

As patients with recurrent lung cancer often do not have measurable disease, we also included change in serum carcinoembryonic antigen (CEA) level (cut off, 5 ng/mL) as an evaluation

criterion to avoid underestimating gefitinib effectiveness. CEA has been reported as a useful clinical therapeutic marker.[19] When the elevated CEA level decreased to a level less than half of the baseline level, gefitinib treatment was judged as effective. On the other hand, gefitinib treatment was judged as ineffective when the tumors showed any growth or a new lesion appeared in the imaging studies, or when the serum CEA level increased. Any patient who did not fit either of these criteria was classified as not assessable. All these evaluations were done before the *EGFR* gene analysis, without knowledge of mutational status of the *EGFR* gene.

Statistical Analysis

For comparisons of proportions, the χ^2 test or Fisher's exact test was used. The Kaplan-Meier method was used to estimate the probability of survival as a function of time, and survival differences were analyzed by the log-rank test. The two-sided significance level was set at $P < .05$. To identify which independent factors had a joint significant influence on gefitinib effectiveness, the logistic regression modeling technique was used, and for multivariate analysis of the overall survival, the Cox proportional hazards modeling technique was applied. All analyses were performed using StatView version 5 (SAS institute Inc, Cary, NC) software on a Macintosh computer.

RESULTS

EGFR *Mutations*

Mutations of the *EGFR* gene were detected in 33 (56%) of 59 patients. Seventeen were deletions, 15 were point mutations, and one was an insertion. Details of these mutations are shown in Figure 1. As previously reported,[15-17] *EGFR* mutations were significantly associated with adenocarcinoma histology, female sex, and never-smoking status (Table 1). However, the mutations were not associated with the age or stage of the patients. Furthermore, median time from the original surgery to

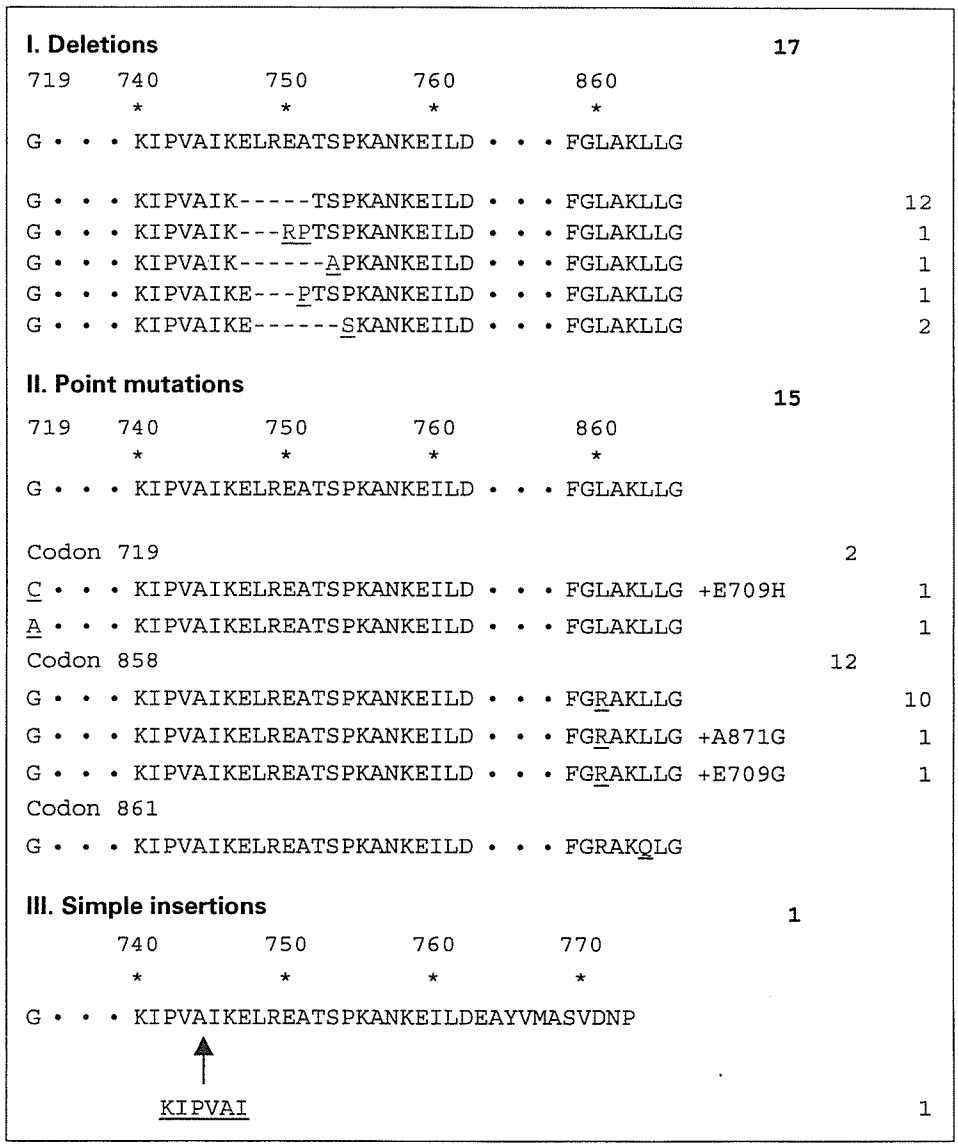

Fig 1. Analysis of 33 epidermal growth factor receptor (EGFR) mutations in tyrosine kinase domain of the *EGFR* gene found in unselected patients with lung cancer.

Table 1. Incidence of EGFR Mutations and Clinical and Pathologic Features

Variable	EGFR Mutation No. of Patients	%	EGFR Wild-Type	P
All cases	33	56	26	
Sex				
Male	14	44	18	.0402
Female	19	70	8	
Age, years				
≤ 64	22	55	18	.8342
> 64	11	58	8	
Histologic type				
Adenocarcinoma	32	64	18	.0033
Nonadenocarcinoma	1	11	8	
Squamous cell carcinoma	0	0	5	
Large-cell carcinoma	0	0	3	
Adenosquamous carcinoma	1	100	0	
Smoking status				
Never smoker	20	71	8	.0227
Former or current smoker	13	42	18	
Stage				
I-II	12	50	12	.4472
III-IV	21	60	14	

Abbreviation: EGFR, epidermal growth factor receptor.

recurrence was almost identical in patients with *EGFR* mutations (362 days) and in those without *EGFR* mutations (363 days; $P = .8265$).

Clinical Improvement After Gefitinib Treatment

Forty-one of 59 patients had measurable disease at recurrence with imaging studies. Of these, 20 showed appreciable tumor shrinkage after gefitinib treatment, whereas 17 tumors increased in size, and there was no change in tumor size in four patients. All of these 20 tumors (pulmonary metastases in 11, pleural disseminated nodules in two, hepatic metastases in two, mediastinal lymph node swelling in two, brain metastases in two, and chest wall tumor in one) showed at least a 30% decrease in diameter. Figure 2 shows representative imaging studies. A computed tomography scan of the chest in patient L703 (73-year-old woman, adenocarcinoma) showed masses in the right-lower lobe and marked improvement 8 weeks after gefitinib initiation. A computed tomography scan of the liver in patient L1492 (52-year-old woman, adenocarcinoma) showed masses in the right lobe of the liver and dramatic improvement 10 days after gefitinib initiation. A large chest-wall mass in the left back of patient L1362 (62-year-old man, adenosquamous carcinoma) before gefitinib treatment almost disappeared 13 weeks after gefitinib initiation. A left-lung tumor in patient L1171 (70-year-old woman, adenocarcinoma) was smaller 6 weeks after gefitinib initiation.

CEA was above the upper normal limit (5 ng/mL) at baseline in 32 patients. Serum CEA level decreased to $< 10\%$, $< 50\%$, and to $> 50\%$ of the baseline level in three, 12, and five patients, respectively, whereas CEA level increased in 12 patients. When we combined the results of imaging studies with CEA and judged according to our criteria, gefitinib treatment was effective in 26 (52%), not effective in 24 (48%), and not assessable in nine patients (Table 2). There was a good correlation between these two examinations. The imaging studies and change in CEA levels did not conflict in any patients. In 17 patients with measurable diseases and whose baseline CEA level was elevated, the CEA level decreased in all 11 patients showing tumor shrinkage and increased in all five patients showing tumor growth, except for one patient whose tumors showed no change in size ($P < .001$, Fisher's exact test), supporting the validity of our criteria.

We searched for a relation between gefitinib effectiveness and various clinical and pathologic features (Table 2). Never-smokers and patients with adenocarcinoma had a significantly higher incidence of gefitinib effect. However, we could not detect significant difference in gefitinib sensitivity by sex or presence of prior chemotherapy, probably because of the small sample size, although there was a trend that female and chemotherapy-naïve patients were more responsive.

Relationship Between Clinical Response to Gefitinib Treatment and EGFR Mutations

The incidence of *EGFR* mutations in terms of response to gefitinib treatment as judged by imaging studies and CEA levels is shown in Table 3. Of 20 patients who showed tumor shrinkage, 19 (95%) had mutations of the *EGFR* gene. On the other hand, two (12%) of 17 patients whose tumors grew after gefitinib treatment harbored *EGFR* mutations ($P < .001$, Fisher's exact test). In Figure 2, patient L703, L1492, and L1362 had *EGFR* mutations (delE746-A750, L858R, and E746-S752insA, respectively). Of three, 12, and five patients whose CEA level decreased to less than 10%, less than 50%, and to more than 50% of the baseline level after gefitinib treatment, three (100%), 10 (83%), and four (80%) had *EGFR* mutations, respectively. On the other hand, of 12 patients whose CEA level increased, three (25%) had *EGFR* mutations ($P = .004$, Fisher's exact test).

When we used our criteria combining the results of imaging studies with CEA, gefitinib was effective in 24 (83%) of 29 patients with *EGFR* mutations, whereas it was effective only in two (10%) of 21 patients without *EGFR* mutations ($P < .0001$; Table 2). There were three patients with *EGFR* mutations (two with L858R and one with G719A) whose CEA level increased after gefitinib treatment but did not have measurable diseases. There were also two patients with *EGFR* mutations, one with L858R+E709H and one with I744-K745 ins KIPVAI whose tumor progressed.

Logistic regression analysis (Table 4) showed that *EGFR* mutation was the only significant factor contributing to gefitinib sensitivity.

On the other hand, patient L1171, who showed a decrease in size of multiple pulmonary metastatic nodules

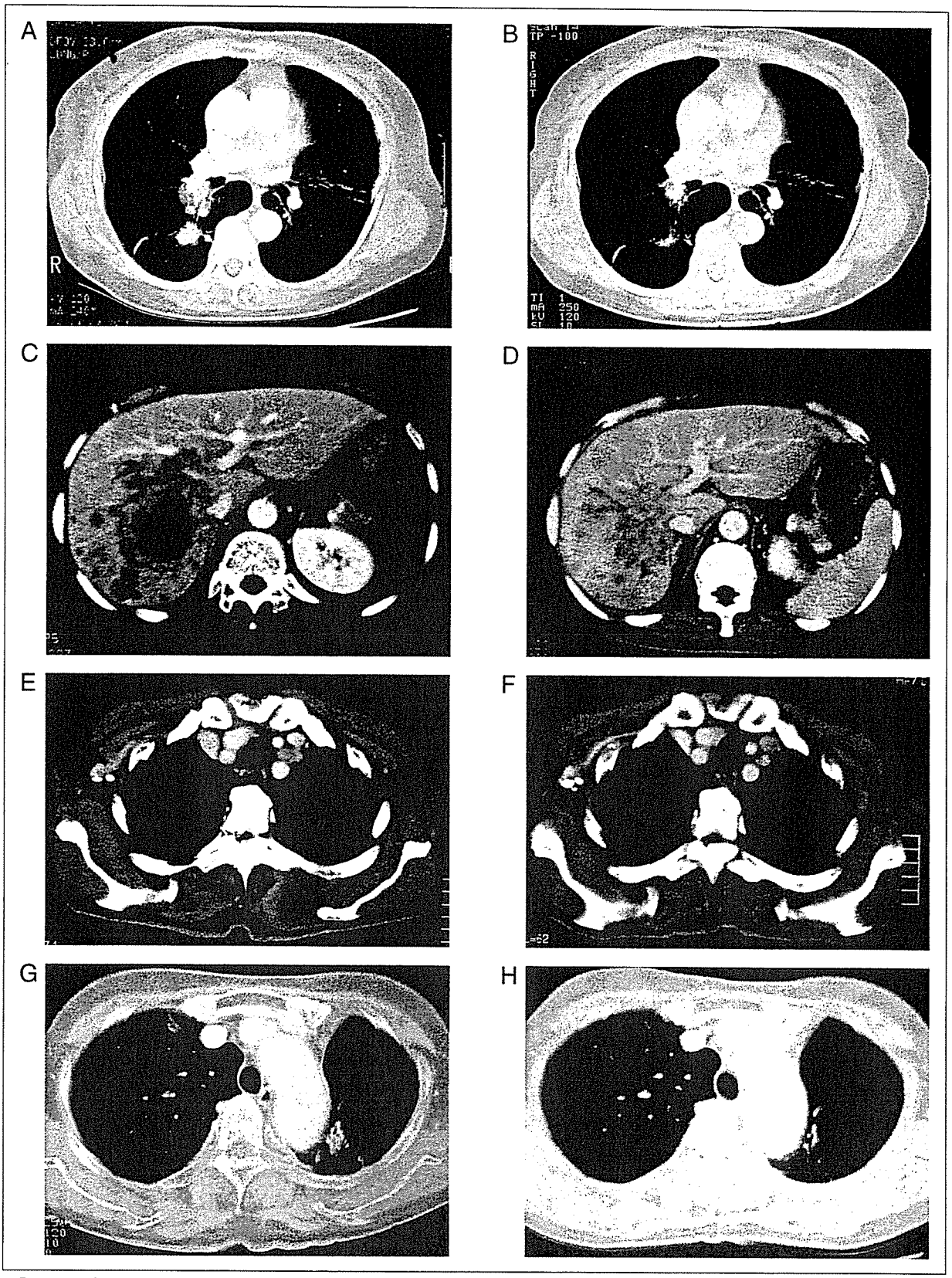

Fig 2. Examples of the response to gefitinib in representative four patients with recurrent non–small-cell lung cancer. Computed tomography (CT) scans before gefitinib treatment (A, C, E, G) and after the gefitinib was initiated (B, D, F, H) are shown. CT scans of patient L703 (A, B), patient L1492 (C, D), patient L1362 (E, F), and patient L1171 (G, H).

Table 2. Relation Between Gefitinib Effectiveness and Various Clinical and Pathologic Features

Variable	Effective: No. of Patients	Effective: %*	Not Effective	Not Assessable	P†
All patients	26	52	24	9	
Sex					
Male	11	41	16	5	.0842
Female	15	65	8	4	
Smoking status					
Never-smoker	17	68	8	3	.0235
Former or current smoker	9	36	16	6	
Histologic type					
Adenocarcinoma	25	58	18	7	.0313
Nonadenocarcinoma	1	14	6	2	
Prior chemotherapy					
Present	17	47	19	4	.2782
Absent	9	64	5	5	
EGFR mutation					
Mutation	24	83	5	4	< .0001
Deletion	16	100	0	1	.0108‡
Insertion	0	0	1	0	
Point mutation	8	67	4	3	
Wild-type	2	10	19	5	

Abbreviation: EGFR, epidermal growth factor receptor.
*Percentages were calculated excluding patients who were not assessable.
†*P* values were calculated excluding patients who were not assessable.
‡*P* value for Fisher's exact test comparing deletion mutants with the other mutants.

(Figs 2G and H) and a decrease in CEA level from 16.8 to 4.3 ng/mL, did not have *EGFR* mutations. In this patient, we extended our search for mutations to exons 22 and 23 of the *EGFR* gene, and still found none. Another patient without *EGFR* mutation in whom gefitinib was effective was a 59-year-old man who showed a decrease in serum CEA level from 10.6 to 1.5 ng/mL after 2 weeks of gefitinib treatment; this low level of CEA was maintained at least for 7 months.

When we further analyzed gefitinib response by classes of *EGFR* mutation, we found that there was a difference of response between patients with deletion mutations and those with the other types of mutations. Gefitinib was effective in all 16 patients with deletions, and effective in eight of 13 with other types of mutation ($P = .0108$).

Effect of EGFR *Mutation on Patient Survival After Gefitinib Treatment*

Patients with *EGFR* mutations survived for a significantly longer time calculated from the day of gefitinib initiation than those without *EGFR* mutations ($P = .0053$, log-rank test; Fig 3). Likewise, 26 gefitinib responders survived for a longer time than 24 nonresponders ($P = .0320$, log-rank test; not shown). Multivariate analysis revealed that *EGFR* mutation was the only factor that significantly and independently affected overall survival (Table 5). *EGFR* mutation class did not affect overall survival (not shown).

DISCUSSION

Recurrence after complete resection of NSCLC often presents as a form of distant metastases.[20] In clinical practice, chemotherapy is given to these patients except for a small number in whom re-resection of the tumor is indicated. Many studies have shown that chemotherapy prolongs survival and improves quality of life in unresectable stage IV tumors.[21] However, patients with unresectable tumors and patients with recurrent diseases may not be the same. There have been no large-scale randomized clinical trials addressing whether chemotherapy improves survival of patients with recurrence. Yoshino et al[22] found that chemotherapy for recurrence only tended to prolong survival in 118 of 468 consecutive patients who had recurrence after pulmonary resections. After introduction of gefitinib to clinical practice in 2002 in Japan, some patients with recurrent disease showed dramatic responses to gefitinib treatment, but many others did not respond. It has been unclear which patients respond to gefitinib and also whether gefitinib treatment prolongs survival in these patients.

Recent studies have showed striking correlation between gefitinib sensitivity and *EGFR* mutations both in vitro and in clinical studies.[15-17] Because this study was a retrospective analysis of response to gefitinib prescribed as routine care, judgment of gefitinib effectiveness tended to be less strict than that in a prospective clinical trial. Yet, changes in serum CEA level never conflicted with imaging studies. We were able to confirm a relation between *EGFR*

Table 3. Response to Gefitinib Treatment in 59 Patients With Recurrent Disease

CEA Level	Imaging Results: Shrinkage	No Change	Not Measurable	Growth	Total
Decreased					
<10% of the baseline	**3 (3)**				3 (3)
<50% of the baseline	**6 (5)**	**1 (1)**	**5 (4)**		12 (10)
>50% of the baseline	**2 (2)**		*3 (2)*		5 (4)
Not assessable	**9 (9)**	*3 (1)*	*3 (1)*	12 (2)	27 (13)
Elevated			7 (3)	5 (0)	12 (3)
Total	20 (19)	4 (2)	18 (10)	17 (2)	59 (33)

NOTE. Numbers in bold indicate that gefitinib treatment resulted in clinical improvement in these patients; numbers with underlines indicate the treatment resulted in progression of the disease; numbers in parentheses show number of patients with EGFR mutations in each category; and italicized numbers indicate that gefitinib treatment could not be assessed.
Abbreviations: EGFR, epidermal growth factor receptor; CEA, carcinoembryonic antigen.

Table 4. Logistic Regression Analysis of Various Factors That Predict EGFR Effectiveness

Variable	Odds Ratio	95% CI	*P*
Sex			
Male/female	1.139	0.130 to 9.953	.9063
Smoking status			
Never/former/current	1.496	0.165 to 13.535	.7202
Histologic type			
Adenocarcinoma/ nonadenocarcinoma	1.727	0.091 to 33.33	.7159
Prior chemotherapy			
Yes/no	0.427	0.060 to 3.027	.3948
EGFR mutation			
Mutant/wild-type	40.000	6.024 to 2750	< .0001

Abbreviation: EGFR, epidermal growth factor receptor.

mutations and gefitinib sensitivity in a slightly different clinical setting. We correlated *EGFR* mutations found in specimens taken at the time of surgery with response to gefitinib, often after several courses of cytotoxic chemotherapy for recurrent disease. Multivariate analysis revealed that *EGFR* mutation was the only independent predictor for gefitinib response among several allegedly contributing factors. As in previous studies, *EGFR* mutation was not a perfect predictor of gefitinib effectiveness.[15-17] Two patients without *EGFR* mutations showed response to gefitinib. It is not clear at this time whether *EGFR* mutations are present in other parts of the gene or whether mechanisms other than *EGFR* mutations govern sensitivity in these patients.

We found a significant difference in gefitinib sensitivity according to classes of *EGFR* mutations. All 16 patients with deletion mutants responded to gefitinib, compared with eight of 12 patients with other mutations (P = .0108). It is not clear whether this difference is based on differences in biologic activity of these mutant proteins.

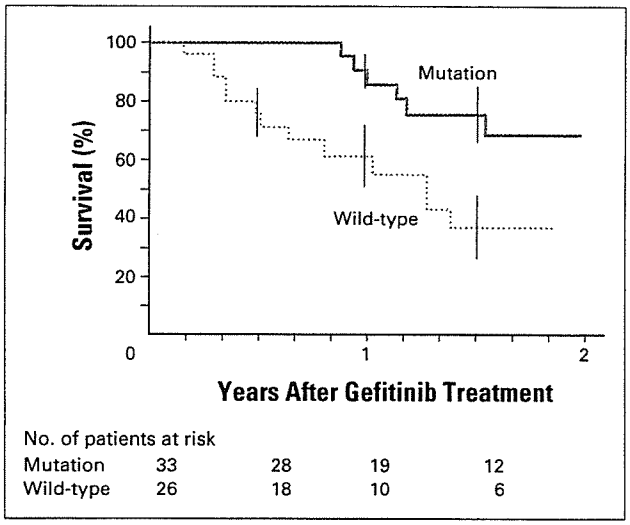

Fig 3. Effect of epidermal growth factor receptor mutations on survival, calculated from the day of initiating gefitinib treatment in patients who had recurrent disease after surgery (P = .0053, log-rank test).

Table 5. Cox Proportional Hazards Model for Survival Analysis

Variable	Hazard Ratio	95% CI	*P*
Sex			
Female/male	0.359	0.068 to 1.900	.2280
Smoking status			
Never/former/current	0.511	0.092 to 2.854	.4445
Histologic type			
Adenocarcinoma/ nonadenocarcinoma	0.335	0.095 to 1.184	.0894
Prior chemotherapy			
Yes/no	0.653	0.222 to 1.923	.4397
Stage			
I-II/III-IV	0.848	0.322 to 2.232	.7380
Age, years			
> 64/≤ 64	0.964	0.342 to 2.717	.9457
EGFR mutation			
Mutant/wild-type	0.342	0.117 to 0.998	.0496

Abbreviation: EGFR, epidermal growth factor receptor.

Gefitinib sensitivity was essentially the same in COS cells transfected with L858R and in cells transfected with del L747-P753insS.[16] A more recent study showed that the tyrosine residue at codon 845 is highly phosphorylated in L858R mutants, but not in deletion mutants after epidermal growth factor binding.[23] This might explain the difference in gefitinib response between tumors with L858R and those with deletions.

Although our criteria for tumor response are soft, these are merely a surrogate marker for the effect on survival. We were able to show, for the first time, that *EGFR* mutation was the only significant and independent predictor for a prolonged survival after gefitinib treatment. In a previous study, we showed that *EGFR* mutation itself is not a predictor for better postoperative survival in 236 unselected patients with adenocarcinoma,[24] and in the present study, median disease-free interval was almost identical in patients with or without *EGFR* mutations. A recent placebo-controlled clinical trial showed that treatment with erlotinib, another oral EGFR TK inhibitor, significantly prolongs survival after first and second chemotherapy for NSCLC,[25] although *EGFR* mutation frequency is reported to be around 10% in Western countries.[15-17] This result is interpreted to mean that a subset of patients without mutations have also benefited from erlotinib therapy. The present study suggests that if patients were selected by presence of *EGFR* mutations, it would be possible to concentrate patients with benefits from gefitinib treatment, avoiding unnecessary adverse reactions such as fatal interstitial lung disease, which is relatively common in Japanese patients.[26] Furthermore, our results provide a basis for postoperative adjuvant gefitinib treatment in NSCLC patients with *EGFR* mutations, as adjuvant treatment is considered the earliest treatment of metastatic disease. These possibilities should be tested in future clinical trials.

It is common for patients to show progressive disease soon after presenting an initial striking response to

gefitinib. However, we could not detect any evidence that differences in classes of *EGFR* mutations are associated with duration of response (data not shown).

In conclusion, tumors with *EGFR* mutations showed good, but not perfect, correlation with clinical response in patients with postoperative recurrence of NSCLC. Furthermore, patients with *EGFR* mutations survived for a significantly longer period than those without *EGFR* mutations. Future clinical trials using gefitinib should examine *EGFR* mutations for effective selection of patients who are most likely to benefit from this molecular-targeted drug.

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Authors' Disclosures of Potential Conflicts of Interest

The following authors or their immediate family members have indicated a financial interest. No conflict exists for drugs or devices used in a study if they are not being evaluated as part of the investigation. Honoraria: Tetsuya Mitsudomi, AstraZeneca Japan, Bristol-Myers Squibb Japan, TAIHO Pharmaceutical. For a detailed description of this category, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and Disclosures of Potential Conflicts of Interest found in Information for Contributors in the front of each issue.

REFERENCES

1. Kuroishi T, Hirose K, Tajima K: Cancer mortality in Japan, in Tominaga S, Oshima A (eds): Gann Monograph Cancer Research. Tokyo, Japan Scientific Societies Press, 1999, pp 1-38
2. Mitsudomi T, Viallet J, Linnoila RI, et al: Mutations of ras genes distinguish a subset of non-small cell lung cancer cell lines from small cell lung cancer cell lines. Oncogene 6:1353-1362, 1991
3. Mitsudomi T, Oyama T, Nishida K, et al: p53 nuclear immunostaining and gene mutations in non-small-cell lung cancer and their effects on patient survival. Ann Oncol 6:S9-13, 1995
4. Nishio M, Koshikawa T, Kuroishi T, et al: Prognostic significance of abnormal p53 accumulation in primary, resected non-small-cell lung cancers. J Clin Oncol 14:497-502, 1996
5. Nishio M, Koshikawa T, Yatabe Y, et al: Prognostic significance of cyclin D1 and Rb expression in combination with p53 abnormalities in primary, resected non-small cell lung cancer. Clin Cancer Res 3:1051-1058, 1997
6. Yatabe Y, Masuda A, Koshikawa T, et al: p27kip1 in human lung cancers: Differential changes in small cell and non-small cell carcinomas. Cancer Res 58:1042-1047, 1998
7. Hida T, Yatabe Y, Achiwa H, et al: Increased expression of cyclooxygenase 2 occurs frequently in human lung cancers, specifically in adenocarcinomas. Cancer Res 58:3761-3764, 1998
8. Arteaga CL: Overview of epidermal growth factor receptor biology and its role as a therapeutic target in human neoplasia. Semin Oncol 29:3-9, 2002
9. Bunn PA, Jr., Franklin W: Epidermal growth factor receptor expression, signal pathway, and inhibitors in non-small cell lung cancer. Semin Oncol 29:38-44, 2002
10. Ciardiello F, Tortora G: A novel approach in the treatment of cancer: Targeting the epidermal growth factor receptor. Clin Cancer Res 7:2958-2970, 2001
11. Kris MG, Natale RB, Herbst RS, et al: Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: A randomized trial. JAMA 290:2149-2158, 2003
12. Fukuoka M, Yano S, Giaccone G, et al: Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer. J Clin Oncol 21:2237-2246, 2003
13. Cappuzzo F, Gregorc V, Rossi E, et al: Gefitinib in pretreated non-small-cell lung cancer (NSCLC): Analysis of efficacy and correlation with HER2 and epidermal growth factor receptor expression in locally advanced or metastatic NSCLC. J Clin Oncol 21:2658-2663, 2003
14. Suzuki T, Nakagawa T, Endo H, et al: The sensitivity of lung cancer cell lines to the EGFR-selective tyrosine kinase inhibitor ZD1839 ('Iressa') is not related to the expression of EGFR or HER-2 or to K-ras gene status. Lung Cancer 42:35-41, 2003
15. Paez JG, Janne PA, Lee JC, et al: EGFR mutations in lung cancer: Correlation with clinical response to gefitinib therapy. Science 304:1497-1500, 2004
16. Lynch TJ, Bell DW, Sordella R, et al: Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 350:2129-2139, 2004
17. Pao W, Miller V, Zakowski M, et al: EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci U S A 101:13306-13311, 2004
18. Therasse P, Arbuck SG, Eisenhauer EA, et al: New guidelines to evaluate the response to treatment in solid tumors. European Organisation for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 92:205-216, 2000
19. Salgia R, Harpole D, Herndon JE, 2nd, et al: Role of serum tumor markers CA 125 and CEA in non-small cell lung cancer. Anticancer Res 21:1241-1246, 2001
20. Mitsudomi T, Nishioka K, Maruyama R, et al: Kinetic analysis of recurrence and survival after potentially curative resection of nonsmall cell lung cancer. J Surg Oncol 63:159-165, 1996
21. Chemotherapy in non-small cell lung cancer: a meta-analysis using updated data on individual patients from 52 randomized trials. Non-Small Cell Lung Cancer Collaborative Group. BMJ 311:899-909, 1995
22. Yoshino I, Yohena T, Kitajima M, et al: Survival of non-small cell lung cancer patients with postoperative recurrence at distant organs. Ann Thorac Cardiovasc Surg 7:204-209, 2001
23. Sordella R, Bell DW, Haber DA, et al: Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. Science 305:1163-1167, 2004
24. Kosaka T, Yatabe Y, Endoh H, et al: Mutations of the epidermal growth factor receptor gene in lung cancer: Biological and clinical implications. Cancer Res 64:8919-8923, 2004
25. Shepherd FA, Pereira J, Ciuleanu TE, et al: A randomized placebo-controlled trial of erlotinib in patients with advanced non-small cell lung cancer following failure of 1st line or 2nd line chemotherapy. Proc Am Soc Clin Oncol 22:622s, 2004 (abstr 7022)
26. Inoue A, Saijo Y, Maemondo M, et al: Severe acute interstitial pneumonia and gefitinib. Lancet 361:137-139, 2003

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PHASE II STUDIES

Phase II study of amrubicin in previously untreated patients with extensive-disease small cell lung cancer: West Japan Thoracic Oncology Group (WJTOG) study

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Summary *Purpose:* To evaluate the efficacy and safety of amrubicin, (+)-(7*S*, 9*S*)-9-acetyl-9-amino-7-[(2-deoxy-β-D-*erythro*-pentopyranosyl)oxy]-7,8,9,10-tetrahydro-6,11-dihydroxy-5,12-naphthacenedione hydrochloride, in previously untreated patients with extensive-disease small cell lung cancer (SCLC).

Patients and methods: A total of 35 previously untreated patients with extensive-disease SCLC were entered into the study. Amrubicin was given by daily intravenous infusion at 45 mg/m^2/day for 3 consecutive days, every 3 weeks. Unless there was tumor regression of 25% or greater after the first cycle, or 50% or greater after the second cycle, treatment was switched to salvage chemotherapy in combination with etoposide (100 mg/m^2, days 1, 2, and 3) and cisplatin (80 mg/m^2, day 1).

Results: Of the 35 patients entered, 33 were eligible and assessable for efficacy and toxicity. Of the 33 patients, 3 (9.1%) had a complete response (95% confidence interval [CI], 1.9–24.3%) and 22 had a partial response, for an overall response rate of 75.8% (95% CI, 57.7–88.9%). Median survival time was 11.7 months (95% CI, 9.9–15.3 months), and 1-year and 2-year survival rates were 48.5% and 20.2%, respectively. The most common toxicity was hematologic. Non-hematologic toxicity of grade 3 or 4 was only seen in 3 patients with anorexia (9.1%) and 1 patient with alopecia (3.0%). Salvage chemotherapy was administered to only 6 patients.

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Conclusion: Amrubicin was active for extensive-disease SCLC with acceptable toxicity. Further studies in combination with other agents for SCLC are warranted.

Keywords Amrubicin · Small cell lung cancer · Anthracycline · Previously untreated patients · Phase II study

Introduction

Small cell lung cancer (SCLC) is a major cause of cancer deaths and accounts for 15 to 20% of all lung cancers [1]. Although this cancer is initially highly responsive to chemotherapy, the vast majority of patients will ultimately relapse and die of recurrent disease within 2 years [2]. Recently, combination chemotherapy with irinotecan and cisplatin for extensive-disease SCLC produced more survival benefit than etoposide and cisplatin, the worldwide standard regimen since 1981 [3, 4]. Median survival time and 2-year survival rate of the standard regimen is 12.8 months and 19.5%, respectively. Clearly, new and more effective agents against SCLC are needed.

Amrubicin is a totally synthetic 9-aminoanthracycline, (+)-(7*S*, 9*S*)-9-acetyl-9-amino-7-[(2-deoxy-β-D-*erythro*-pentopyranosyl)oxy]-7, 8, 9, 10-tetrahydro-6, 11-dihydroxy-5,12-naphthacenedione hydrochloride, with a chemical structure similar to that of doxorubicin (Fig. 1) [5]. Amrubicin showed more potent antitumor activity than doxorubicin in several human tumor xenografts implanted in nude mice [6]. Acute toxicity of amrubicin is qualitatively similar to that of doxorubicin [7], however, amrubicin shows almost no delayed toxicity (e.g. cardiotoxicity) [8, 9].

Amrubicin is converted to an active metabolite, amrubicinol, by reduction of its C-13 ketone group to a hydroxy group. *In vitro* cytotoxic activity of amrubicinol was almost equipotent to that of doxorubicin and 20 to 220 times more potent than that of its parent compound, amrubicin [10]. Amrubicinol is considered to be closely associated with the efficacy and toxicity of amrubicin [11].

Despite their similarity in chemical structure, amrubicin has a different mode of action to doxorubicin [12]. Amrubicin and its active metabolite, amrubicinol, are inhibitors of DNA topoisomerase II. Amrubicin and amrubicinol exert cytotoxic effects by stabilizing topoisomerase II-mediated cleavable complexes, while doxorubicin does not inhibit this step of the catalytic cycle of topoisomerase II at concentrations for which it demonstrates cytotoxicity. Doxorubicin is a potent DNA intercalator, and its cytotoxicity is thought to be mainly due to this. Amrubicin and amrubicinol are about one-tenth weaker DNA intercalators than doxorubicin. Therefore, they are similar to etoposide in terms of inhibition of topoisomerase II by stabilizing the cleavable complexes, although etoposide does not show any DNA intercalating activity.

In a phase I–II study in patients with non-small cell lung cancer, amrubicin was administered as a 5-min intravenous infusion for 3 consecutive days [13]. The maximum tolerated dose (MTD) was 50 mg/m^2/day and the dose-limiting toxicities were leukopenia, neutropenia, thrombocytopenia, and gastrointestinal complications. The recommended dose for the phase II study was 45 mg/m^2/day for 3 consecutive days every 3 weeks.

Based on these experimental data and preliminary clinical reports indicating that amrubicin may be active against lung cancer, the West Japan Thoracic Oncology Group (WJTOG) evaluated it for use in SCLC. The WJTOG conducted a phase II study in previously untreated extensive-disease SCLC patients as a first-line therapy. Salvage chemotherapy with etoposide and cisplatin and an early cessation rule were set in place as precautionary measures.

Patients and methods

Eligibility criteria

Eligibility criteria included histologically or cytologically proven small cell lung cancer with extensive-disease defined as distant metastasis and/or disease involving the

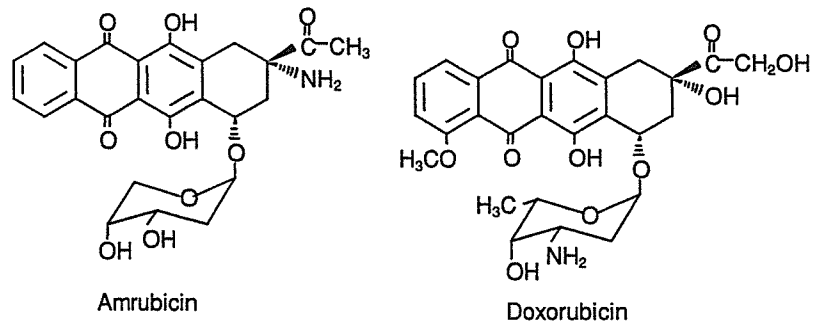

Fig. 1 Chemical structures of amrubicin and doxorubicin

contralateral hilar lymph nodes; no prior treatment; life expectancy of at least 2 months; the Eastern Cooperative Oncology Group (ECOG) performance status of 0 to 2; at least one bidimensionally measurable lesion; age less than 80; adequate organ function, such as white blood cell (WBC) count of 4000×10^6/L or greater, hemoglobin level 10 g/dL or greater, platelet count 100×10^9/L or greater, AST and ALT less than 100 IU/L, bilirubin level 1.5 mg/dL or less, creatinine concentration 1.2 mg/dL or less, electrocardiogram (ECG) findings within normal range, and left ventricular ejection fraction (LVEF) of echocardiogram 60% or greater. All patients gave written informed consent. Ineligibility criteria were: brain or bone metastases requiring radiation; continuous long-term treatment with non-steroidal anti-inflammatory drugs and glucocorticoids; pulmonary fibrosis; serious complications and other active malignancy; or pregnant or nursing subjects.

This study was approved by the institutional review boards at each participating center.

Study design

Amrubicin (Sumitomo Pharmaceuticals Co., Ltd, Osaka, Japan) was dissolved in 20 mL normal saline and administered once intravenously as a 5-min infusion at a dose of 45 mg/m^2/day on days 1 to 3, every 3 weeks.

Before treatment, all patients underwent a medical history, physical examination, hematology and serum biochemistry tests, urinalysis, ECG, LVEF, and baseline tumor measurements (chest radiography, CT scans, bone scintigraphy, and other measurements as appropriate). All measurable and assessable lesions were evaluated within 2 weeks before treatment. ECG and LVEF were undertaken within 1 month before treatment.

Complete and differential blood cell counts, platelet counts, hematocrit analysis, biochemical analysis including AST, ALT, alkaline phosphatase, LDH, total bilirubin, BUN, creatinine, serum bilirubin, albumin, total protein, and electrolyte levels (Na, K, Cl, and Ca), and urinalysis (including protein, glucose, urobilinogen, and occult blood) were performed weekly as a rule. When severe myelosuppression was observed, complete and differential blood cell counts plus platelet counts were performed 2 times or more per week. ECG was undertaken every treatment cycle and LVEF every other cycle. Chest radiography and CT scans were carried out every cycle as a rule.

Subjective and objective symptoms were observed and recorded as appropriate.

Dose modifications were made according to WBC and platelet counts. If the WBC count nadir was lower than $1{,}000 \times 10^6$/L for 4 days or longer and/or the platelet count nadir was lower than 50×10^9/L, a dose reduction of 5 mg was stipulated in the subsequent treatment course. Treatment was postponed until the WBC and platelet counts recovered to $\geq 3{,}000 \times 10^6$/L and $\geq 100 \times 10^9$/L, respectively.

In patients who demonstrated tumor regression of 25% or greater after the first course of chemotherapy, amrubicin treatment was continued. After the second course, patients had to have achieved tumor regression of 50% or greater to continue to receive the drug up to a maximum of 6 courses. Treatment of combination chemotherapy with etoposide (100 mg/m^2 on days 1, 2, and 3) and cisplatin (80 mg/m^2 on day 1) was recommended for patients who failed to fulfill any of the above criteria.

Evaluation of response and toxicity

Response was assessed according to the "Criteria for the evaluation of the clinical effects of solid cancer chemotherapy" of the Japan Society for Cancer Therapy [14], which are virtually identical to those of the World Health Organization [15]. A complete response (CR) was defined as disappearance of all lesions for a minimum of 4 weeks. A partial response (PR) was defined as a 50% or greater decrease in the sum of the products of the diameters of measurable lesions for a minimum period of 4 weeks and no new lesions. No change (NC) was defined as a decrease in the tumor mass of less than 25% or any increase of less than 25%. Progressive disease (PD) was defined as an increase in the size of any measurable lesion by 25% or greater or the appearance of new lesions.

Toxicity grading was recorded based on the side effect record form in the "Criteria for the evaluation of the clinical effects of solid cancer chemotherapy" of the Japan Society for Cancer Therapy [14].

Statistical analyses

The estimated sample size was 30 to guarantee that the lower limits of 95% confidence interval would be at least 20% at 40% of expected response rate. An early cessation rule was in place to terminate the study if at least 4 responses had not been seen among 15 patients evaluated. Median overall survival was estimated using the product-limit (Kaplan-Meier) method [16].

Results

Patient characteristics

Of 35 patients entered into this study between May 1995 and January 1997, 33 patients were eligible and assessable for efficacy and toxicity. There were 2 ineligible patients because of serious complications before treatment (cardiac