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KEYWORDS
EKB-569;
Non-small cell lung cancer;
EGFR mutation;
Resistance to gefitinib;
Irreversible inhibitor of EGFR

Summary EKB-569 is a potent, low molecular weight, selective, and irreversible inhibitor of epidermal growth factor receptor (EGFR) that is being developed as an anticancer agent. A phase 1, dose-escalation study was conducted in Japanese patients. EKB-569 was administered orally, once daily, in 28-day cycles, to patients with advanced-stage malignancies known to overexpress EGFR. Two patients with advanced non-small cell lung cancer with EGFR mutations and acquired gefitinib resistance from the phase 1 study are described in detail. *Case #1* is a 63-year-old man with smoking history. He received treatment from 4 March 2004. Because he had no severe adverse events, a total of 10 courses of therapy were completed through December 16. Grade 2 skin rash and ALT elevation, and grade 1 diarrhea and nail changes developed. A chest CT scan on 4 August 2003 revealed multiple pulmonary metastases that had decreased in size. *Case #2* is a 49-year-old woman with no smoking history. She received therapy from 9 February 2004. She received a total of five courses of the therapy until 22 June 2004. Grade 3 nausea and vomiting

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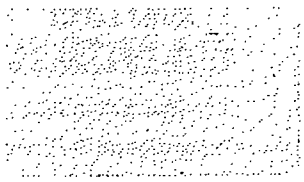

and grade 1 diarrhea and dry skin developed. A chest CT scan on March 3 revealed multiple pulmonary metastases that had decreased in size. A brain MRI on March 4 showed that multiple brain metastases also had decreased in size. Based on RECIST criteria, they had stable disease but radiographic tumor regression was observed.

1. Introduction

1.1. Efficacy of gefitinib

The epidermal growth factor receptor (EGFR) autocrine pathway contributes to a number of processes important to cancer development and progression, including cell proliferation, apotosis, angiogenesis, and metastatic spread [1]. EGFR-tyrosine kinase has become a particularly promising drug targeting for treating non-small cell lung cancer. Gefitinib is an orally active, selective EGFR tyrosine kinase inhibitor that blocks signal transduction pathways implicated in proliferation and survival of cancer cells [2]. Responsiveness characteristics include distinct subgroups of women, patients who have never smoked, patients with adenocarcinoma, and Asians [3–5]. Molecular predictive markers have also been investigated. It is suggested that MAPK is a predictive marker for survival after treatment with gefitinib in chemo-naive patients with bronchioloalveolar carcinoma [6]. Patients with P-Akt-positive tumors who received gefitinib had a better response rate, disease control rate, and time to progression than patients with P-Akt-negative tumors, suggesting that gefitinib may be most effective in patients with basal Akt activation [7]. However, it was not possible to predict gefitinib sensitivity by the level of EGFR overexpression as determined by immunohistochemistry [8] or immunoblotting [9]. Recently it has been reported that somatic mutations in the tyrosine kinase domain of the *EGFR* gene occur in a subset of patients with lung cancer who showed a dramatic response to the EGFR tyrosine kinase inhibitors gefitinib and erlotinib [10–12]. All of these mutations were within exons 18 through 21 of the kinase domain of the *EGFR* gene.

1.2. Drug summary

EKB-569 (Wyeth Research, Collegeville, PA) is a potent, low molecular weight, selective, and irreversible inhibitor of EGFR that is being developed as an anticancer agent. EGFR is a receptor tyrosine kinase that is activated by a variety of growth factors. Upon binding ligands, including epidermal growth factor (EGF) or transforming growth factor alpha (TGF-α), EGFR dimerizes and its intracellular kinase domain is activated, leading to the recruitment and phosphorylation of a number of proteins that ultimately lead to cell growth [13,14]. Several features of EKB-569 may provide certain advantages over other EGFR inhibitors. First, EKB-569 is an orally available, small-molecule EGFR inhibitor, whereas antibody-targeted EGFR inhibitors require intravenous (IV) administration. Second, EKB-569 is an irreversible inhibitor of EGFR, while other small-molecule EGFR inhibitors bind EGFR reversibly [15].

1.3. Effects in humans (Japanese)

A phase 1, open-label, dose-escalation study to assess the safety, tolerability, and pharmacokinetics of EKB-569 was conducted in Japanese patients. EKB-569 was administered orally, once daily, in 28-day cycles, to patients (pts) with advanced-stage malignancies known to overexpress EGFR. Enrollment and treatment are completed; 15 pts (six men, nine women) were treated with 25 mg (3 pts), 35 mg (8 pts), or 50 mg (4 pts) of EKB-569. Their median age was 62 years (range 47–72); ECOG performance status varied: 0 = 4/15 (26.7%) or 1 = 11/15 (73.3%).

The most frequently occurring tumor types included non-small cell lung (10 pts) and breast (2 pts). The remaining tumors were renal, leiomyosarcoma, and malignant thymoma (1 pt each). The most frequently reported EKB-569-related adverse events were diarrhea (86.7%), rash (53.3%), anorexia (40.0%), and dry skin (40.0%). Dose-limiting toxicities were observed at the 50-mg dose level with grade 4 interstitial lung disease and grade 3 diarrhea, stomatitis, and increased blood calcium levels. Thus, the maximum tolerated dose was 35 mg EKB-569 per day.

1.4. Molecular analysis of lung cancer specimens

We obtained appropriate approval from the institution and written informed consent from the patients for the comprehensive use of tumor samples for molecular and pathologic analyses. Surgically resected tumor samples were obtained retrospectively before the patients received

any systemic treatment. All of these tumors were formalin fixed and paraffin embedded by the Department of Pathology. To minimize non-neoplastic tissue contamination, the tumor portion was first selected and marked on an H&E-stained tissue section slide by a pathologist. Only the tumor portion was dissected from the unstained tissue section and sent for DNA extraction.

DNA was extracted from the paraffin section containing a representative portion of each tumor, using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany). For mutational analysis of the kinase domain of the *EGFR* coding sequence, exons 19, 20, and 21 were amplified with three pairs of primers (exon 19, F: 5'-TCACAATTGCCAGTTAACGTCT-3'-(this is the convention for writing a primer), R: 5#-cagcaaagcagaaactcacatc; exon 20, F: 5#-tgaaact-caagatcgcattcat, R: 5#-catggcaaactcttgctatcc; exon 21, F: 5#-gagcttcttcccatgatgatct, R: 5#-gaaaatgctggctgacctaaag). The PCR conditions were one cycle at 95 °C for 11 min, 46 cycles at 95 °C for 30 s, 60 °C for 30 s, 72 °C for 40 s, followed by one cycle at 72 °C for 7 min. PCR products were diluted and cycle-sequenced using the Big Dye Terminator v3.1/1.1 cycle sequencing kit (Applied Biosystems, Forster City, CA) according to the manufacturer's instructions. Sequencing products were electrophoresed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). All sequencing reactions were performed in both forward and reverse directions and chromatograms were reviewed manually and analyzed by BLAST (basic local alignment search tool). High-quality sequence variations found in both directions were scored as candidate mutations.

2. Clinical cases

Two patients from the Japanese phase 1 study are described in detail.

2.1. Case #1

A 63-year-old man with smoking history (BI: 720) who was treated for hyperlipidemia and hypertension showed an abnormal chest X-ray in February 1996. Further examinations including a chest computed tomography (CT) scan and bronchoscopy revealed an adenocarcinoma of the lung, c-T1N0M0, stage Ia, in the right upper lobe. He had undergone a right upper lobectomy with mediastinal lymph node dissection in July 1996 and was proven to have a well-differentiated adenocarcinoma, p-T1N0M0, stage Ia. After further follow-up, multiple pulmonary metastases in both lungs were found in January 2000. Then he was given first-line chemotherapy of cisplatin and docetaxel beginning in May 2000. After two courses of this regimen, multiple pulmonary metastases had not increased in size by CT scan; however skin metastases were found. He was started on oral gefitinib 250 mg/day on November 2000. After 4 weeks, a CT scan indicated a reduction of multiple pulmonary metastases. During this treatment, grade 2 rash and grade 1 nail changes, AST/ALT elevations, and diarrhea were observed. On June 2002, multiple pulmonary metastases had increased, and this treatment was discontinued. The patient entered a phase I study of a new EGFR tyrosine kinase inhibitor (TAK-165), starting treatment on October 2002. After 2 weeks of treatment, grade 3 anorexia was observed and the therapy was stopped. On February 2003, multiple pulmonary metastases had more increased, and on March 2003, he entered a phase I study of EKB-569, receiving treatment from 4 March 2004. EKB-569 (25 mg) was administered orally, once daily, in 28-day cycles. Because he had no severe adverse events, a total of 10 courses of therapy were completed through December 16. Grade 2 skin rash and ALT elevation, and grade 1 diarrhea and nail changes developed during this therapy. Based on RECIST criteria, the patient had stable disease (SD) but radiographic tumor regression was observed on 4 August 2003 (day 27 in the sixth course) (Fig. 1). The size of multiple pulmonary metastases increase by CT scan on 8 December 2003, and the treatment was stopped on 17 December 2003.

A lung cancer specimen was obtained at surgery and studied by immunohistochemistry. EGFR overexpression was detected. In addition, we found the heterozygous in-frame deletion E746-A750 in exon 19 of the *EGFR* gene by direct sequencing of the specimen.

2.2. Case #2

A 49-year-old woman with no smoking history, who was treated for Basedow's disease, insomnia, and bronchial asthma, had an abnormal chest X-ray in October 2000. Further examinations including a chest CT scan and bronchoscopy revealed lung cancer in the left upper lobe. She was diagnosed with adenocarcinoma, c-T1N0M0, stage Ia. She had a left-upper lobectomy with mediastinal lymph node dissection, which revealed a well-differentiated adenocarcinoma, p-T4N2M1, stage IV. She was then given first-line chemotherapy of carboplatin and paclitaxel beginning in January 2001. After two courses of therapy, she discontinued treatment because of adverse events. Right supraclavicular lymph node metastases were found on August

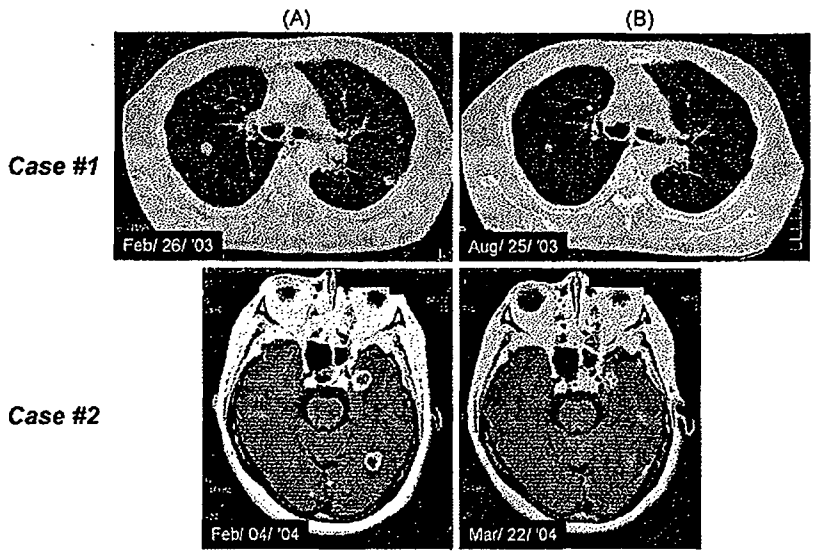

Fig. 1 *Clinical case* #1: a 63-year-old man with adenocarcinoma of lung. CT scan before treatment (A) and after initiation of EKB-569 (B). *Clinical case* #2: a 49-year-old woman with adenocarcinoma of brain metastasis. MRI scan before treatment (A) and after initiation of EKB-569 (B).

2001. Radiotherapy for the metastases (60 Gy/30 fractions) was done, and they decreased in size. On March 2002, right supraclavicular lymph node metastases increased and left clavicular lymph node metastases were found. On April 2002, the patient enrolled in a phase II trial of cisplatin, gemcitabine, and irinotecan for non-small-cell lung cancer. After two courses of therapy, bone metastases were found and pulmonary metastases had grown slowly so the treatment was stopped. She entered a phase I study of a new EGFR tyrosine kinase inhibitor (TAK-165) and started treatment on July 2002. The treatment was stopped after a week later due to grade 3 fatigue. In September 2002, the patient was started on oral gefitinib 250 mg/day. While she was taking 250 mg gefitinib daily for 15 months, the size of multiple pulmonary and bone metastases did not increase by CT scan and she had SD. On December 2003, the patient developed grade 3 oral mucositis and discontinued treatment. On January 2004, the size of multiple pulmonary and bone metastases increase by CT scan. She then entered a phase I study of EKB-569 and received therapy from 9 February 2004. EKB-569 (35 mg) was administered orally, once daily, in 28-day cycles. She received a total of five courses of the therapy until 22 June 2004. Grade 3 nausea and vomiting and grade 1 diarrhea and dry skin developed during the therapy. A chest CT scan on March 3 (day 24 in the first course) revealed multiple pulmonary metastases that had decreased in size. A brain MRI on March 4 (day 25 in the first course) showed that multiple brain metastases also had decreased in size (Fig. 1). The response was SD by RECIST criteria, although tumor regression was observed. The size of bone metastases increase by CT scan on 18 June 2004, and the treatment was stopped on 22 June 2004.

A lung cancer specimen was obtained by surgery and studied by immunohistochemistry. EGFR overexpression was detected. This lung cancer specimen had a heterozygous point mutation in exon 21 (L858R, CTG to CGG) of the *EGFR* gene.

3. Discussion

This is the first case report to describe the effects of EKB-569 on patients with adenocarcinoma of the lung. Case 1 is a 63-year-old man with a smoking history (BI: 720), and case 2 is a 49-year-old woman with no smoking history. Case 1 had an exon 19 deletion of E746-A750, and case 2 had an exon 21-point mutation. These patients underwent surgery and were treated with platinum-based chemotherapy and EGFR tyrosine kinase inhibitors. The treatment with EKB-569 was effective in these two patients after resistance to gefitinib and cytotoxic chemotherapy. These cases suggest that EKB-569 is effective in patients with *EGFR* mutations as has been reported for gefitinib and erlotinib. Despite initial responses to these EGFR inhibitors, patients eventually progress by unknown mechanisms of "acquired" resistance.

Recently, a second mutation in the *EGFR* kinase domain, which is associated with acquired resistance of non-small cell lung cancer to gefitinib or erlotinib, was reported [16,17]. Pao et al. showed that in two of five patients with acquired resistance

to gefitinib or erlotinib, progressing tumors contained, in addition to a primary drug-sensitive mutation in EGFR, a secondary mutation in exon 20. This mutation leads to a substitution of methionine for threonine at position 790 (T790M) in the kinase domain [16]. Kobayashi et al. reported the case of a patient with EGFR-mutant, gefitinib-responsive, advanced non-small cell lung cancer who relapsed after two years of complete remission during treatment with gefitinib. The DNA sequence of the EGFR gene in his tumor biopsy specimen at relapse also revealed the presence of the secondary point mutation, T790M [17]. Kurata et al. reported an interesting case in which acquired resistance to gefitinib could be overcome [18]. In this case, the patient received gefitinib, then a combination of nedaplatin and gemcitabine, and then gefitinib again. The cytotoxic agents may have altered the *EGFR* gene or associated genes to produce acquired sensitivity to gefitinib.

Kobayashi et al. also found that CL-387,785, a specific and irreversible, anilinoquinoline EGFR inhibitor [19], strongly inhibited the EGFR kinase in cells transfected with DNA containing the L747-S752 deletion in the *EGFR* gene or a double mutation with the L747-S753 deletion and the T790M point mutation. They speculated that CL-387,785 inhibited the EGFR kinase of the double mutant because of its altered binding to the kinase domain or its covalent binding to EGFR [17]. Kwak et al. used a bronchoalveolar cancer cell line with an L746-A750 deletion in the *EGFR* gene to isolate gefitinib-resistant clones. These clones had not acquired secondary EGFR mutations but were sensitive to the irreversible, anilinoquinoline EGFR inhibitor EKB-569 [20].

We have shown that EKB-569 had clinical activity in two patients with advanced non-small cell lung cancer with EGFR mutations and acquired gefitinib resistance. Thus, irreversible EGFR inhibitors may be an effective therapy for patients with EGFR-mutant advanced non-small cell lung cancer who have relapsed after treatment with gefitinib.

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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′-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

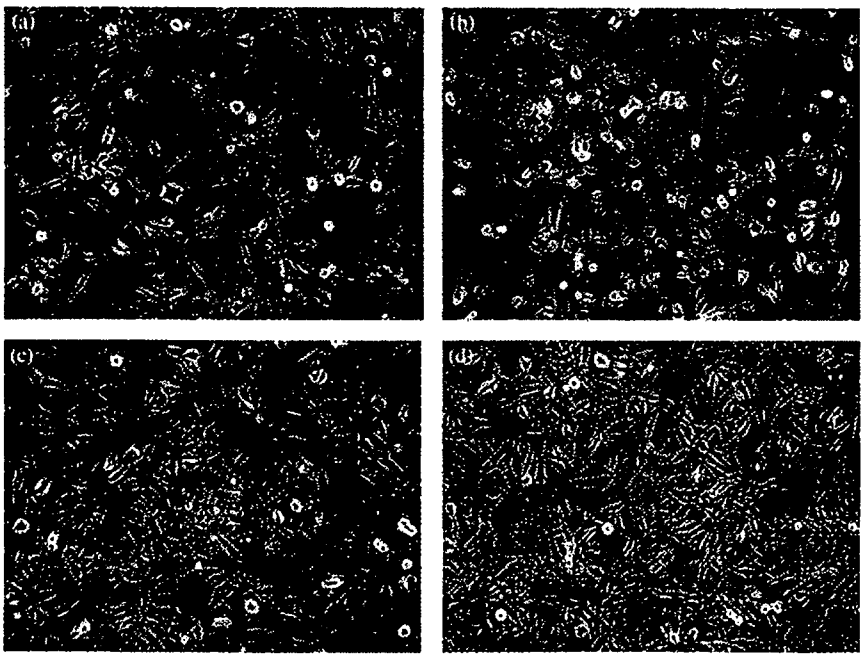

Fig. 1. Morphology of the four malignant pleural mesothelioma (MPM) cell lines. Micrographs of cultured (a) ACC-MESO-1 cells, (b) ACC-MESO-4 cells, (c) Y-MESO-8A cells, and (d) Y-MESO-8D cells (original magnifications ×100).

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.

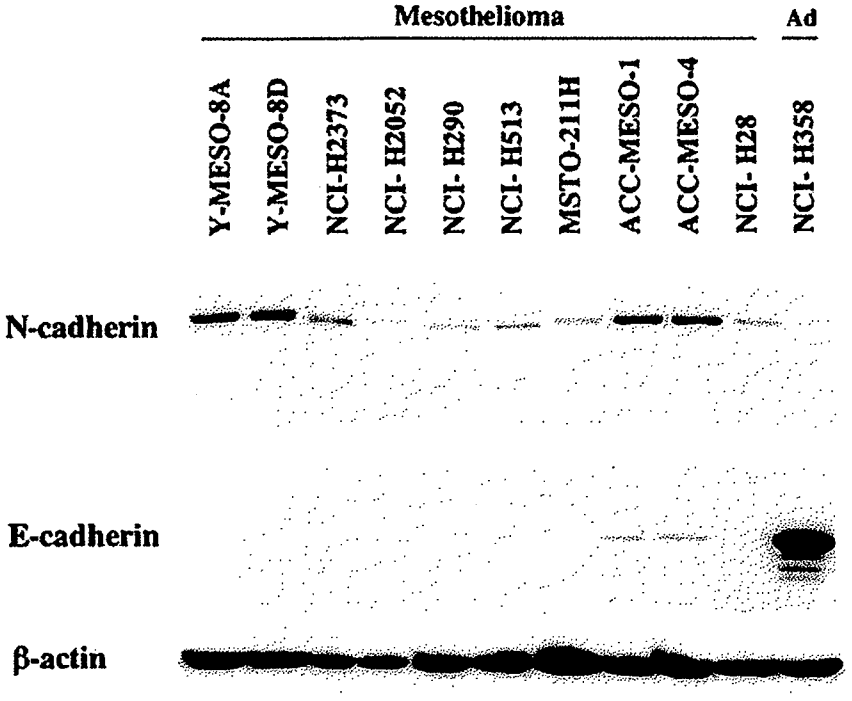

Fig. 2. Western blot analysis of E-cadherin and N-cadherin. Each lane was loaded with 15 μg of total cell lysate from Y-MESO-8A (lane 1), Y-MESO-8D (lane 2), NCI-H2373 (lane 3), NCI-H2052 (lane 4), NCI-H290 (lane 5), NCI-H513 (lane 6), MSTO-211H (lane 7), ACC-MESO-1 (lane 8), ACC-MESO-4 (lane 9), NCI-H28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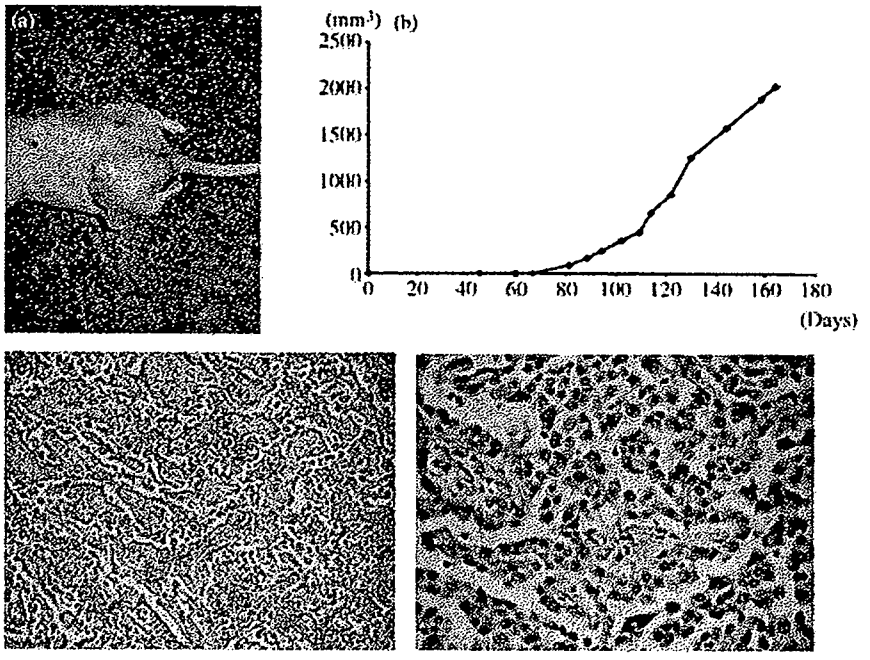

Fig. 3. (a) Xenografted tumor of Y-MESO-8A. (b) Growth curve of the xenografted tumor in nude mice. (c) Histology of xenografted Y-MESO-8A tumor (haematoxylin and eosin, original magnification x100 and x400). As in epithelioid mesothelioma, the tumor shows papillary proliferation consisting of atypical 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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JOURNAL OF CLINICAL ONCOLOGY ORIGINAL REPORT

First-Line Single Agent Treatment With Gefitinib in Patients With Advanced Non–Small-Cell Lung Cancer: A Phase II Study

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ABSTRACT

Purpose
We conducted a phase II study of single agent treatment with gefitinib in chemotherapy-naïve patients with advanced non–small-cell lung cancer (NSCLC) to assess its efficacy and toxicity.

Patients and Methods
Patients received 250 mg doses of gefitinib daily. Administration of gefitinib was terminated if partial response (PR) was not achieved within 8 weeks or if tumor reduction was not observed within 4 weeks. In these cases, platinum-based doublet chemotherapy was given as a salvage treatment. We evaluated mutation status of the epidermal growth factor receptor (EGFR) gene in cases with available tumor samples.

Results
Forty-two patients were enrolled between March and November 2003, with 40 of these patients being eligible. The response rate was 30% (95% CI, 17% to 47%). The most common toxicity included grade 1 or 2 acne-like rash (50%) and grade 1 diarrhea (18%). Grade 2 or 3 hepatic toxicity was observed in 8% of patients. Four patients developed grade 5 interstitial lung disease (ILD). Thirty patients received second-line chemotherapy. Median survival time was 13.9 months (95% CI, 9.1 to 18.7 months), and the 1-year survival rate was 55%. Tumor samples were available in 13 patients, including four cases of PR, six cases of stable disease, and three cases of progressive disease. *EGFR* mutations (deletions in exon 19 or point mutations [L858R or E746V]) were detected in four tumor tissues. All four patients with *EGFR* mutation achieved PR with gefitinib treatment.

Conclusion
Single agent treatment with gefitinib is active in chemotherapy-naïve patients with advanced NSCLC, but produces unacceptably frequent ILD in the Japanese population.

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INTRODUCTION

Previous meta-analysis demonstrated that cisplatin-based chemotherapy yielded a modest but significant survival benefit over best supportive care in advanced non–small-cell lung cancer (NSCLC).[1-4] In the 1990s, new agents, including vinorelbine, gemcitabine, paclitaxel, docetaxel, and irinotecan became available for the treatment of NSCLC. Several phase III trials comparing doublet platinum-based chemotherapies demonstrated no significant difference with respect to response rate, survival, or quality of life.[5,6] Nonplatinum or triplet platinum-based combination chemotherapies have been investigated, but none of these produced longer survival than standard doublet platinum-based chemotherapy.[7-9]

Recently, molecular-targeted agents have been introduced for the treatment of NSCLC. Gefitinib is an orally active epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, which displays activity against recurrent NSCLC after platinum-based chemotherapy. Two international, randomized phase II trials in patients with advanced or metastatic NSCLC after platinum-based chemotherapy demonstrated response rates of 12% to 18% (28% in the Japanese population).[10,11] Two international, randomized, double-blinded, placebo-controlled phase III trials investigated the role of gefitinib combined with platinum-based chemotherapy regimens, including carboplatin and paclitaxel, or cisplatin and gemcitabine in chemotherapy-naïve patients with advanced NSCLC.[12,13] Surprisingly, there were no improvements in overall survival,

time to progression, or response rate. There are no data available regarding first-line treatment with single agent gefitinib against NSCLC in the Japanese population. Here, we conducted a phase II study of single agent treatment with gefitinib in chemotherapy-naïve patients with advanced NSCLC. If a failure with gefitinib treatment was perceived, standard platinum-based doublet chemotherapy was performed as salvage. The primary end point of this phase II trial was response rate, and the secondary end points were toxicity, survival, and response rate of salvage chemotherapy.

PATIENTS AND METHODS

Patient Population

Patients were required to have histologically or cytologically confirmed stage IIIB (malignant pleural or pericardial effusion and/or metastasis in the same lobe) or stage IV NSCLC. Recurrences after surgical resection were permitted. Other criteria included: (1) age 20 years or older, but younger than 75 years; (2) Eastern Cooperative Oncology Group performance status (PS) 0 or 1; (3) measurable disease; (4) PaO2 $\geq$ 60 mmHg; (5) adequate organ function (ie, total bilirubin $\leq$ 2.0, AST and ALT $\leq$ 100 U/L, serum creatinine $\leq$ 1.5 mg/dL, leukocyte count 4,000 to 12,000/mm^3, neutrophil count $\geq$ 2,000/mm^3, hemoglobin $\geq$ 9.5 g/dL, and platelets $\geq$ 100,000/mm^3); (6) no prior chemotherapy or thoracic radiotherapy; (7) no interstitial pneumonia or pulmonary fibrosis, as determined by chest x-ray; (8) no paralytic ileus or vomiting, (9) no symptomatic brain metastases, (10) no active infection; (11) no active concomitant malignancy; (12) no pregnancy or breast-feeding; (13) no severe allergy to drugs. Patients with PaO2 less than 60 mmHg were excluded, because those patients might have pulmonary fibrosis, which is a risk factor of interstitial lung disease (ILD).[14] All patients were required to provide written informed consent and the institutional review board at the National Cancer Center approved the protocol.

Treatment Plan

Treatment was started within a week after enrollment in the study. Patients received 250 mg of gefitinib orally daily. In the event of grade 3 or more and/or unacceptable toxicities, gefitinib was postponed until these toxicities were improved to grade 2 or less. Dose reduction was not performed. If treatment was postponed four times or more, the treatment was terminated. Therapy was continued unless the patient experienced unacceptable toxicity or progressive disease, partial response (PR) was not achieved within 8 weeks, or the sum of the longest diameters of the target lesions decreased less than 10% within 4 weeks. If the gefitinib treatment failed according to these criteria, platinum-based doublet chemotherapy was performed as a salvage regimen.

Previous trials of gefitinib for pretreated patients with NSCLC reported that most responding patients showed rapid tumor regression within 4 or 8 weeks.[11] Furthermore, most responses by gefitinib were extreme shrinkage of the tumor. Minor response, as frequently seen by the treatment with cytotoxic agents, was seldom experienced. Stable disease with gefitinib corresponded to no tumor reduction or slight progression. If patients with stable disease continued the treatment with gefitinib until progressive disease became obvious, those patients might not be able to receive platinum-based salvage chemotherapy because of poor PS due to progressive disease. Platinum-based combination chemotherapy is the standard care for patients with advanced NSCLC and good PS. Platinum-based chemotherapy was thought to be essential for patients with no response from the first-line single agent treatment with gefitinib. Therefore, we implemented these early stopping criteria for treatment with gefitinib.

Study Evaluations

Pretreatment evaluations consisted of a complete medical history, determination of performance status, physical examination, hematologic and biochemical profiles, arterial blood gas examination, ECG, chest x-ray, bone scan, and computed tomography (CT) scan of the chest, ultrasound or CT scan of the abdomen, and magnetic resonance imaging or CT scan of the whole brain. Evaluations performed included a weekly chest x-ray for 4 weeks, and once every 2 weeks for biochemistry, complete blood cell, platelet, leukocyte differential counts, physical examination, determination of performance status, and toxicity assessment. Imaging studies were scheduled to assess objective response every month.

Response and Toxicity Criteria

Response evaluation criteria in solid tumors (RECIST) guidelines were used for evaluation of antitumor activity.[15] The target lesions were defined as $\geq$ 2 cm in the longest diameter on CT scans. A complete response (CR) was defined as the complete disappearance of all clinically detectable tumors for at least 4 weeks. A PR was defined as an at least 30% decrease in the sum of the longest diameters of the target lesions for more than 4 weeks with no new area of malignant disease. Progressive disease (PD) indicated at least a 20% increase in the sum of the longest diameter of the target lesions or a new malignant lesion. Stable disease was defined as insufficient shrinkage to qualify for PR and insufficient increase to qualify for PD. Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria version 2.0.

Mutation Analysis of the EGFR Gene

Tumor specimens were obtained during diagnostic or surgical procedures. Biopsied or surgically resected specimens were fixed with formalin or 100% methanol, respectively. Tumor genomic DNA was prepared from paraffin-embedded sections using laser capture microdissection in biopsied specimens or macrodissection in surgically resected specimens at Mitsubishi Chemical Safety Institute LTD. Exons 18, 19, and 21 of the *EGFR* gene were amplified and sequenced as previously described.[16]

Statistical Analysis

In accordance with the minimax two-stage phase II study design by Simon,[17] the treatment program was designed to refuse response rates of 10% (P_0) and to provide a significance level of .05 with a statistical power of 80% in assessing the activity of the regimen as a 25% response rate (P_1). The upper limit for first-stage drug rejection was two responses in the 22 assessable patients; the upper limit of second-stage rejection was seven responses within the cohort of 40 assessable patients. Overall survival was defined as the interval between enrollment in this study and death or the final follow-up visit. Median overall survival was estimated by the Kaplan-Meier analysis method.[18] Fisher's exact test was used in a contingency table.

RESULTS

Patient Population

A total of 42 patients were enrolled in this study between March and November, 2003, with 40 of these patients being eligible. One patient was found ineligible due to anemia, the other because spinal magnetic resonance imaging could not confirm a positive bone scan. Patient characteristics are listed in Table 1. Sixty percent of patients were male; median age was 61 years. The most common histologic subtype was adenocarcinoma (75%). Most patients (93%) had stage IV disease or recurrence after surgical resection. Eighty percent of patients were current or former smokers.

Efficacy

One patient (3%) has been receiving gefitinib after 22 months. Four patients suspended gefitinib for 11, 14, 27, or 29 days, because of liver dysfunction (n = 3) and fever due to urinary tract infection (n = 1). Thirty-nine patients terminated gefitinib because of progressive disease (n = 20), no tumor reduction within 4 weeks (n = 12), not achieving PR within 8 weeks (n = 1), toxicities including pulmonary (n = 3), nausea and vomiting (n = 1), rash (n = 1), or hepatic dysfunction (n = 1).

There were 12 PRs in 40 eligible patients, and the objective response rate was 30% (95% CI, 17% to 47%; Table 2). All but one

Table 1. Patient Characteristics

Characteristic	No. of Patients
Patients enrolled	42
Patients eligible	40
Sex	
Male	24
Female	16
Age, years	
Median	61
Range	44-74
Performance status	
0	14
1	26
Stage	
IIIB	3
IV	34
Recurrence after surgery	3
Histologic type	
Adenocarcinoma	30
Squamous cell carcinoma	3
Large cell carcinoma	7
Smoking history	
Current	27
Former	5
Never	8

patient from this subgroup achieved PR within 4 weeks, with the remaining patient achieving PR within 8 weeks. The background of the 12 responding patients was as follows: nine females, three males; 11 adenocarcinomas, one large-cell carcinoma; six individuals who never smoked, five current smokers, and one former smoker. Response rates based on patient characteristics were as follows: three of 24 (13%) males, nine of 16 (56%) females ($P = .0050$); 11 of 30 (37%) individuals with adenocarcinoma, one of 10 (10%) individuals with squamous or large-cell carcinoma ($P = .0048$); six of 32 (19%) current or former smokers, and six of eight (75%) individuals who never smoked ($P = .0048$).

The median follow-up time was 23 months, and nine patients were still alive at the most recent follow-up. The median survival time was 13.9 months (95% CI, 9.1 to 18.7 months), and the 1-year survival rate was 55% (Fig 1).

Safety and Toxicity

Toxicity was evaluated in all eligible patients. The most common toxicity was rash (Table 3). Thirty-eight percent and 13% of patients

Table 2. Efficacy of Single Agent Treatment With Gefitinib in Patients With Stage IIIb or IV Non-Small-Cell Lung Cancer

Type of Response	No. of Patients	% of Patients
Complete	0	0
Partial	12	30
CR + PR	12	30
95% CI	17 to 47	
Stable disease	16	40
Progression	12	30

Abbreviations: CR, complete response; PR, partial response.

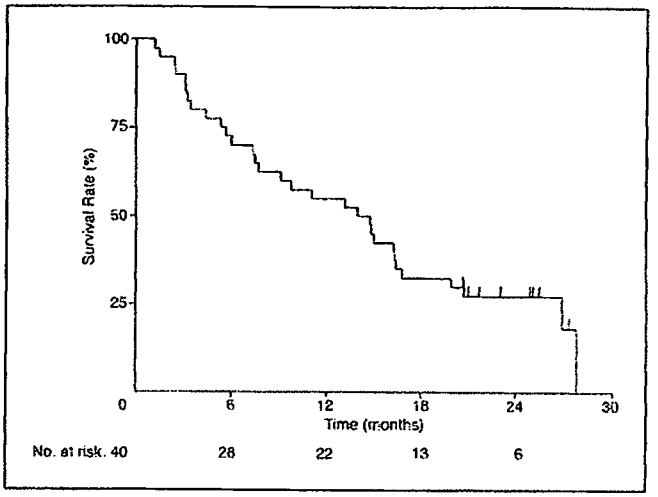

Fig 1. Overall survival of all eligible patients (n = 40) was calculated according to the Kaplan-Meier method. The median survival time was 13.9 months (95% CI, 9.1 to 18.7 months), and the 1-year survival rate was 55%.

experienced grade 1 or 2 rash, respectively. One patient experienced grade 3 nausea and vomiting, leading to gefitinib treatment being terminated. Grade 3 hepatic toxicity was observed in one patient, also causing termination of gefitinib treatment.

The most problematic toxicity was ILD. We reviewed the medical records, chest x-rays, and CT films of all the cases, which were suspected as ILD by the physician in charge. ILD was diagnosed on the basis of standard or high-resolution CT findings of the chest (diffuse ground-glass opacity, consolidation, or infiltrate) and no response to antibiotics. We diagnosed that four patients experienced grade 5 ILD during or after first-line treatment with gefitinib. The first patient was a 61-year-old man. He developed dyspnea and fever elevation (38.1°C) on day 23 of the treatment with gefitinib and administration of gefitinib was terminated. Chest CT demonstrated bilateral diffuse ground-glass opacity, and PaO2 was 43.7mmHg in the room air. KL-6 antigen, a serum marker of interstitial pneumonia, was not elevated

Table 3. Maximum Toxicity Grades Associated With Single Agent Treatment With Gefitinib in 40 Patients With Non-Small-Cell Lung Cancer

	Toxicity Grade									
	1		2		3		4		5	
Toxicity	No. of Patients	%	No. of Patients	%	No. of Patients	%	No. of Patients	%	No. of Patients	%
Rash	15	38	5	13	0	0	0	0	0	0
Dry skin	4	10	0	0	0	0	0	0	0	0
Diarrhea	7	18	0	0	0	0	0	0	0	0
Nausea	3	8	0	0	1	3	0	0	0	0
Mucositis	6	15	0	0	0	0	0	0	0	0
Alopecia	4	10	0	0	0	0	0	0	0	0
Hyponatremia	24	60	0	0	3	8	0	0	0	0
Hypokalemia	12	30	0	0	0	0	0	0	0	0
Hepatic	11	28	2	5	1	3	0	0	0	0
Renal	4	10	1	3	0	0	0	0	0	0
ILD	0	0	0	0	0	0	0	0	4	10

Abbreviation: ILD; interstitial lung disease.

(351 U/mL) on day 24, but elevated on day 31 (1,400 U/mL). Beta-D-glucan, a serum marker of fungal infection and *Pneumocystis carinii* pneumonia, was also negative. Methylprednisolone and antibiotics were administered, with temporal improvement of ILD. However, subsequently, pulmonary function gradually deteriorated, leading to death. Autopsy revealed alveolar damage with organization around the bronchus and vessels in both neoplastic and non-neoplastic lesions, compatible with drug-induced ILD. The second patient was a 64-year-old man. Chest CT on day 27 showed stable disease, but administration of gefitinib was continued (protocol violation). Periodic chest x-ray film on day 45 showed abnormal shadow in the left lung field. High-resolution CT of the chest on the same day revealed reticular shadow on bilateral upper lobe. The treatment with gefitinib was terminated on day 45. KL-6 antigen was not elevated on day 49 (276 U/mL). Methylprednisolone and antibiotics were administered, but were not effective, leading to death. The third patient was a 67-year-old man. Chest CT on day 30 demonstrated enlargement of primary lesion and bilateral reticular shadow in subpleural lesions. Gefitinib was terminated on day 30. The patient developed dyspnea without fever elevation on day 37. Pao2 in the room air fell to 61.0 mmHg from 82.4 mmHg at pretreatment. Chest x-ray showed that the bilateral diffuse reticular shadow deteriorated. Methylprednisolone and antibiotics were administered, but were not effective, leading to death. Autopsy revealed severe fibrotic thickness of alveolar septum, compatible with severe interstitial pneumonia. There was no pathological evidence of carcinomatous lymphangiosis. The fourth patient was a 59-year-old woman. Chest x-ray showed consolidation in the left lung on day 21. Slight fever (37.9°C) developed on day 22. Blood culture was negative. Antibiotics were administered, but consolidation deteriorated and spread to both lungs on day 25. Gefitinib was terminated on day 25. KL-6 antigen was elevated to 3,590 U/mL. Methylprednisolone was administered, but was not effective, leading to death (Table 4). Four other patients experienced ILD after second-line or third-line chemotherapy. Two patients received second-line treatment with cisplatin plus vinorelbine (one and four courses), one patient received treatment with cisplatin plus gemcitabine (one course), and one patient received third-line treatment with docetaxel (four courses). Three of four patients received steroids, with temporal improvement of ILD being observed in two patients. However, ILD deteriorated during tapering of steroid treatment, with three patients subsequently dying. One patient stopped the third-line treatment with docetaxel, with the associated ILD showing improvement in this case without steroid treatment (Table 4).

We retrospectively reviewed the pretreatment chest x-rays and CT films of all patients. Interstitial shadow was not detected on pretreatment chest x-ray films in any patients. However, six patients showed evidence of interstitial shadow on pretreatment chest CT films. Three of the six patients with interstitial shadow, as determined by pretreatment chest CT, experienced ILD either during or following administration of gefitinib or second-line chemotherapy. None of the six patients responded to gefitinib treatment. On the other hand, four of 34 patients who showed no interstitial shadow on pretreatment chest CT films experienced ILD. Interstitial shadow as determined by pretreatment chest CT was not a statistically significant risk factor of ILD ($P = .0819$; Table 5).

Second-Line Chemotherapy

A total of 30 patients received second-line chemotherapy. Twenty-seven patients received platinum-based chemotherapy (cisplatin plus vinorelbine; n = 17), carboplatin plus paclitaxel (n = 5), cisplatin plus gemcitabine (n = 3), cisplatin plus docetaxel (n = 1), and cisplatin plus irinotecan (n = 1). The remaining three patients received vinorelbine plus gemcitabine or vinorelbine alone. Nine of 30 patients achieved PR with these second-line chemotherapies. The objective response rate of second-line chemotherapy was 30% (95% CI, 15% to 50%).

Mutation Status of the EGFR Gene

Out of 42 enrolled patients, 16 patients were diagnosed pathologically, 22 were diagnosed cytologically, and four patients recurred after surgical resection. Biopsied specimens were available in nine patients. Therefore, tissue samples were available in a total of 13 patients. These 13 patients included four PRs, six with stable disease, and three PDs. *EGFR* mutations were detected in four tumor tissues, including the in-frame nucleotide deletions in exon 19 (n = 3) and an L858R mutation in exon 21 (n = 1). One tumor had an in-frame deletion and

Table 4. Four Patients Developed Interstitial Lung Disease During First-Line Chemotherapy With Gefitinib, With Another Four Patients Showing ILD During Either Second- or Third-Line Chemotherapy

Age (years)	Sex	Smoking Index	Pathology	Onset of ILD	Response to Gefitinib	Death From Chemotherapy
61	M	1,520	AD	Day 23*	PD	Day 74
64	M	880	AD	Day 45*	SD	Day 51
67	M	1,880	SQ	Day 37†	PD	Day 45
59	F	0	AD	Day 21*	PD	Day 35
61	M	820	AD	Day 131‡	SD	Day 154
68	M	2,000	LA	Day 37‡	PD	Day 106
68	M	705	AD	Day 22§	PR	Day 87
59	M	1,170	AD	Day 108‖	SD	Alive

Abbreviations: ILD, interstitial lung disease; M, male; F, female; AD, adenocarcinoma; SQ, squamous cell carcinoma; LA, large-cell carcinoma; PD, progressive disease; SD, stable disease; PR, partial response.
*During gefitinib administration.
†One week after discontinuation of gefitinib.
‡ After 2nd-line chemotherapy of cisplatin and vinorelbine.
§ After 2nd-line chemotherapy of cisplatin and gemcitabine.
‖ After 3rd-line chemotherapy of docetaxel.

Table 5. Interstitial Shadow on Pretreatment Chest Computed Tomography Films and ILD

Interstitial Shadow on Pretreatment Chest Computed Tomography Scans	No ILD	ILD
No existence	29	5
Existence	3	3

NOTE. $P = .0819$.
Abbreviation: ILD interstitial lung disease.

an E746V mutation in exon 19. All four PR patients had *EGFR* mutations (Table 6).

DISCUSSION

This phase II study was designed to evaluate the efficacy and safety of first-line single agent treatment with gefitinib in patients with advanced NSCLC. There is no other paper that evaluates single agent treatment with gefitinib prospectively in patients with advanced NSCLC. The observed response rate of 30% (95% CI, 17% to 47%), median survival of 13.9 months and 1-year survival of 55% are promising. However, grade 5 ILD occurred in 10% (95% CI, 3% to 24%) of patients. This high rate of ILD was not acceptable. The incidence of ILD was seen to be less than 1% in two randomized controlled studies comparing gefitinib with placebo in combination with gemcitabine and cisplatin or paclitaxel and carboplatin.[12,13] The reason for the high incidence of ILD observed in our study is unknown. The West Japan Thoracic Oncology Group analyzed 1,976 patients receiving gefitinib retrospectively. In this case, the incidence of ILD was 3.2% (95% CI, 2.5% to 4.6%) and the death rate due to ILD was 1.3% (95% CI, 0.8% to1.9%). Multivariate analyses found that risk factors included being male, individuals who smoked, and complication of interstitial pneumonia.[14] Our retrospective analyses revealed that three of six patients with interstitial shadow on pretreatment chest CT films, but not detected on chest x-ray films developed ILD; on the other hand, five of 34 patients without interstitial shadow developed ILD. Interstitial shadow on pretreatment chest CT was a marginally significant risk factor of ILD ($P = .0819$). It might be suggested that patients with interstitial shadow on pretreatment chest CT films be excluded from administration of gefitinib; however, our analyses were biased because we analyzed retrospectively and did not blind patient clinical information. Prospective analysis is needed to evaluate interstitial shadow by chest CT before treatment with gefitinib.

The Southwest Oncology Group conducted a phase II trial to evaluate gefitinib in patients with advanced bronchioloalveolar carcinoma (SWOG 0126). Previously untreated (n = 102) and treated (n = 36) patients were entered and eligible in SWOG 0126. The response rate was 19% and the median survival time was 12 months in the untreated population.[19] These subset analyses were comparable to our results.

Recently, mutations in the tyrosine kinase domain of EGFR were found to be associated with gefitinib sensitivity in patients with NSCLC.[16,20,21] Our retrospective analyses demonstrated that *EGFR* mutations were detected in four of 13 patients, and those four patients achieved PR in the single agent treatment of gefitinib. These results were compatible with previous reports.[16,20,21]

Thirty patients received second-line chemotherapy, including platinum-based (n = 27) and nonplatinum-based (n = 3) regimens; the response rate was 30%. Pretreatment with gefitinib does not seem to adversely affect the response of second-line chemotherapy. However, our small-scale study does not suggest the best second-line regimen. Platinum combined with any third-generation agents including paclitaxel, docetaxel, vinorelbine,

Table 6. Mutation Status of the *EGFR* Gene

Sex	Age (years)	Pathologic Type	Smoking Status	Overall Survival (months)	*EGFR* Gene	Effect of Mutation	Response to Gefitinib	Response to Second Line Chemotherapy
M	68	AD	Current	14.9	Deletion of 15 nucleotides (2236-2250)	In-frame deletion (E746-A750)	PR	PD
F	67	AD	Current	16.2	Deletion of 15 nucleotides (2236-2250)	In-frame deletion (E746-A750)	PR	PD
F	54	AD	Current	5.6	Deletion of 18 nucleotides (2238-2255) and substitution of T for A at nucleotides 2237	In-frame deletion (L747-S752) and amino acid substitution (F746V)	PR	NR
F	57	AD	Never	25.4	Substitution of G for T at nucleotide 2573	Amino acid substitution (L858R)	PR	SD
M	61	AD	Current	7.5	Wild	—	SD	SD
M	54	AD	Current	9.7	Wild	—	SD	SD
M	45	AD	Current	16.2	Wild	—	SD	PR
M	59	AD	Current	14.7	Wild	—	SD	PR
M	67	SQ	Current	2.4	Wild	—	SD	NR
M	59	AD	Current	24.9	Wild	—	SD	PR
M	61	AD	Current	2.4	Wild	—	PD	NR
F	61	SQ	Current	3.4	Wild	—	PD	PD
F	61	AD	Current	16.3	Wild	—	PD	PR

Abbreviations: EGFR, epidermal growth factor receptor; M, male; F, female; AD, adenocarcinoma; SQ, squamous cell carcinoma; PR, partial response; SD, stable disease; PD, progressive disease; NR, not received.