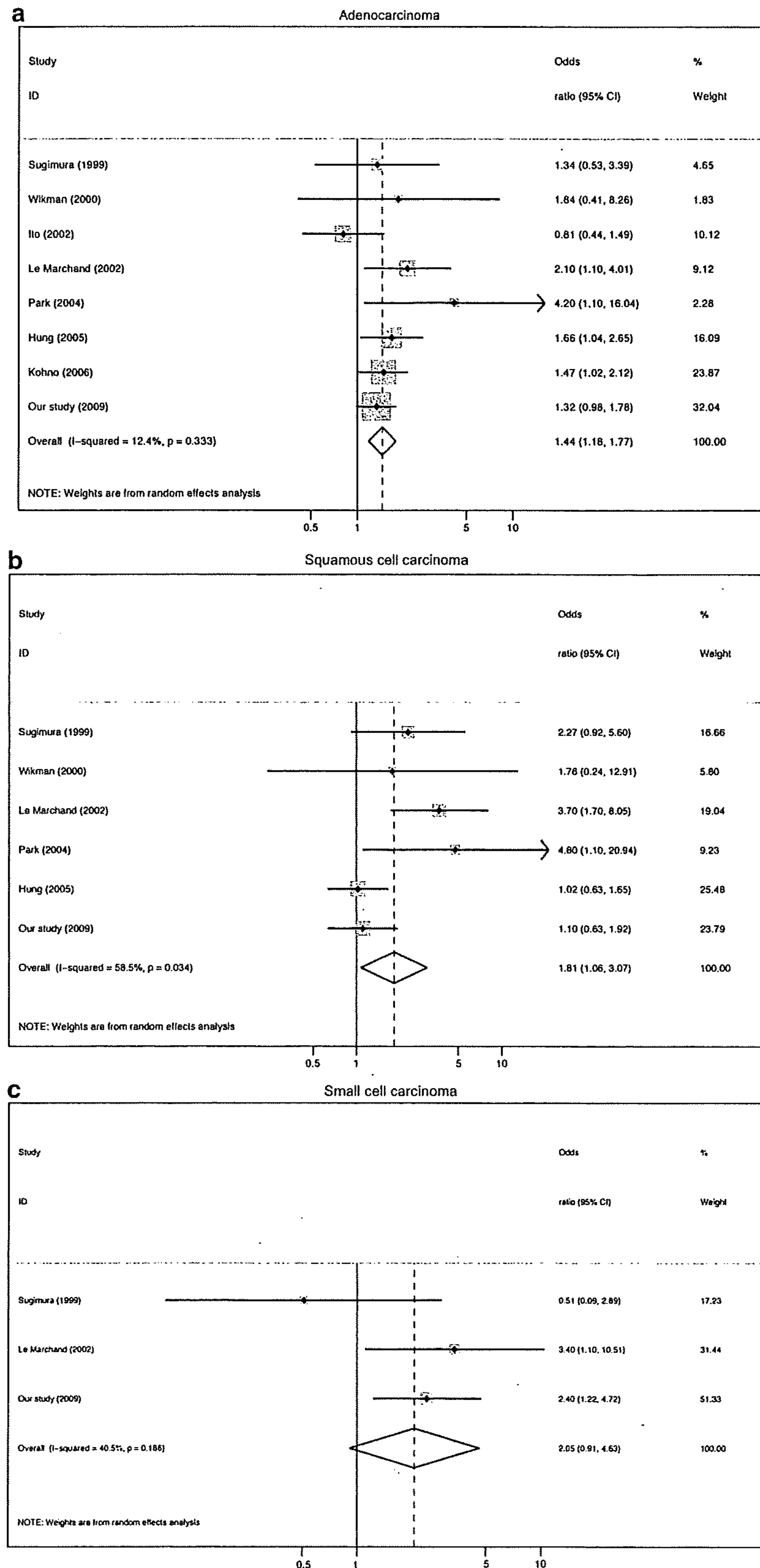

Figure 1 Meta-analysis for human 8-oxoguanine DNA glycosylase 1 (hOGG1) polymorphism according to histological subtype. A meta-analysis was conducted of the studies listed in Table 4. We extracted the ORs and 95% CIs for the Cys/Cys homozygotes of *hOGG1* relative to the Ser/Ser according to histological subtype from each study. We applied a random-effect model. An OR >1.0 indicates a higher risk with the Cys/Cys genotype than with Ser/Ser homozygotes. I^2 indicates the proportion of variation in summary estimates attributable to heterogeneity. All analyses were performed using the 'metan' command in STATA (version 10.1).

we also identified a potentially increased risk for these types of lung cancer.

Results of a number of studies examining the role of the *hOGG1* Ser326Cys polymorphism in lung cancer susceptibility conducted to date have been inconsistent.[7–12,17] Our case–control study showed a significant association between *hOGG1* Ser326Cys polymorphism and lung cancer overall, supporting the potential effect of this polymorphism on lung cancer susceptibility. Because the question of whether the effect of this polymorphism differed by histology remained unanswered, we also conducted a meta-analysis with consideration to histology. To the best of our knowledge, this is the first report to summarize the association between *hOGG1* polymorphism and susceptibility by histological type. Results of our meta-analysis indicated that the effect is consistent for adenocarcinoma, but not for squamous- or small-cell carcinoma. This inconsistency might be due to the heterogeneity of populations and distribution of subtypes across studies. The subjects included in the analyses were mainly Japanese and Caucasian. The most common subtype was adenocarcinoma in Japanese but squamous-cell carcinoma in Caucasians. Given that the magnitude of effect of smoking on risk differs by histological subtype,[28] the magnitude of effect of the *hOGG1* polymorphism might also differ across subtypes and populations. Even within the same histological subtype, the effect of smoking differs with the presence of certain gene mutations in cancer.[29] A comprehensive understanding of the *hOGG1* polymorphism will thus require further study, with particular focus on squamous- and small-cell carcinomas.

Our case–control study had several potential limitations. One methodological issue was the selection of hospital-based patients without cancer as controls. However, because cases and controls were selected from the same hospital and almost all patients lived in the Tokai area of central Japan, the internal validity of this case–control study is likely acceptable. External validity (generalizability of the results) has been confirmed in our previous study.[30] In addition, to dilute any bias that might have resulted from the inclusion of a specific diagnostic group that is related to the exposure, we did not set eligibility criteria for control diseases. As for allele frequencies in the subjects, given that our frequencies were comparable to those previously reported in public databases such as HapMap JPT,[31] bias in the distribution of selected polymorphisms was negligible. Second, the self-reported values for lifestyle factors considered as potential confounders may be inaccurate. If present, however, any such misclassification would likely be nondifferential, and would likely underestimate the causal association. The meta-analysis was based on published data, and the potential for publication selection bias could not be ruled out even if heterogeneity across the studies was limited for adenocarcinoma.

In conclusion, we found a positive association between lung cancer and Cys/Cys individuals in a Japanese population. The association was clear for small-cell carcinoma and adenocarcinoma of the lung in this population. Further systematic evaluation revealed that associations with the locus were conclusive for adenocarcinoma. Further studies are needed to clarify the effect of genotype on squamous-cell carcinoma and small-cell carcinoma.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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Activation of the PI3K-AKT pathway in human malignant mesothelioma cells

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Abstract. Malignant mesothelioma (MM) is a highly aggressive neoplasm, which is associated with asbestos exposure. The dysregulated phosphatidylinositol 3-kinase (PI3K)-AKT pathway plays an important role in cell proliferation, survival and motility in various cancers. In this study, we analyzed the activation status and underlying mechanisms of this pathway in MM cells using 21 cell lines. AKT activation was observed in 13 (62%) of the 21 MM cell lines under serum-starved conditions. Two cell lines, ACC-MESO-1 and Y-MESO-25, showed no expression of PTEN protein, while 7 other cell lines showed low expression of PTEN mRNA and protein compared to expression levels in an immortalized normal mesothelial cell line, MeT-5A. We found that PTEN inactivation in the ACC-MESO-1 and Y-MESO-25 lines was due to a 39.4-kb deletion including *PTEN* exon 2, and to a 7.7-kb deletion including exon 1, respectively. Re-expression of PTEN in these cells reduced the activity of colony formation *in vitro*. In contrast, no mutation of *PIK3CA* or *LKB1* was found in any of the MM cell lines. These findings suggest that AKT is frequently activated in MM cells, in part due to the downregulation of *PTEN*. Thus, the PI3K-AKT signaling pathway is a potential therapeutic target for MM.

Introduction

Malignant mesothelioma (MM), a highly aggressive neoplasm of the pleura, peritoneum or pericardium with a very poor prognosis (1), is presently a worldwide problem due to its increasing incidence (2,3). In Japan, a recent study using an age-cohort model reported that there will be approximately 100,000 deaths due to MM in the next 40 years (4). Patients with MM are usually diagnosed at advanced stages, and the disease is refractory to conventional therapy. Therefore, the survival rate of patients with MM is very poor, with a median survival of 7-11 months following diagnosis, especially in advanced stage patients. This is despite the recent advancement in chemotherapeutic modalities that combines cisplatin and antifolate (5,6).

The long latency period between asbestos exposure and tumor development implies that multiple, and likely diverse, genetic changes are required for the malignant transformation of mesothelial cells. Many studies have been conducted to determine the key underlying genetic and epigenetic events responsible for the development of MM. Karyotyping, allele typing and comparative genomic hybridization analysis have demonstrated that most cases of MM have multiple chromosomal alterations, which include chromosome 9p21 and 22q (7-14). Inactivation of the *p16^{INK4a}/p14^{ARF}* locus at the 9p21 locus is found in over 70% of MM samples (15-17). The *NF2* gene at the 22q12 locus, which is responsible for a familiar cancer syndrome of neurofibromatosis type II, has also been shown to be inactivated in 40-50% of MMs, mainly with homozygous deletion or nonsense mutation (18,19). In contrast, mutation of the *p53* gene, one of the most frequently mutated genes in human malignancies, is relatively uncommon (20).

The phosphatidylinositol 3-kinase (PI3K)-AKT pathway regulates a number of normal cellular processes, including cell proliferation, survival and motility (21). AKT, also known as protein kinase B (PKB), is a serine/threonine kinase located downstream of PI3K. Activated PI3K generates a lipid second messenger, phosphatidylinositol-3,4,5-tri-phosphate (PIP3), which is essential for the translocation of AKT to the plasma membrane. There, AKT is phosphorylated and activated by phosphoinositide-dependent kinase 1 (PDK1). In tumor cells, AKT can be activated by a variety of mechanisms, including the loss of *PTEN*, mutation of the PI3K catalytic subunit gene *PIK3CA*, and activation of PI3K via autocrine or paracrine

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Key words: malignant mesothelioma, phosphatidylinositol 3-kinase, AKT, *PTEN*

stimulation of the receptor tyrosine kinases (22,23). Recently, the *LKB1* tumor suppressor gene encoding a serine/threonine kinase has also been implicated in the regulation of PTEN activity (24).

There have been a limited number of studies of MM seeking to analyze this signaling cascade. For example, Altomare *et al* reported that elevated phospho-AKT staining was observed in 17 (65%) of 26 MM specimens, and loss of *PTEN* was detected in 1 of 9 human MM cell lines (25). Papp *et al* investigated *PTEN* point mutations in 18 mesothelioma specimens with single-strand polymorphism analysis, but no mutations were detected (26). To the best of our knowledge, there have been no detailed reports analyzing *PIK3CA* and *LKB1* mutation status in MM. Thus, although activation of the PI3K-AKT pathway is likely to be significantly involved in MM development, the molecular mechanism by which AKT is activated in MM remains unclear. In the present study, we carried out mutation and expression analyses of the genes mainly associated with the PI3K-AKT pathway in MM cells. We observed frequent dysregulation of the PI3K-AKT pathway in MMs, with PTEN inactivation being one of the important mechanisms underlying its activation.

Materials and methods

Cell lines. The study employed 21 MM cell lines and 1 non-MM cell line (MeT-5A). In addition to 4 cell lines, including ACC-MESO-1, ACC-MESO-4, Y-MESO-8A and Y-MESO-8D that have previously been reported (27), 11 cell lines, including Y-MESO-9, Y-MESO-12, Y-MESO-14, Y-MESO-21, Y-MESO-22, Y-MESO-25, Y-MESO-26B, Y-MESO-27, Y-MESO-28, Y-MESO-29 and Y-MESO-30, were established in our laboratory and will be described in a future report. Y-MESO-8A and Y-MESO-8D, established from the same patient, showed distinct morphological patterns (27). NCI-H28, NCI-H290, NCI-H2052, NCI-H2373 and NCI-H2452 were gifts from Dr Adi F. Gazdar. MSTO-211H and MeT-5A were purchased from the American Type Culture Collection (Rockville, MD, USA), and MeT-5A was cultured according to their instructions. All MM cell lines were maintained in RPMI-1640 medium (Sigma-Aldrich, Irvine, UK) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and 1X antibiotic-antimycotic (Invitrogen) at 37˚C in a humidified incubator with 5% CO_2. MM samples for the establishment of cell lines and clinical data were collected after obtaining appropriate institutional review board approval and written informed consent from all patients.

Extraction of DNA and RNA. Genomic DNA was extracted using a standard phenol-chloroform method. Total RNA was prepared using RNeasy Plus RNA Extraction Kit (Qiagen K.K., Tokyo, Japan) according to the manufacturer's protocol. Random-primed, first-strand cDNA was synthesized from 3 μg total RNA using Superscript II according to the manufacturer's instructions (Invitrogen).

Real-time reverse transcription (RT)-PCR. Expression levels of PTEN mRNA were measured by means of quantitative real-time fluorescence detection. Briefly, PTEN-qRTS6, 5'-GGAAGTCTATGTGATCAAGAAACAGT-3' (sense) and PTEN-qRTAS8, 5'-CAGAAGTTGAACTGCTAGCCTCT GGA-3' (antisense) primers, respectively located at PTEN exon 6 and exon 8, were synthesized. Quantitative RT-PCR with the primers and Power SYBR Green PCR Master Mix (Applied Biosystems, Foster, CA, USA) was carried out with an ABI 7500 Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. *GAPDH* served as an endogenous control; the expression levels of PTEN mRNA in each of the samples were normalized on the basis of the corresponding *GAPDH* content and recorded as relative expression levels.

RT-PCR of PTEN exon 1- 5 was carried out using a primer set of PTEN-RT-S1, 5'-ATGACAGCCATCATCAAAGAG ATC-3' (sense) and PTEN-RT-AS1, 5'-AGCTGTGGTGGG TTATGGTCTTCA-3' (antisense).

Mutation analysis. Sequencing of *PIK3CA*, *PTEN* and *LKB1* was carried out using an Applied Biosystems Model 3100 DNA Sequencer with a PCR primer and a BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems) following the PCR amplification of genomic DNA. The primer sets covering the entire coding region of *PIK3CA* and *PTEN* have been previously reported (22,28). The primer sets of *LKB1* are available on request.

Cloning of PTEN homozygous deletion region breakpoints. Genomic PCR of *PTEN* covering exon 1 or exon 2 was carried out using the primer sets PTEN-S1, 5'-GTGACCTCCTT CGGAAAGTA-3' (sense) and PTEN-AS1, 5'-CTTTACTGG ACAGATAAGCTTATT-3' (antisense), or PTEN-S2, 5'-CC TTCTTGTGGGGTGCTAATGAAA-3' (sense) and PTEN-AS2, 5'-CTACTTAAGGGAGTGTAGTAGTGG-3' (antisense), respectively. PCR products were analyzed by gel electrophoresis on 2% agarose gel and sequenced.

Western blot analysis. Preparation of total cell lysates and Western blotting were carried out as described previously (27). 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 (30 μg) was subjected to SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). Following blocking with 3% non-fat dry milk, the filters were incubated with the primary antibody, washed with PBS with 0.5% Tween-20, reacted with the secondary antibody, and then detected with ECL (Amersham Biosciences, Buckinghamshire, UK). Anti-AKT, anti-phosphorylated-AKT (Ser473) and anti-PTEN antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA) and anti-ß-actin antibody from Sigma (St. Louis, MO, USA).

Constructs. A 1.2-kilobase (kb) fragment covering the entire human *PTEN* coding region was amplified using RT-PCR and introduced into the pcDNA3.1-V5/HIS expression vector (Invitrogen) to generate a wild-type PTEN expression construct (pcDNA-PTENwt-V5/HIS). A *PTEN* mutant-type (H123Y) expression construct (pcDNA-PTENmt123Y-V5/HIS) was synthesized using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according

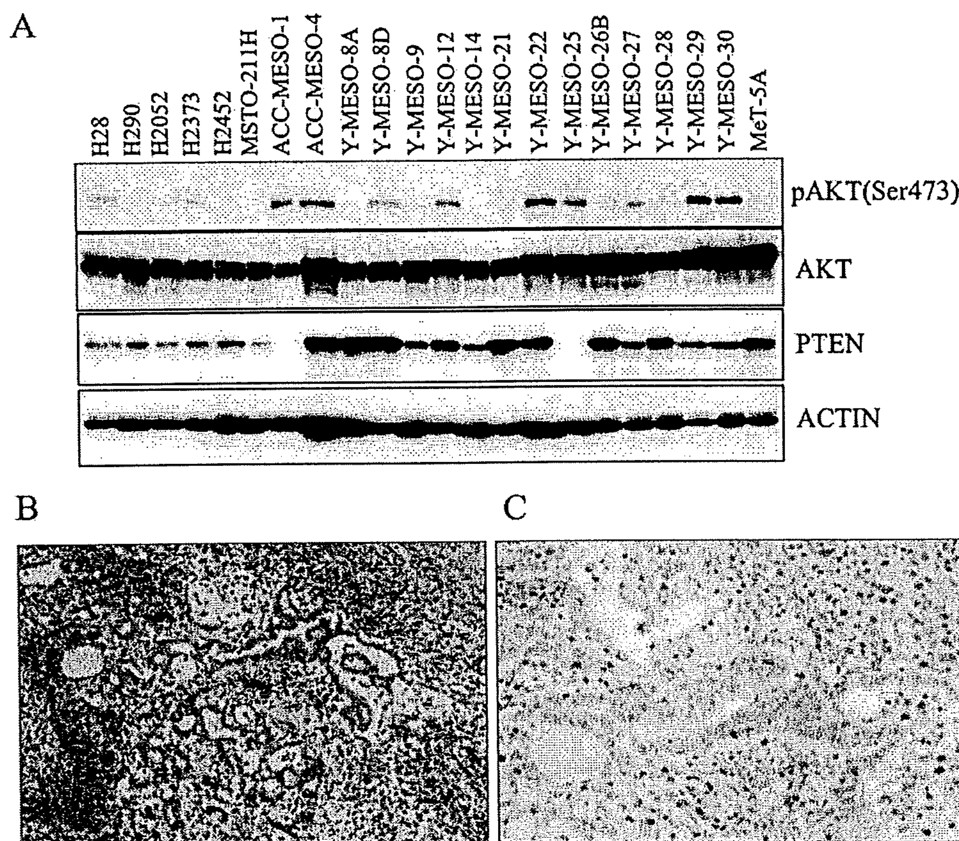

Figure 1. Expression of AKT and PTEN in MM cells. (A) Western blot analysis of AKT and PTEN in 21 MM cell lines and normal mesothelial cell line MeT-5A. Activation of AKT was studied with anti-phospho-AKT (Ser473) antibody. Expression of ß-actin was used as the control. (B) The primary tumor cells of the Y-MESO-25 cell line showed a papillotubular pattern with hematoxylin and eosin staining. (C) Immunohistochemical staining of PTEN showed no expression in the tumor cells, but was positive in stromal cells (original magnification, x200).

to the manufacturer's instructions. The sequences of all constructs were confirmed. The expression vector of the dominant negative form of AKT (AKT DN) has been described previously (29).

Colony formation assay. Cells were cultured to 70% confluence on 6-well plates and transfected with the wild-type PTEN, mutant-type PTEN, or pcDNA3.1-V5/HIS vector along with FuGENE 6 reagent following the manufacturer's protocol (Roche Diagnostics, Basel, Switzerland). After 24 h, G418 was added to the medium for a final concentration of 400 μg/ml. After 16-18 days, the cells were stained with methylene blue.

Results

Activation of the PI3K-AKT pathway in malignant mesothelioma cell lines. Dysregulated activation of the PI3K-AKT signaling pathway has been demonstrated in a variety of human malignancies. To determine the frequency and mechanisms underlying the activation of this pathway in MM, we analyzed 21 MM cell lines as well as the MeT-5A cell line, an immortalized cell line from normal mesothelial cells. Western blot analysis was performed for AKT and PTEN, a tumor suppressor involved in the PI3K-AKT pathway. Under serum-starved conditions, we found that AKT was phosphorylated (activated) at a high level in 6 cell lines (ACC-MESO-1, ACC-MESO-4, Y-MESO-22, Y-MESO-25, Y-MESO-29 and Y-MESO-30) and at mid-level in 7 cell lines (NCI-H28, NCI-H2052, NCI-H2373, Y-MESO-8D, Y-MESO-12, Y-MESO-26B and Y-MESO-27), but was undetectable in another 8 cell lines and MeT-5A (Fig. 1A). Thus, overall elevated AKT activation was shown in 13 (62%) MM cell lines.

Mutational analyses of PIK3CA, LKB1 and PTEN in malignant pleural mesothelioma cell lines. We performed mutation analyses for the *PIK3CA* and *LKB1* genes, since their activating mutations are known to be responsible for AKT activation. All 20 exons of the *PIK3CA* coding region and 9 exons of *LKB1* were sequenced, but no mutations were found (data not shown).

Next, we studied whether PTEN can be altered in malignant pleural mesothelioma (MPM), since PTEN inactivation is also known to be one of the major causes of AKT activation. Western blot analysis revealed that PTEN expression was completely lost in two cell lines, ACC-MESO-1 and Y-MESO-25 (Fig. 1A). Furthermore, compared to the Met-5A level, 11 other cell lines clearly exhibited a relatively low level of PTEN, including NCI-H28, H290, H2052, H2373, H2452, MSTO-211H, Y-MESO-9, Y-MESO-14, Y-MESO-27, Y-MESO-29 and Y-MESO-30. Since the original pathological specimen of the Y-MESO-25 cell line was available, we performed

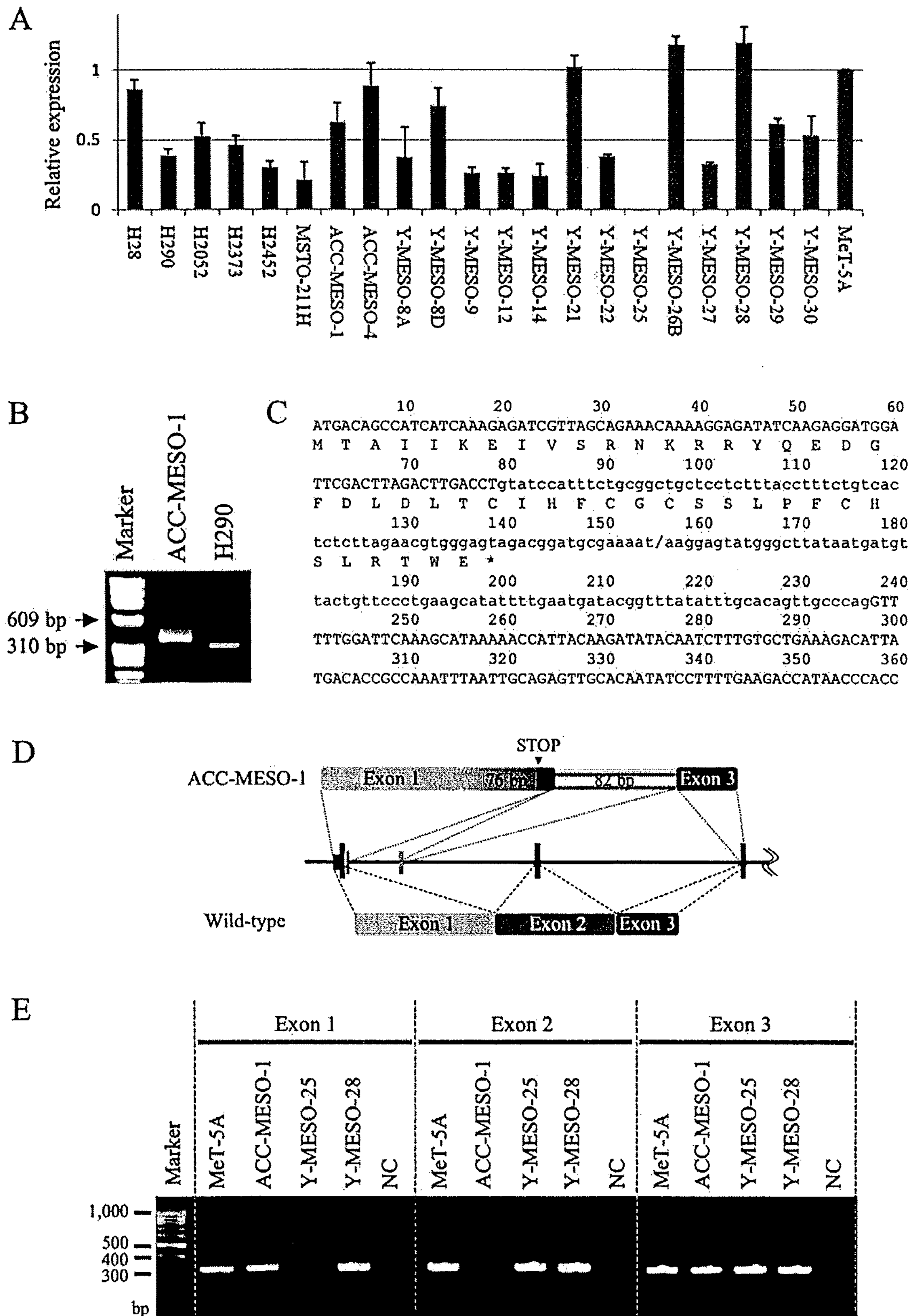

Figure 2. Expression analysis of PTEN mRNA in MM cell lines. (A) Quantitative RT-PCR analysis of PTEN with a primer set covering exon 6 to 8. The relative expression of MeT-5A was arbitrarily set to 1.0. Data represent mean ± SD. Y-MESO-25 showed an undetectable level of PTEN mRNA expression and 10 other cell lines exhibited lower expression under 0.5. (B) RT-PCR analysis with a *PTEN* primer set covering exon 1 to 6 showed an aberrant sized band in ACC-MESO-1. (C) Sequence analysis of the amplified PTEN PCR product of ACC-MESO-1. Seventy-six nucleotides (indicated by lowercase letters from 80 to 155) were intron 1 sequence which followed the exon 1. Eighty-two nucleotides (lowercase letters from 156 to 237) were also from intron 1. (D) Schematic representation of the aberrant transcript of PTEN in ACC-MESO-1 (above) and the wild-type (below). (E) Genomic PCR analysis of Y-MESO-25 and ACC-MESO-1 cells detected no PCR products of PTEN exon 1 and exon 2, respectively. NC, negative control (water blank).

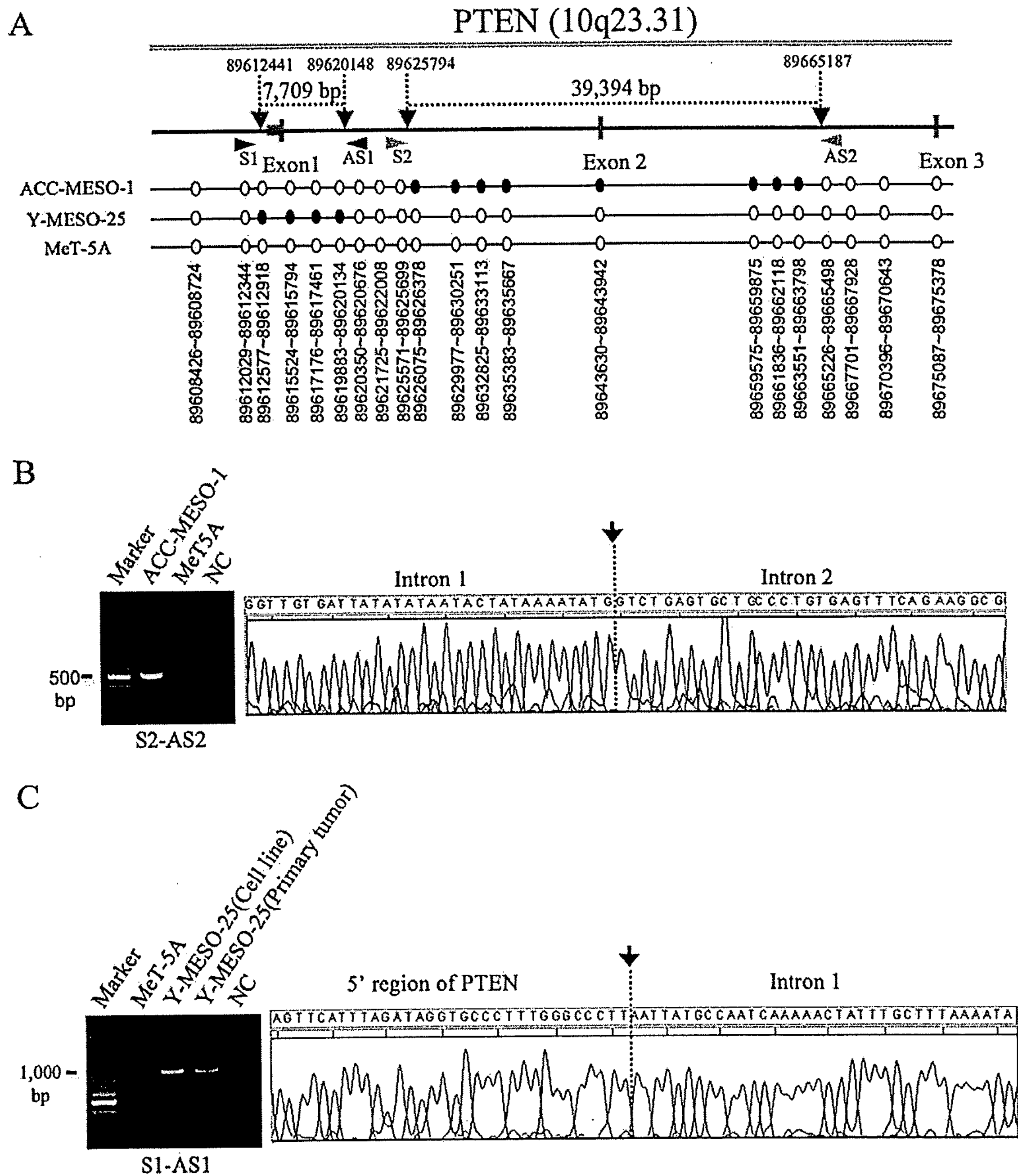

Figure 3. Cloning of the homozygous deletion breakpoints of the PTEN locus in ACC-MESO-1 and Y-MESO-25. (A) Results of PCR analysis for each locus are shown by open ovals (retention) and closed ovals (homozygous deletion). Nucleotide numbers of each location are indicated according to the March 2006 human reference sequence (NCBI Build 36.1). (B) PCR amplification with the S2 and AS2 primers yielded an aberrant 534-bp product with genomic DNA from ACC-MESO-1, but not from MeT-5A (left). The sequence of the PCR product detected the breakpoint indicated by an arrow (right). (C) PCR amplification with the S1 and AS1 primers yielded an aberrant 940-bp product with genomic DNA from Y-MESO-25 and its primary tumor, but not from MeT-5A (left). The nucleotide sequence of the PCR product detected the breakpoint indicated by the arrow (right).

immunohistochemical analysis and found additional defective expression of PTEN in the primary tumor cells (Fig. 1B and C).

PTEN expression was examined by quantitative real-time PCR (qRT-PCR) analysis using a primer set covering exons 6-8. While, as in other cell lines, the PTEN mRNA transcript in ACC-MESO-1 was detectable, a complete loss of PTEN expression was observed in Y-MESO-25 (Fig. 2A). Ten other cell lines also showed low expression of PTEN mRNA (less than half of the MeT-5A level), including NCI-H290, H2373, H2452, MSTO-211H, Y-MESO-8A, Y-MESO-9, Y-MESO-12, Y-MESO-14, Y-MESO-22 and Y-MESO-27 (Fig. 2A). RT-PCR of PTEN was then performed using various primer sets. When a primer set encompassing exons 1-5 was used, an aberrant-sized PTEN transcript (~370 bp) was detected in ACC-MESO-1 (Fig. 2B). Sequence analysis of this cDNA fragment revealed an abnormal splicing pattern with the addition of a 76-bp intron 1-fragment following exon 1 and another 82-bp intron 1-fragment between exons 1 and 3, while

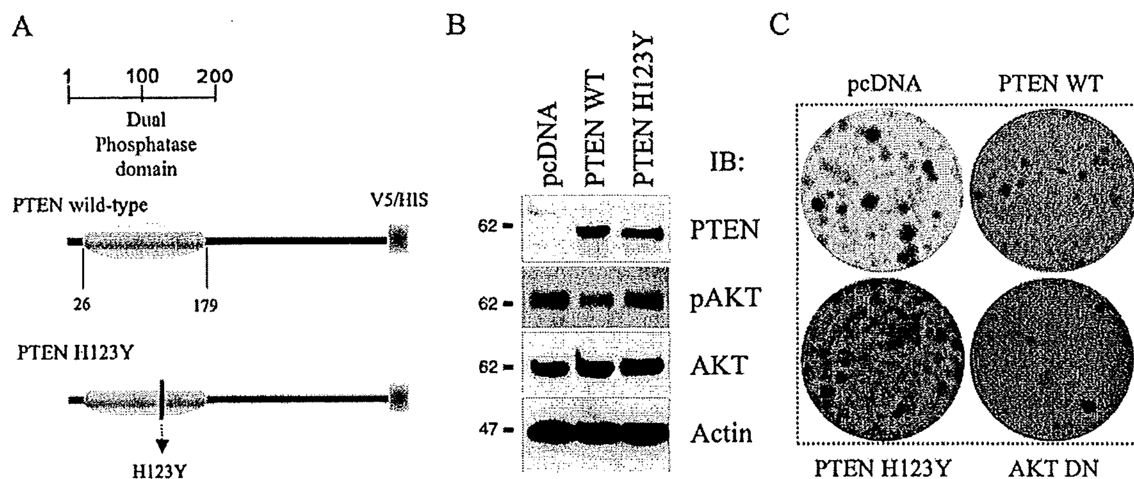

Figure 4. Re-expression of PTEN suppresses AKT activation and reduces the colony formation of malignant pleural mesothelioma cells. (A) Schematic representation of V5-His tagged wild-type and mutant-type (H123Y) PTEN constructs. (B) Western blot analyses of PTEN, pAKT and AKT after transfection of wild-type and mutant-type PTEN into Y-MESO-25 cells. ß-actin antibody was used as the control. (C) G418-resistant colonies of ACC-MESO-1 were detected with methylene blue staining 16-18 days after cells were transfected with control (pcDNA3.1), wild-type PTEN (pcDNA-PTENwt-V5/HIS), mutant-type PTEN (pcDNA-PTENmt123Y-V5/HIS) or AKT dominant negative (AKT DN) construct.

skipping exon 2 (Fig. 2C and D). This splicing alteration caused the addition of an extra 158 bp with the deletion of exon 2, resulting in a premature stop (Fig. 2C and D).

To determine whether a genetic mutation was responsible for PTEN inactivation, we sequenced 9 exons covering the entire coding region of the *PTEN* gene. Although no mutations were detected in the other 19 cell lines, no amplification of exon 1 in Y-MESO-25 or exon 2 in ACC-MESO-1 was found, indicating that these cell lines harbored a homozygous deletion of *PTEN* (Fig. 2E).

Homozygous deletion on the PTEN locus. We further carried out a genomic PCR analysis for these two cell lines using multiple primer sets to determine the break-points of the homozygous deletion regions in more detail. The results of the PCR analysis are summarized in Fig. 3A. Genomic PCR with the S2 and AS2 primer demonstrated a 530-bp PCR product in ACC-MESO-1 (Fig. 3B), which resulted from an ~40-kb deletion including exon 2. Meanwhile, in Y-MESO-25, genomic PCR with the S1 and AS1 primers detected an ~900-bp PCR product in DNA derived from the cell line as well as from its primary tumor (Fig. 3C). The Y-MESO-25 cell line was demonstrated to have a 7.7-kb homozygous deletion including the promoter region and exon 1 of *PTEN*.

Re-expression of PTEN or AKT dominant negative constructs reduces colony formation of MM cells. To determine whether the loss of *PTEN* plays a pro-oncogenic role for ACC-MESO-1 and Y-MESO-25 cell lines, we transfected a wild-type PTEN expression vector into these cell lines. In addition to the wild-type *PTEN*, a mutant-type construct with a tyrosine for histidine substitution at amino acid position 123 was also synthesized. This mutation has been shown to abrogate the phosphatase function of PTEN (30) (Fig. 4A). Western blot analysis showed that the wild-type but not the H123Y mutant-type of *PTEN* suppressed the phosphorylation status of AKT (Fig. 4B). Colony formation assay showed that wild-type, but not mutant-type, *PTEN* reduced the number of colonies, indicating that PTEN inactivation is critical for the cell survival or proliferation of these cell lines (Fig. 4C). AKT dominant negative construct also significantly inhibited colony formation (Fig. 4C).

Discussion

In this study, we examined the frequency and underlying mechanisms of the dysregulated PI3K-AKT pathway in MM cells. Twenty one MM cell lines were used, including 15 cell lines established in our laboratory from Japanese patients. Our results indicate that AKT was highly phosphorylated in 13 (62%) of 21 MM cell lines under serum-starved conditions, suggesting that constitutively activated AKT may be observed in most MMs. Two cell lines harbored a homozygous deletion of *PTEN*, and 11 cell lines showed low expression of PTEN protein, although no mutation of *PIK3CA* or *LKB1* was detected. Re-expression of *PTEN* in the cell lines with *PTEN* homozygous deletion suppressed colony formation, indicating that *PTEN* inactivation was critical for cell survival or proliferation in these cell lines.

Previous immunohistochemical studies demonstrated the elevated activation of AKT in 65-84% of MM samples (25,31). Thus the frequency of AKT activation in MMs in our study using cell lines seems to be very similar to that of previous studies. Whereas one previous study reported that no *PTEN* mutation was detected in 18 MM specimens, another study demonstrated that a homozygous deletion of *PTEN* was detected in 1 of 9 MM cell lines (25,26). Thus, our finding that a *PTEN* homozygous deletion, but not point mutation, was present in 2 (9.5%) of 21 MM cell lines is compatible with the results of previous studies.

The loss of PTEN expression has been attributed to allelic loss or promoter hypermethylation in some malignancies (32,33). In non-small cell lung cancer, the homozygous deletion and mutation of *PTEN* occur rarely, although loss of hetero-

zygosity (LOH) has been reported to occur in 26% to more than 50% of tumors (34). While we did not study the LOH or methylation status of the *PTEN* gene, among another 19 cell lines excluding ACC-MESO-1 and Y-MESO-25, over half the lines also exhibited low PTEN mRNA and protein expression levels. Ten cell lines expressed PTEN mRNA at less than half the level observed in MeT-5A, and 11 cell lines showed significantly low expression of PTEN, with 7 cell lines (NCI-H290, H2373, H2452, MSTO-211H, Y-MESO-9, Y-MESO-14 and Y-MESO-27) showing simultaneous suppression at the mRNA and protein level. This suggests that these cell lines had only one normal allele or suffered from hypermethylation of the other allele of the *PTEN* gene, resulting in low levels of PTEN mRNA and protein expression. From this standpoint, Garland *et al* reported that, while no PTEN expression was noted in 3 (16%) of 19 primary MMs, 15 (79%) showed only a mild intensity of staining (31). Opitz *et al* also reported negative PTEN expression in 62% of MPMs from an immunohistochemical study using tissue microarray containing 341 cases, with 14% being weak, 9.5% moderate, and 14.5% strong (35). Taken together with other studies, our results suggest that, as one of the underlying mechanisms, activation of the PI3K-AKT pathway is due to a *PTEN* homozygous deletion in ~10% of MMs, and to the suppression of PTEN expression in a significant proportion of MM cases, probably caused either by the allelic loss or epigenetic silencing of the *PTEN* gene.

Meanwhile, several cell lines showed a distinct difference between the expression of PTEN mRNA/protein and AKT phosphorylation status in each MPM cell line. For example, although NCI-H290, H2452, MSTO-H211 showed low expression of PTEN at both the mRNA and protein level, AKT phosphorylation was not very obvious. Thus, significant down-regulation of PTEN does not necessarily lead directly to AKT activation. In this regard, recent evidence suggests that PTEN has other functions as a tumor suppressor, such as chromosomal instability (36). Thus, other functions of PTEN that are not involved in the dephosphorylation of PIP3 may be responsible for a pro-oncogenic role of MM cells when PTEN is suppressed.

For PI3K-AKT pathway activation, it should also be considered that the activation of upstream molecules, including receptor tyrosine kinases, may play an important role. For example, hepatocyte growth factor-MET receptor signaling activation has been demonstrated to be accompanied by AKT activation in MPM cell lines (25,37). Activation of HGF/MET signaling has also been shown to induce the proliferation of MM cells via a PI3K/MEK5/Fra-1 pathway (38). Meanwhile, overexpression of EGFR and PDGFR ß has been implicated in the tumorigenesis of mesothelioma (39-42). Thus, more detailed investigation of the activation status and underlying genetic abnormalities of these receptor tyrosine kinases is warranted in future, in order to reveal other possible underlying mechanisms of PI3K-AKT activation in MM cells.

Finally, exogenous expression of wild-type PTEN in the MM cell lines with a PTEN homozygous deletion clearly triggered significant cell-growth suppression, while the H123Y mutant lacking both dual-phosphatase activities was ineffective. Expression of the AKT dominant negative form also exhibited growth suppression. These results suggest that the protein phosphatase activity of PTEN likely contributes to its tumor suppressor function in a subset of MMs. This is supported by a previous study, which reported that PTEN overexpression transfected with PTEN adenoviral vectors engendered apoptotic cell death in two human MM cell lines by AKT hypophosphorylation (43).

In conclusion, our data strongly suggest that the PI3K-AKT pathway is significantly involved in MM carcinogenesis, and that elucidation of the downstream targets that dictate cellular response to this signaling pathway may have important implications for the development of future MM treatment strategies.

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ORIGINAL REPORT

Randomized Phase III Trial of Platinum-Doublet Chemotherapy Followed by Gefitinib Compared With Continued Platinum-Doublet Chemotherapy in Japanese Patients With Advanced Non–Small-Cell Lung Cancer: Results of a West Japan Thoracic Oncology Group Trial (WJTOG0203)

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See accompanying editorial on page 713 and article on page 744

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Clinical Trials repository link available on JCO.org.

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ABSTRACT

Purpose
Gefitinib is a small molecule inhibitor of the epidermal growth factor receptor tyrosine kinase. We conducted a phase III trial to evaluate whether gefitinib improves survival as sequential therapy after platinum-doublet chemotherapy in patients with advanced non–small-cell lung cancer (NSCLC).

Patients and Methods
Chemotherapy-naïve patients with advanced stage (IIIB/IV) NSCLC, Eastern Cooperative Oncology Group performance status of 0 to 1, and adequate organ function were randomly assigned to either platinum-doublet chemotherapy up to six cycles (arm A) or platinum-doublet chemotherapy for three cycles followed by gefitinib 250 mg orally once daily, until disease progression (arm B). Patients were stratified by disease stage, sex, histology, and chemotherapy regimens. The primary end point was overall survival; secondary end points included progression-free survival, tumor response, safety, and quality of life.

Results
Between March 2003 and May 2005, 604 patients were randomly assigned. There was a statistically significant improvement in progression-free survival in arm B (hazard ratio [HR], 0.68; 95% CI, 0.57 to 0.80; $P < .001$); however, overall survival results did not reach statistical significance (HR, 0.86; 95% CI, 0.72 to 1.03; $P = .11$). In an exploratory subset analysis of overall survival by histologic group, patients in arm B with adenocarcinoma did significantly better than patients in arm A with adenocarcinoma (n = 467; HR, 0.79; 95% CI, 0.65 to 0.98; $P = .03$).

Conclusion
This trial failed to meet the primary end point of OS in patients with NSCLC. The exploratory subset analyses demonstrate a possible survival prolongation for sequential therapy of gefitinib, especially for patients with adenocarcinoma.

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INTRODUCTION

Lung cancer is the most common cancer worldwide, with an estimated 1.2 million new cases globally (12.3% of all cancers) and 1.1 million deaths (17.8% of all cancer deaths) in 2000.[1] The estimated global incidence of non–small-cell lung cancer (NSCLC) in 2000 was approximately 1 million, which accounted for approximately 80% of all cases of lung cancer.[1] Treatment of advanced NSCLC is palliative; the aim is to prolong survival without leading to deterioration in quality of life.[2] The recommended first-line treatment of advanced NSCLC currently involves up to six cycles of platinum-based combination chemotherapy, with no single combination recommended over another.[3,4] Recently, combination chemotherapy of pemetrexed plus cisplatin was significantly superior to gemcitabine plus cisplatin in nonsquamous NSCLC.[5]

Gefitinib is an orally active epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) that blocks the signal transduction pathways

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implicated in the proliferation and survival of cancer cells.[6] In two phase II trials in patients with pretreated advanced NSCLC (Iressa Dose Evaluation in Advanced Lung Cancer [IDEAL] 1 and 2), gefitinib 250 mg/d showed response rates of 12% and 18% and a median survival time (MST) of 7.0 and 7.6 months in IDEAL1 and 2, respectively; in addition, the toxicity profile was not severe.[7,8] This favorable tolerability profile, coupled with a mechanism of action that is distinct from that of cytotoxic agents, provides a strong rationale for use of gefitinib in combination with standard cytotoxic regimens. Platinum-doublet chemotherapy added to gefitinib in untreated patients with NSCLC was evaluated in two large-scale, placebo-controlled, randomized trials (INTACT-1 and -2).[9,10] Gefitinib showed no survival benefit over placebo when combined with standard platinum-doublet chemotherapy in both trials. Furthermore, gefitinib did not improve time to progression or objective tumor response over chemotherapy alone. These results were disappointing and surprising because of the significant antitumor activity of gefitinib when given alone to pretreated patients with NSCLC.

First, it is possible that each of the agents is working against a susceptible subpopulation of tumor cells so that the effect is redundant rather than additive, or that one agent results in the loss of an intermediary molecule that is essential to the function of the other agent, resulting in an antagonistic effect. Second, patients included in these studies were not selected on the basis of a specific biomarker, such as target EGFR expression, gene amplification, or mutations. Clinical profiles of females, never smokers, adenocarcinoma histology, and Asian ethnicity have all been recognized as favorable subgroups that respond to gefitinib.[11-14]

Because no additive effect was observed by administering gefitinib continuously in combination with chemotherapy, possible alternatives could be the administration of gefitinib in the interval between chemotherapy cycles or as sequential treatment after chemotherapy. This could also potentially prevent the problem of drug interference or antagonism. We conducted a randomized phase III trial to evaluate whether gefitinib improves survival as sequential therapy after platinum-doublet chemotherapy in chemotherapy-naive patents with NSCLC.

PATIENTS AND METHODS

Patients

Eligible patients were 20 to 75 years of age, with histologically or cytologically confirmed stage IIIB (with malignant pleural effusion or contralateral hilar lymph node metastases) or stage IV NSCLC who had not previously received any chemotherapy. Patients who had recurrence after complete surgical resection were permitted. Patients treated with either adjuvant or neoadjuvant chemotherapy were excluded in this trial. Additional criteria included a Eastern Cooperative Oncology Group performance status of 0 to 1, and adequate organ function as indicated by WBC count ≥ 4,000/μL, absolute neutrophil count ≥ 2,000/μL, hemoglobin ≥ 9.5 g/dL, platelets ≥ 100,000/μL, AST/ALT ≤ 2.5 times the upper limit of normal, total bilirubin ≤ 1.5 mg/dL, serum creatinine ≤ 1.2 mg/dL, and PaO_2 in arterial blood ≥ 70 mmHg. Asymptomatic brain metastases were allowed provided that they had been irradiated and were clinically and radiologically stable. Patients were excluded from the study if they had radiologically and clinically apparent interstitial pneumonitis or pulmonary fibrosis. All patients provided written informed consent, and the study protocol was approved by the West Japan Thoracic Oncology Group Protocol Review Committee and the institutional review board of each participating institution.

Treatment Plan

Eligible patients were centrally registered at West Japan Thoracic Oncology Group Data Center and were randomly assigned to receive either platinum-doublet chemotherapy up to six cycles (arm A) or three cycles of platinum doublet followed by gefitinib 250 mg/d orally, until disease progression (arm B). Patients who achieved disease control (response or stable disease) treated with three cycles of platinum-doublet went for gefitinib treatment phase in arm B. Each physician selected his/her chemotherapy options before randomization. Platinum-doublet chemotherapy options included any of the following: (1) carboplatin area under the curve 6, day 1, and paclitaxel 200 mg/m^2, day 1, every 3 weeks; (2) cisplatin 80 mg/m^2, day 1, and irinotecan 60 mg/m^2, days 1, 8, 15, every 4 weeks; (3) cisplatin 80 mg/m^2, day 1, and vinorelbine 25 mg/m^2, days 1, 8, every 3 weeks; (4) cisplatin 80 mg/m^2, day 1, and gemcitabine 1,000 mg/m^2 days 1, 8, every 3 weeks; or (5) cisplatin 80 mg/m^2, day 1, and docetaxel 60 mg/m^2 day 1, every 3 weeks. The dose of carboplatin was calculated using Calvert's formula, and the glomerular filtration rate was estimated by the Cockcroft-Gaut formula. These treatment schedules and doses are used as standard platinum-doublet regimens for advanced NSCLC in Japan.[15,16]

Randomization was stratified according to the institution, type of histology (adenocarcinoma *v* nonadenocarcinoma), clinical stage (IIIB *v* IV), and selected platinum-doublet regimens with the use of a minimization procedure. Patients receiving platinum-doublet chemotherapy received standard supportive treatments, including hydration and antiemetics, according to each institutional standard guideline. After withdrawing from the trial as a result of disease progression or intolerable toxicity, any systemic treatment, including with EGFR-TKI, was permitted in both arms.

Baseline and Follow-Up Assessments

Pretreatment evaluation included a complete medical history and physical examination, a CBC with differential and platelet count, standard biochemical profile, ECG, chest radiographs, computed tomography (CT) scans of the chest, abdomen, and brain, magnetic resonance imaging, and a whole-body bone scan. During treatment, a CBC and biochemical tests were performed at least every 2 weeks. A detailed medical history was taken and a complete physical examination with clinical assessment was performed every 2 weeks to assess disease symptoms and treatment toxicity, and chest

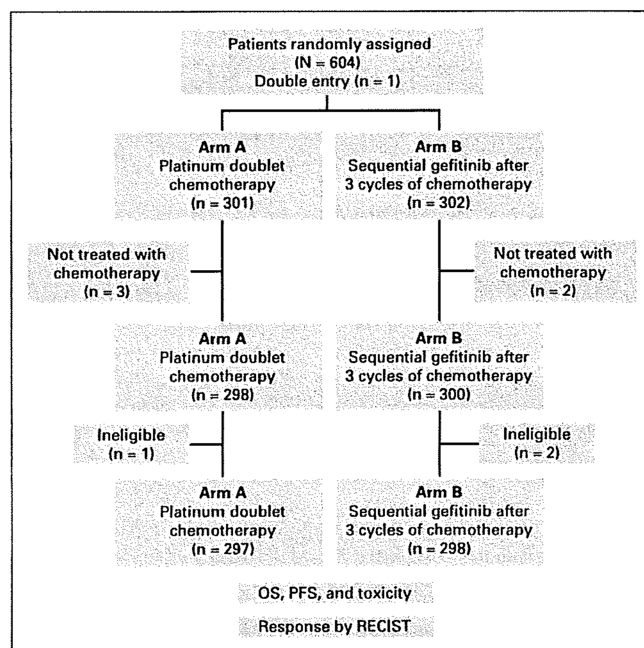

Fig 1. CONSORT diagram for the study. OS, overall survival; PFS, progression-free survival; RECIST, Response Evaluation Criteria in Solid Tumors.

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radiographs were done every treatment cycle. Toxicity was evaluated according to the National Cancer Institute Cancer Common Toxicity Criteria (NCI-CTC) version2.[17]

All patients were assessed for response by CT scans monthly during treatment. Response Evaluation Criteria in Solid Tumors (RECIST) were used for the evaluation of response.[18]

Disease-related symptoms were assessed using the Lung Cancer Subscale (LCS) of the Functional Assessment of Cancer Therapy-Lung quality of life instrument (version 4.0).[19] Patients were asked to complete the instrument at the time of enrollment and at 12 weeks and 18 weeks after initiation of treatment. The maximum attainable score on the LCS was 28, where the patient was considered asymptomatic.

Statistical Analysis

The primary end point was OS; secondary end points included PFS, tumor response, safety, and quality of life. Based on previous trials evaluating platinum-doublet chemotherapy, the MST was approximately a range of 8 to 11 months.[3] In IDEAL-1, which was the trial of gefitinib alone in patients with previously treated NSCLC, median time to treatment failure was 98 days.[7] This trial was designed to detect a 3-month difference in MST. To attain 80% power at a two-sided significance level of .05, assuming a MST in the chemotherapy alone arm of 9 months with 2 years of follow-up after 3 years of accrual, 225 patients in each treatment group were required. Both the OS and PFS were estimated with the Kaplan-Meier method. Comparisons of OS and PFS between arms were assessed by the stratified log-rank test. Two interim analyses were planned after half the patients were registered and at the end of registration.

At the first interim analysis, 14% of patients in arm B unexpectedly withdrew from sequential gefitinib treatment after the three cycles of platinum-doublet chemotherapy at their own request because of hearing the news of interstitial lung disease (ILD) as a result of the use of gefitinib in Japan. If 15% of patients treated with sequential gefitinib withdrew, 284 patients in each arm were required to attain an 80% power at a two-sided significance level of .05, assuming a MST of the chemotherapy alone arm of 9 months with 2 years of follow-up after 3 years of accrual. Consequently, a protocol amendment was performed in April 2004.

For symptom analysis, comparisons of LCS between arms were conducted using a linear mixed-effects model in which the missing data depend on the observed LCS, using the MIXED procedure in SAS version 9 (SAS Institute, Cary, NC).

RESULTS

Patient Characteristics

From March 2003 to May 2005, 604 patients with advanced NSCLC from 39 institutions were enrolled (Appendix, online only). Patients were randomly assigned to platinum-doublet chemotherapy up to 6 cycles (n = 302, arm A) or sequential gefitinib after three cycles of platinum-doublet chemotherapy (n = 302, arm B). One patient was double entry in arm A, and three patients in arm A and two in arm B did not receive any chemotherapy. Therefore, a total of 598 patients (298 in arm A and 300 in arm B) were included in the analysis of patients' profiles and the assessment for toxicity. In addition, three patients did not meet the entry criteria; thus, 297 patients with measurable lesions by RECIST in arm A and 298 eligible patients in arm B were assessable for OS, PFS, and response. Figure 1 shows the CONSORT diagram. Table 1 presents baseline patient characteristics and lists the platinum-doublet chemotherapy regimen selected by each physician.

Table 1. Patients' Characteristics and Selected Platinum-Doublet Chemotherapy Regimens

Parameter	Arm A		Arm B		P
	No. of Patients	%	No. of Patients	%	
Patients enrolled	298		300		—
Median age, years	63		62		.114
Range	35-74		25-74		
Sex					
Male	191	34.6	192	64.0	.981
Female	107	67.8	108	36.0	
ECOG PS					
0	103	30.8	90	30.0	.778
1	195	69.2	210	70.0	
Histology					
Adenocarcinoma	232	77.9	237	79.0	.733
Nonadenocarcinoma	66	22.1	63	21.0	
Clinical stage					
IIIB	54	18.1	55	18.3	.946
IV	244	81.9	245	81.7	
Smoking status					
Smoker	202	67.8	210	70.0	.559
Nonsmoker	96	32.2	90	30.0	
Selected platinum-doublet chemotherapy regimens					
CP	193	64.8	195	65.0	.987
IP	8	2.7	10	3.3	
VP	44	14.8	45	15.0	
GP	45	15.1	42	14.0	
DP	8	2.7	8	2.7	

NOTE. Differences between two arms were tested by χ^2 test, excluding age (Wilcoxon test), ECOG PS.
Abbreviations: ECOG PS, Eastern Cooperative Oncology Group performance status; CP, carboplatin and paclitaxel; IP, irinotecan and cisplatin; VP, vinorelbine and cisplatin; GP, gemcitabine and cisplatin; DP, docetaxel and cisplatin.

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Treatment Delivery

The median number of chemotherapy cycles was three (range, 1 to 6) in arm A, and three (range, 1 to 3) in arm B. One hundred seventy-two patients (57.3%) in arm B were treated with gefitinib after completion of three cycles of platinum-doublet. The median treatment duration of gefitinib was 69.5 days, and the maximum treatment duration was 1,324 days. As presented in Figure 2, EGFR-TKIs, which included gefitinib, erlotinib, and vandetanib, were used in 54.5% and 75.2% of patients in arm A and B, respectively, at any time during treatment of NSCLC. In arm B, gefitinib treatment did not take place because of early disease progression before the completion of three cycles of platinum-doublet chemotherapy in 93 patients (31.2%), and 33 (11.1%) in arm B rejected the use of gefitinib after platinum-doublet because of publication of a news report about gefitinib-induced ILD.

Treatment Efficacy

At the time of final analysis, 247 (83.2%) and 232 patients (78.0%) had died in arm A and arm B, respectively. The MST was 12.9 months for chemotherapy alone and 13.7 months for chemotherapy followed by gefitinib (hazard ratio [HR] according to Cox's regression model, 0.86; 95% CI, 0.72 to 1.03; $P = .11$ stratified log-rank test, Fig 3A). The PFS was 4.3 months in arm A and 4.6 months in arm B (HR, 0.68; 95% CI, 0.57 to 0.80; $P < .001$, Fig 3B).

When exploratory subset analysis were performed, sequential therapy with gefitinib after three cycles of platinum-doublet chemotherapy prolonged OS significantly in the subset of patients with adenocarcinoma (HR, 0.79; 95% CI, 0.65 to 0.98; $P = .03$; Fig 4A). There was no significant difference in OS due to the small subset of patients with nonadenocarcinoma (HR, 1.24; 95% CI, 0.85 to 1.79; $P = .25$; Fig 4B). In addition to the OS plots, the PFS plots for adenocarcinoma and nonadenocarcinoma were showed in Figure 4C and 4D, respectively. Furthermore, results of the subset analysis were summarized for forest plots in Figure 5. Another subset of smokers had a survival advantage with chemotherapy followed by gefitinib over chemotherapy alone. There was no difference between the two treatment groups in the subset of never smokers. Never smokers with NSCLC had a prolonged survival of about 23.5 months in arm A and 21.7 months in arm B.

The overall response rate was 29.3% for chemotherapy alone and 34.2% for chemotherapy followed by gefitinib. There was no significant difference between treatment arms ($P = .20$; Fisher's exact test). The overall disease control rate (response and stable disease) were 71.0% and 75.5% in arm A and in arm B, respectively ($P = .22$).

Toxicity

Toxicity was assessed according to NCI-CTC version 2 in all patients who received at least one treatment cycle of platinum-doublet chemotherapy (Table 2). Grade 3 or 4 anemia developed in 21.8% of patients in arm A and 13.3% of patients in arm B. There was a significant difference between the two arms ($P = .006$). Grade 3 or 4

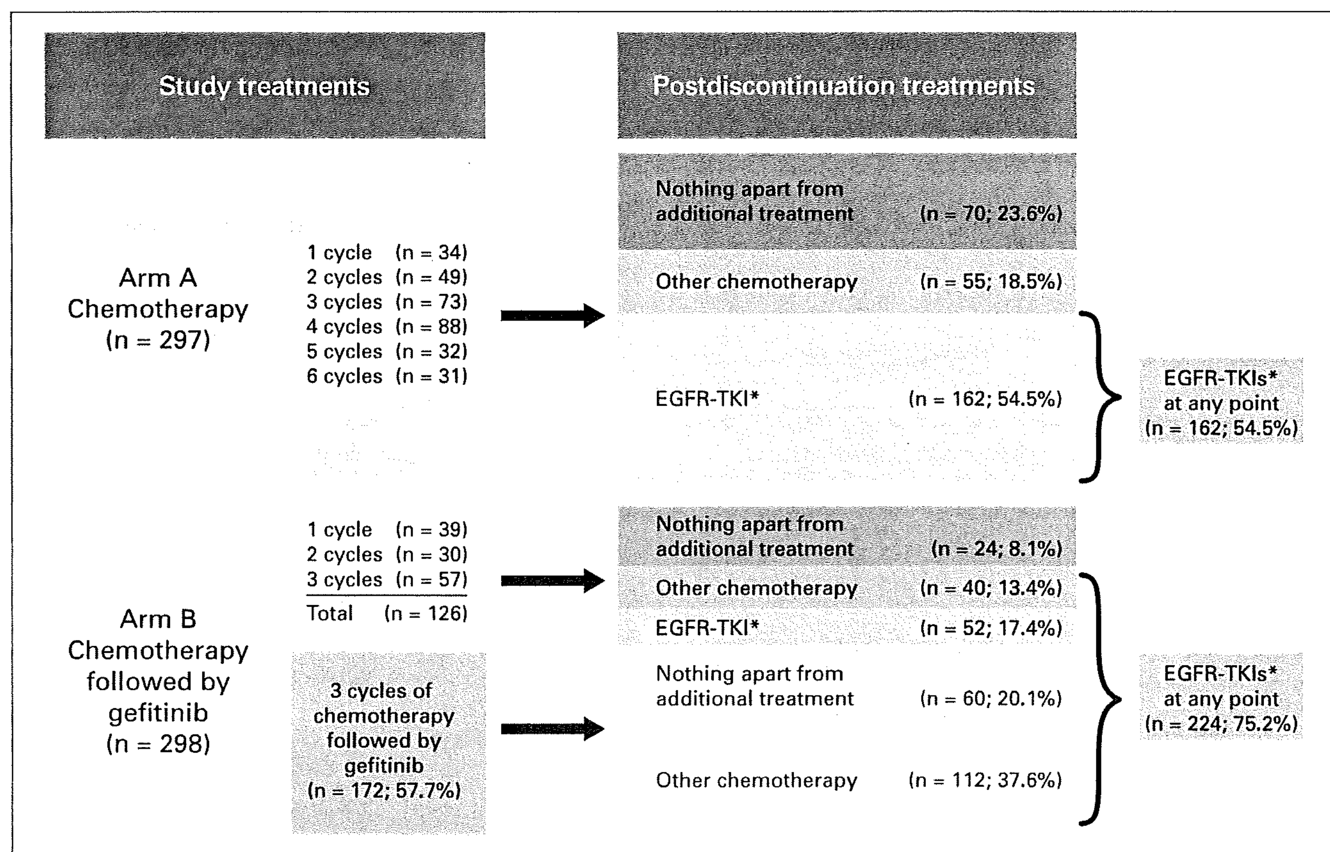

Fig 2. Exposure to active epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI), including postdiscontinuation treatments in the full analysis set population (n = 595).

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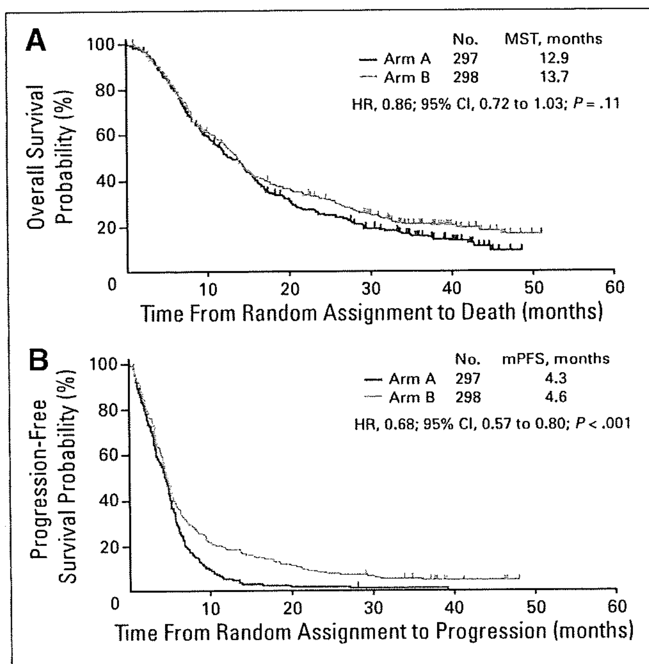

Fig 3. (A) Overall survival and (B) progression-free survival (n = 598). MST, median survival time; HR, hazard ratio; mPFS, median progression-free survival.

thrombocytopenia occurred in 10.7% of patients in arm A and 6.3% of patients in arm B, but differences did not reach significance ($P = .054$). Conversely, grade 3 or 4 AST/ALT elevation in arm B was severer than in arm A ($P = .002$). Severe ILD induced by gefitinib, which many patients feared developing, was observed in two patients in this study.

Disease-Related Symptoms Assessment

All 595 patients completed baseline LCS questionnaires; questionnaire completion rates were 81.0% at 12 weeks and 70.3% at 18 weeks. LCS data were missing in 111 surveys because of death or severe impairment of the patient's general condition; this accounted for 6.2% of the total number of surveys scheduled. The adjusted mean of initial summed scores of LCS were 20.3 for arm A and 20.6 for arm B, respectively. The adjusted LCS scores at 12 and 18 weeks were 21.0 and 20.9 for arm A, and 21.8 and 21.2 for arm B, respectively. Sequential gefitinib seemed to provide better symptom relief, although differences did not reach statistical significance ($P = .10$).

DISCUSSION

Sequential gefitinib therapy after three cycles of standard platinum-doublet chemotherapy showed no survival benefit over platinum-doublet chemotherapy up to six cycles in previously untreated patients with advanced NSCLC. However, sequential gefitinib was associated with significantly prolonged PFS. Recently, positive results with maintenance or sequential chemotherapy have been reported in clinical trials in PFS or time to progression; however, OS was not significantly lengthened.[20,21] More recently, pemetrexed administered to NSCLC patients without progression after four cycles of first-line treatment with platinum-doublet provided significant improvement in PFS compared with placebo (HR, 0.60; 95% CI, 0.49 to 0.73; $P < .00001$).[22]

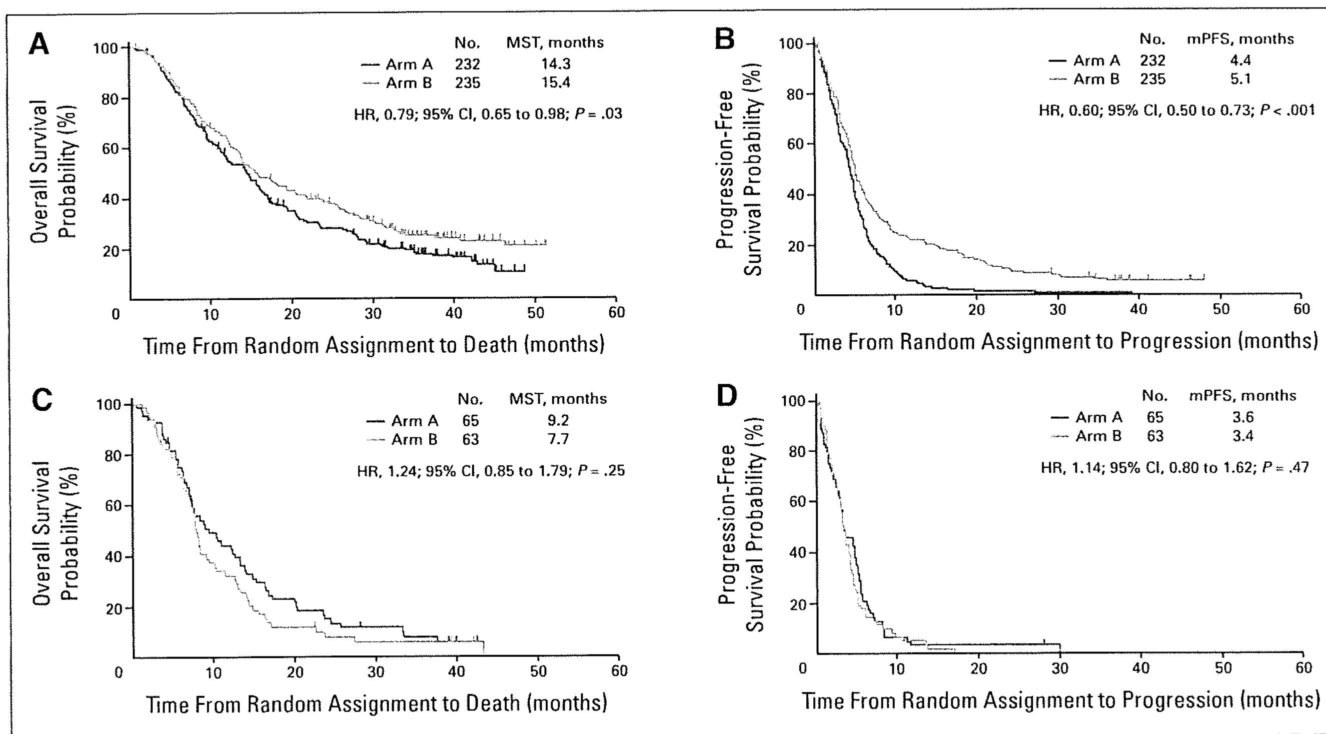

Fig 4. (A) Overall survival in the subset groups of patients with adenocarcinoma (n = 467), (B) progression-free survival in the subset groups of patients with adenocarcinoma (n = 467), (C) overall survival in the subset groups of patients with nonadenocarcinoma (n = 128), and (D) progression-free survival in the subset groups of patients with nonadenocarcinoma (n = 128). MST, median survival time; HR, hazard ratio; mPFS, median progression-free survival.

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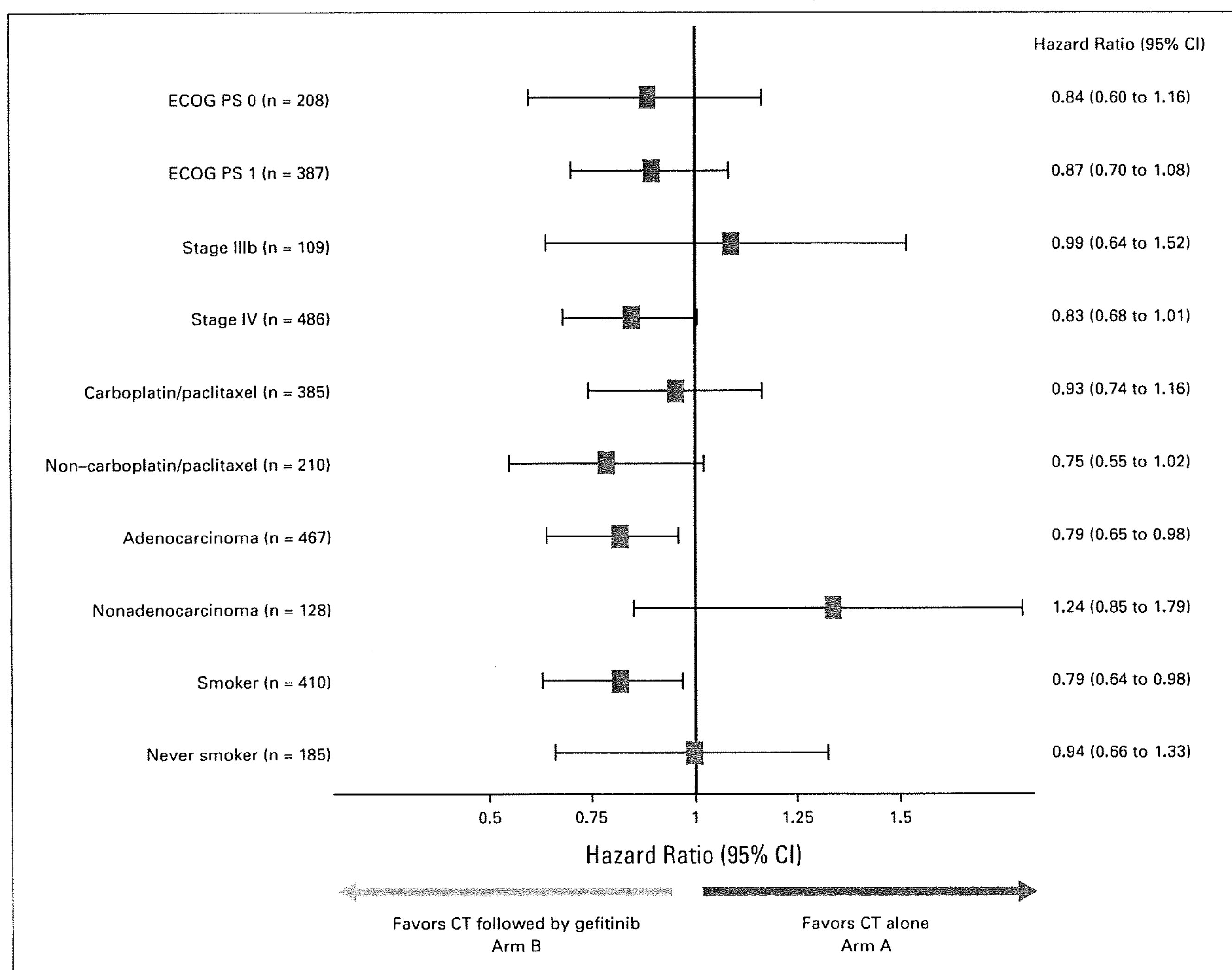

Fig 5. Forest plot subgroup analysis according to patients' backgrounds. CT, chemotherapy; ECOG PS, Eastern Cooperative Oncology Group performance status.

It was the first randomized, double-blind, placebo controlled trial to demonstrate a significant OS prolongation for maintenance treatment with pemetrexed in patients with advanced NSCLC (HR, 0.79; 95% CI, 0.65 to 0.95; $P = .012$).[22] The results of the Sequential Erlotinib in Unresectable NSCLC (SATURN) study, which was a randomized, double-blind, placebo controlled trial with erlotinib as maintenance, were presented this year. Erlotinib maintenance treatment had improvement in PFS of 41% compared with placebo.[23] Maintenance or sequential chemotherapy strategy after standard treatment has lately been receiving considerable attention. As a result, our trial was considered a consolidation therapy using other agent without progression after front-line treatment rather than maintenance.

Although the median number of chemotherapy cycles was three in both arms, 47.5% of patients received more than four cycles in Arm A. The number of treatment cycles was lower in Japanese than in whites; however, comparability was to be kept between the two arms in this randomized trial. These results were consistent with Japanese data on the median number of cycles of platinum-doublet chemotherapy.[15]

Toxicity results were consistent with previous Japanese studies of advanced NSCLC patients who received platinum-doublet chemotherapy.[15,16] Furthermore, no significant severe adverse events were seen that were not predictable from the safety profiles of gefitinib in sequential therapy after platinum-doublet chemotherapy. Recently published data suggested that gefitinib might be associated with ILD in Japanese patients[11]; however, in our study, the overall incidence of ILD was less than 1%, and no imbalance was identified between the two treatment arms in terms of ILD.

It was interesting that sequential gefitinib therapy had a significant survival prolongation in patients with adenocarcinoma histology (HR, 0.79; 95% CI, 0.65 to 0.98; $P = .03$). There was no difference also in PFS or OS for patients with nonadenocarcinoma. It was possible that these patients just did not benefit from an ineffective therapy of sequential gefitinib. In patients with NSCLC, adenocarcinoma histology, nonsmoker, and Japanese or Asian ethnicity are favorable predictive factors for a response to gefitinib treatment.[11-14] When the analysis was performed in the most favorable subset population that responded to gefitinib—that is, among those with both adenocarcinoma histology and nonsmokers—the MST was 23.5 months in arm A and 25.1 months and in arm B, respectively. Indeed, more than three quarters of the patients with favorable profiles in arm A received gefitinib after the protocol treatment, because physicians recognized

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Table 2. Toxicity According to National Cancer Institute Common Toxicity Criteria Version 2

	Arm A (n = 298)				Arm B (n = 300)				
	Grade 3		Grade 4		Grade 3		Grade 4		
Toxicity	No.	%	No.	%	No.	%	No.	%	χ^2 Test *P* for Grade 3 + 4
Hematologic									
Leukopenia	98	32.9	21	7.0	97	32.3	14	4.7	.461
Neutropenia	90	30.2	136	45.6	79	26.3	133	44.3	.153
Febrile neutropenia	33	11.1	5	1.7	38	12.8	0	0	.297
Anemia	57	19.1	8	2.7	35	11.7	5	1.7	.006
Thrombocytopenia	32	10.7	0	0	18	6.0	1	0.3	.054
Nonhematologic									
Anorexia	43	14.4	0	0	33	11.0	2	0.7	.316
AST/ALT	11	3.7	1	0.3	32	10.7	0	0	.002
Constipation	25	8.4	0	0	20	6.7	1	0.3	.631
Creatinine	1	0.3	0	0	0	0	0	0	.315
Diarrhea	6	2.0	0	0	5	1.7	0	0	.152
Dyspnea	3	1.0	5	1.7	4	1.3	5	1.7	.816
Fatigue	22	7.4	7	2.3	18	6.0	4	1.3	.294
Hypersensitivity	1	0.3	1	0.3	2	0.7	2	0.7	.417
Infection	36	12.1	1	0.3	26	8.7	0	0	.135
Nausea	38	12.8	0	0	29	9.7	0	0	.232
Neuropathy									
Motor	5	1.7	1	0.3	4	1.3	1	0.3	.991
Sensory	12	4.0	1	0.3	7	2.3	0	0	.260
Performance status	27	9.1	8	2.7	23	7.7	9	3.0	.676
Pneumonitis (ILD)	2	0.7	0	0	4	1.3	0	0	.417
Rash	2	0.7	0	0	1	0.3	0	0	.559
Stomatitis/pharingitis	0	0	0	0	2	0.7	0	0	.482
Vomiting	12	4.0	1	0.3	15	5.0	2	0.7	.465

Abbreviation: ILD, interstitial lung disease.

these patients were more likely to respond to gefitinib. Patients who were nonsmokers with adenocarcinoma in arm A resulted in subsequent gefitinib therapy as well as in arm B.

Activating mutations in the gene for *EGFR* appear in a subset of adenocarcinoma of lung cancer.[24,25] A higher response to EGFR-TKIs is noted in specific subgroups that include females, never smokers, patients with adenocarcinoma histology, and East Asians.[12] Higher EGFR mutation rates are also noted in these subgroups and are also related to a better response to EGFR-TKIs[24,25] and longer survival.[12] Patients with these mutations exhibit objective response rates in the range of 75% to 95%.[12-14,26,27]

Patients included in this study were not selected on the basis of the target *EGFR* mutation status, because when this study was planned, we had not recognized the *EGFR* mutation as a predictive factor to respond to gefitinib. In Japanese patients with adenocarcinoma, a higher incidence of *EFGR* mutations, are estimated compared with white patients. It seems that more than 40% of Japanese patients with adenocarcinoma have an *EGFR* mutation.[12] Complex results in this study can be explained by analyzing the *EGFR* mutation status of participating patients. It may be important to select patients who are known to receive a clinical benefit with treatment using an EGFR-TKI.

In conclusion, this trial failed to meet the primary end point of OS in patients with advanced NSCLC. The exploratory subset analyses demonstrate a possible survival prolongation for sequential therapy of gefitinib, especially for patients with adenocarcinoma. Further investigations are warranted to confirm the best sequential therapy after platinum-based chemotherapy for patients with advanced NSCLC.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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CLINICAL INVESTIGATION

THE IMPACT OF RADIATION DOSE AND FRACTIONATION ON OUTCOMES FOR LIMITED-STAGE SMALL-CELL LUNG CANCER

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Purpose: To review the treatment outcomes of limited-stage small-cell lung cancer (LS-SCLC) patients and to compare the outcomes among three groups in which the total radiation doses were 45 Gy with accelerated hyperfractionation (AHF), <54 Gy with standard fractionation (SF), and ≥54 Gy with SF.
Methods and Materials: LS-SCLC patients that had been treated with chemoradiotherapy between 1997 and 2007 at Aichi Cancer Center Hospital were reviewed in this study. Of the 127 eligible patients, there were 37 patients in the AHF group, 29 in the SF <54 Gy group, and 61 in the SF ≥54 Gy group.
Results: Fifty-five patients (43%) were alive at the time of this analysis, and the median follow-up time of the surviving patients was 33 months. The median survival times were 30.0 months (95% confidence interval [CI] 16.3–43.7) for the AHF group, 14.0 months (CI 6.6–21.4) for the SF <54 Gy group, and 41.0 months (CI 33.9–48.1) for the SF ≥ 54 Gy group. As for the local control rates, and the overall and progression-free survival rates, all outcomes were significantly lower in the SF <54 Gy group than in the other two groups, although no significant difference was found between the AHF and SF ≥54 Gy groups.
Conclusions: These results suggest the importance of a high dose of radiation when using once-daily regimen. This study will support future prospective studies to establish optimal radiation doses and fractionation. © 2009 Elsevier Inc.

Small-cell lung cancer, Radiation therapy, Radiation dose, Fractionation, Accelerated hyperfractionation.

INTRODUCTION

Chemoradiotherapy is currently the standard treatment for limited-stage small-cell lung cancer (LS-SCLC) (1). Although thoracic radiotherapy (TRT) has been established as an integral component of the treatment platform for LS-SCLC, some questions regarding the optimal radiotherapy approach have also arisen. With regard to fractionation, Turrisi *et al.* determined that accelerated hyperfractionation (AHF) is superior to standard fractionation (SF) in an Intergroup Phase III study (2). However, despite the significant improvement in long-term survival, a pattern of care study found that only 10% of patients with LS-SCLC received a twice-daily regimen because of the inconvenience of twice-daily treatment sessions and the increased rate of severe esophageal toxicity seen with this regimen, whereas more than 80% received once-daily TRT (3). Although traditionally modest doses of TRT (45–50 Gy) are often used in once-daily 1.8- to 2-Gy fractions (4, 5), the optimal total dose for a once-daily regimen has not been proven. In addition, it is also still unclear whether twice-daily TRT of 45 Gy in 3 weeks is superior to a higher total dose than traditional modest doses delivered with a once-daily regimen. In this study, we reviewed the treatment outcomes of LS-SCLC patients that were treated with chemoradiotherapy at Aichi Cancer Center Hospital and compared the outcomes among three groups in which the total radiation doses were 45 Gy with a twice-daily regimen, less than 54 Gy with a once-daily regimen, and equal or greater than 54 Gy with a once-daily regimen.

METHODS AND MATERIALS

Patient selection

LS-SCLC patients that had been treated with chemoradiotherapy between 1997 and 2007 at Aichi Cancer Center Hospital and who met the eligibility criteria were enrolled into this retrospective study. The diagnosis of SCLC was confirmed by histologic or cytologic findings in all cases. Limited-stage was defined as disease confined to one hemithorax with or without bilateral supraclavicular node metastasis. The eligibility criteria consisted of no previous treatment and an Eastern Cooperative Oncology Group performance status

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