reniformis luciferase. The next day, cells were incubated in RPMI1640 with or without the inhibitors. After 16 or 48 hours, cells were lysed in Reporter Lysis Buffer (Promega), and luciferase activity was assessed using the Dual-Glo luciferase assay system (Promega) with an ARVO MX luminometer (PerkinElmer, Norwalk, CT).

TGF-β1, TGF-β2, and TGF-β3 Enzyme-Linked Immunosorbent Assay

Cells (3×10^5 cells/well) were seeded in 6-well plates and incubated for 30 hours. Then, the cells were incubated in freshly replaced FBS-free medium for an additional 60 hours, and the supernatant culture medium was collected and centrifuged. To quantify the amount of TGF-β1, TGF-β2 and TGF-β3 in the conditioned medium, a human TGF-β1 Quantikine ELISA kit, a human TGF-β2 Quantikine ELISA kit and a human TGF-β3 DuoSet kit were used, respectively. These ELISA kits were purchased from R&D Systems. Next, acid activation was performed according to the manufacturer's instructions. The obtained raw data were normalized by cell number.

Quantitative Real-Time Polymerase Chain Reaction

Cells (1×10^6 cells/dish) were seeded into 60-mm dishes (Corning Coaster) and incubated for 30 hours. After the cell-culture medium was replaced with FBS-free medium, cells were incubated for 12 hours in FBS-free medium. Total RNAs were isolated using an RNeasy Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. cDNA template synthesis was performed with total RNA (1 μg), Oligo(dT)$_{12-18}$ Primer (Invitrogen), and an Omniscript RT Kit (QIAGEN) according to the manufacturer's instructions. Quantitative real-time-polymerase chain reaction (PCR) amplification and detection were performed on the StepOnePlus Real time PCR system (Applied Biosystems, Foster City, CA) using SYBR Premix Ex TaqII (Tli RNaseH Plus) (TAKARA BIO, Shiga, Japan) and Perfect Real Time PCR primers for each gene (TAKARA BIO).

Small Interfering RNA Transfection

Cells (3×10^5 cells/well) were seeded into 6-well plates. After incubation for 48 hours, cells were transfected with 250 pmol/well of small interfering RNA (siRNA) targeting *SMAD2* (VHS41107) and *TGFB2* (HSS110687) or scramble control siRNA (Stealth RNAi Negative Control Low GC Duplex) using Lipofectamine 2000 according to the manufacturer's instructions. These siRNAs were purchased from Invitrogen.

RESULTS

EGFR TKI-Resistant PC-9ER Cells Exhibit Enhanced Motility

PC-9ER1 and PC-9ER4 cells generated for this study displayed resistance to erlotinib with approximately 100-fold higher 50% inhibitory concentration (IC_{50}) values than PC-9 cells (Fig. 1A, Supplementary Table S2, Supplemental Digital Content 1, http://links.lww.com/JTO/A361) and also displayed resistance to gefitinib (Fig. 1A, Supplementary Table S2, Supplemental Digital Content 1, http://links.lww.com/JTO/A361). Acquired resistance to EGFR TKIs in PC-9ER cells was not caused by previously reported mechanisms, such as the EGFR T790M mutation (Supplementary Fig. S1A, Supplemental Digital Content 2, http://links.lww.com/JTO/A362),[7] KRAS mutations around codons 12, 13, and 61 (Supplementary Fig. S1A, Supplemental Digital Content 2, http://links.lww.com/JTO/A362),[8] *MET* amplification (Supplementary Fig. S4),[9] phosphatase and tensin homolog (PTEN) loss (Supplementary Fig. S1B, Supplemental Digital Content 2, http://links.lww.com/JTO/A362),[12] IGF-1Rβ activation (data not shown),[10] or hepatocyte growth factor (HGF) overexpression (data not shown).[11] In PC-9ER cells, erlotinib suppressed the phosphorylation of EGFR and extracellular signal-regulated kinases 1/2 (ERK 1/2), but not that of Akt and S6 (Supplementary Fig. S1B, Supplemental Digital Content 2, http://links.lww.com/JTO/A362). These results suggest that persistent activation of phosphatidylinositol-3-kinase (PI3K)/v-akt murine thymoma viral oncogene homolog (Akt) pathway may confer resistance to EGFR TKIs in PC-9ER cells.

PC-9ER cells underwent a distinct morphological change to an epithelial cobblestone-like phenotype, whereas PC-9 parental cells exhibited an elongated mesenchymal-like morphology (Fig. 1B). We then compared the motility of PC-9 and PC-9ER cells and found that PC-9ER cells migrated faster to close the wound than PC-9 cells did, and enhanced cell migration was also confirmed by the transwell assay (Fig. 1C). Enhanced motility is frequently observed in mesenchymal cells that have undergone an EMT.[25] However, PC-9ER cells lost vimentin (mesenchymal marker) expression but retained E-cadherin (epithelial marker) expression, thus suggesting that the enhanced cell motility of PC-9ER cells is independent of EMT (Supplementary Fig. S1B, Supplemental Digital Content 2, http://links.lww.com/JTO/A362).

TGF-β/Smad Pathway is Activated in PC-9ER Cells

Next, we examined the status of the TGF-β/Smad pathway, which plays an important role in cell motility and migration. Enzyme-linked immunosorbent assay (ELISA) was performed to quantify the production of TGF-β ligands secreted in an autocrine manner in the culture medium. PC-9ER cells were found to secret significantly higher amounts of TGF-β2, but not TGF-β1 or TGF-β3, than PC-9 cells did (Fig. 2A; the amount of TGF-β3 was below the detection limit in all cell lines). TGF-β2 mRNA levels in PC-9ER cells were approximately 10-fold higher than those in PC-9 cells (Fig. 2B), whereas the difference in TGF-β1 mRNA levels was not statistically significant. These results indicated that the higher levels of TGF-β2 secretion observed in PC-9ER cells were caused by an increase in TGF-β2 mRNA expression. Massive phosphorylation of Smad2, which is a downstream effector of the TGF-β pathway, was detected in PC-9ER cells (Fig. 2C). Subsequent Smad-mediated transcriptional regulatory activity was also significantly enhanced in PC-9ER cells according to a transcriptional reporter assay (Fig. 2C, Supplementary Fig. 2A, Supplemental Digital Content 3, http://links.lww.com/JTO/A363).

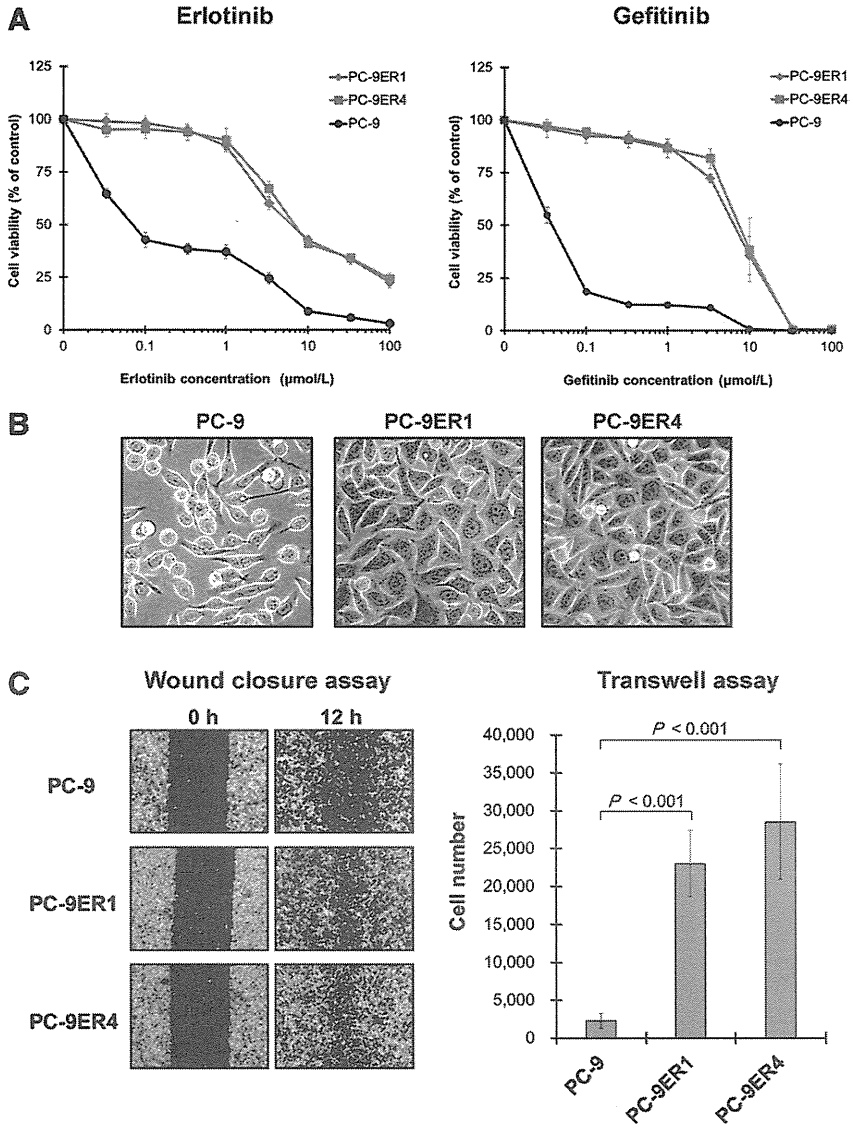

FIGURE 1. Erlotinib-resistant PC-9ER cells exhibit enhanced cell motility. *A*, PC-9 parental cells and erlotinib-resistant PC-9ER1 and PC-9ER4 cells were exposed to erlotinib or gefitinib at the indicated concentrations, and the viability of cells was measured after 72 hours of treatment by using the MTT assay. *B*, Phase-contrast microscopy of PC-9 and PC-9ER cells. *C*, Wound closure was monitored 12 hours after scraping (left panel). Cells that migrated through the transwell filter and attached to the bottom of the lower chamber were trypsinized and counted 24 hours after cell seeding. The error bars indicate SDs of the mean (right panel). MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium.

These results indicate that the TGF-β/Smad pathway is activated in PC-9ER cells.

Silencing of TGF-β2 or Smad2 Suppresses the Enhanced Motility of PC-9ER Cells

To confirm the involvement of the TGF-β/Smad pathway in the enhanced motility of PC-9ER cells, we depleted TGF-β2 and Smad2 with specific siRNAs targeting each gene. Smad2 siRNA completely suppressed Smad2 expression and Smad2 phosphorylation in PC-9ER cells (Fig. 3A). TGF-β2-siRNA significantly suppressed Smad2 phosphorylation and TGF-β2 secretion in PC-9ER cells (Fig. 3A and B). Silencing TGF-β2 or Smad2 also attenuated the enhanced motility of PC-9ER cells (Fig. 3C), confirming that TGF-β/Smad pathway activation and the increased secretion of TGF-β2 contribute to the enhanced motility of PC-9ER cells.

Stimulation of PC-9 Cells with TGF-β2 Induces an Increase in Motility and Activates the TGF-β/Smad Pathway

To further investigate whether the increased secretion of TGF-β2 contributed to the enhanced motility of PC-9ER

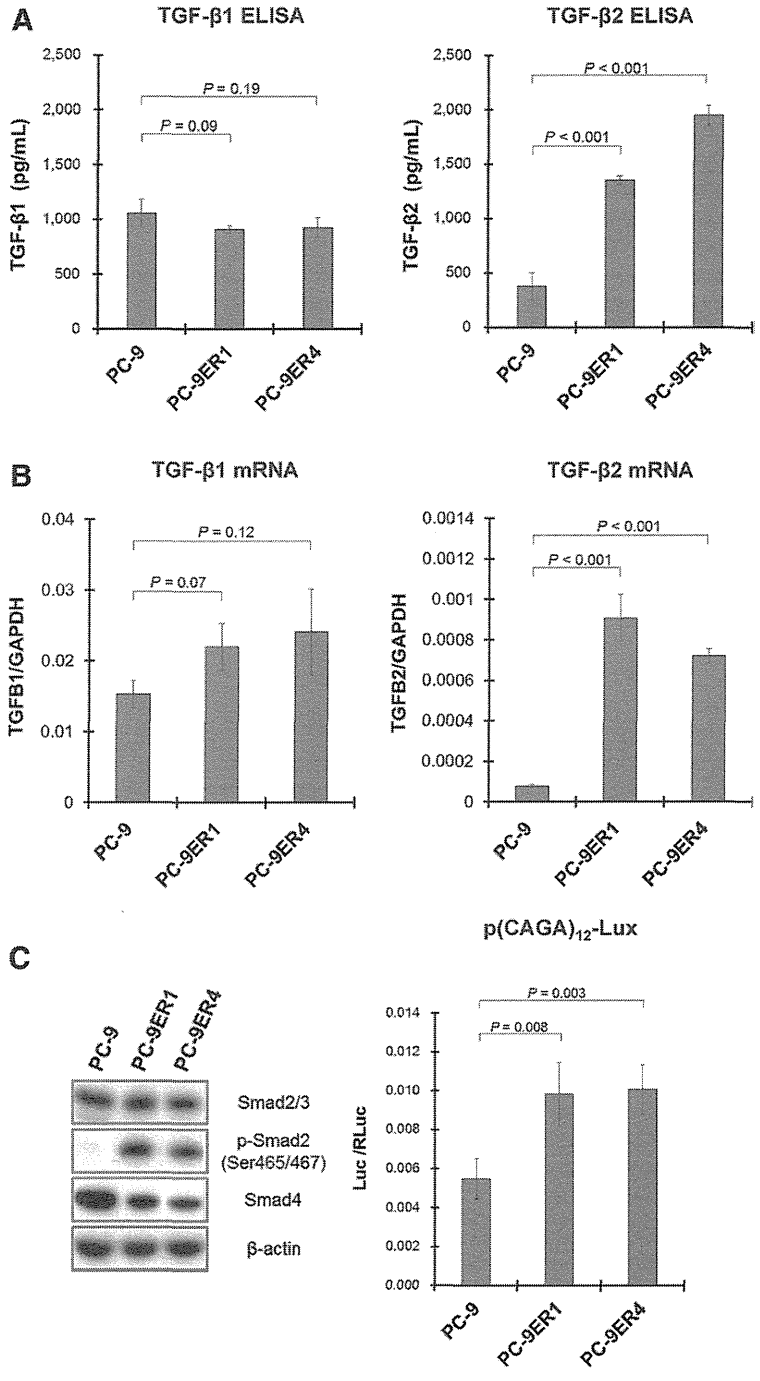

FIGURE 2. The TGF-β/Smad signaling pathway is activated in PC-9ER cells. *A*, Cells were seeded and incubated in 6-well plates for 60 hours in FBS-free medium. Conditioned medium was collected, and the total TGF-β1 and TGF-β2 levels were measured using enzyme-linked immunosorbent assay. The error bars indicate SDs of the mean. *B*, The cellular mRNA levels of TGF-β1 and TGF-β2 were measured using quantitative real-time polymerase chain reaction. The error bars indicate SDs of the mean. *C*, Immunoblot analysis of total Smad2/3, phospho-Smad2, and total Smad4 (left panel). A luciferase assay was performed to assess the Smad-mediated transcriptional regulatory activity by using the Smad-dependent reporter $p(CAGA)_{12}$-Lux. The values were normalized relative to the *Renilla* luciferase activity of a cotransfected pRL-CMV plasmid. The error bars indicate SDs of the mean (right panel). TGF, transforming growth factor beta; FBS, fetal bovine serum.

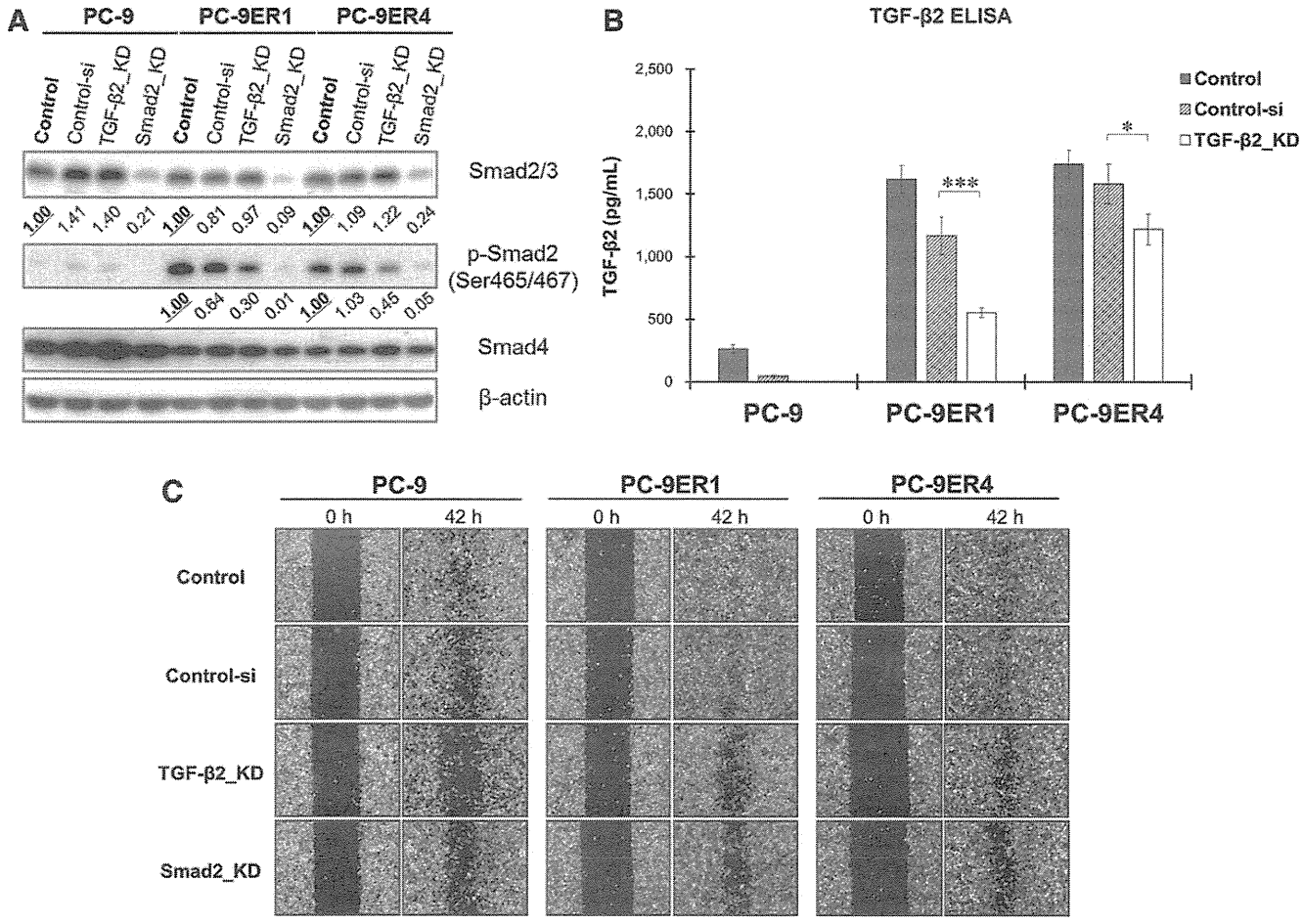

FIGURE 3. Depletion of TGF-β2 and Smad2 suppresses the enhanced motility of PC-9ER cells. *A*, After transfecting negative-control, TGF-β2-specific, or Smad2-specific siRNA into the cells, the cells were washed and incubated with FBS-free medium for 24 hours. Cell lysates were immunoblotted to detect the indicated proteins. The relative value of each band to the control value according to densitometric analysis is indicated. *B*, After transfecting negative-control or TGF-β2-specific siRNA into the cells, the cells were washed and incubated with FBS-free medium for 24 hours. TGF-β2 enzyme-linked immunosorbent assay was performed according to the manufacturer's instructions. $*p < 0.05$; $***p < 0.001$ (Student's *t* test). The error bars indicate SDs of the mean. *C*, After transfecting negative-control, *TGFB2*-specific, or *SMAD2*-specific siRNA into the cells, the cells were washed and incubated with FBS-free medium for 8 hours. Then, a wound was made with a 200-μl pipette tip, and the cells were incubated with FBS-free medium for 42 hours. TGF, transforming growth factor beta; siRNA, small interfering RNA; FBS, fetal bovine serum.

cells, we examined the effect of TGF-β2 stimulation on the motility and TGF-β/Smad pathway activity of PC-9 cells. TGF-β2 stimulation increased cell motility and Smad2 phosphorylation, which were abrogated by the addition of LY364947 (TGF-βRI inhibitor) (Fig. 4A and B). Smad-mediated transcriptional regulatory activity was also significantly induced by TGF-β2 stimulation, and it was suppressed by LY364947 (Fig. 4C, Supplementary Fig. S2B, Supplemental Digital Content 3, http://links.lww.com/JTO/A363). Surprisingly, erlotinib alone attenuated the cell motility induced by TGF-β2, suggesting potential crosstalk between the EGFR and TGF-β/Smad pathways (Fig. 4A). Indeed, modest erlotinib-induced Smad2 phosphorylation and subsequent Smad-mediated transcriptional regulatory activity were observed (Fig. 4B and C), which may be a compensatory response to EGFR pathway inhibition.

Combined Treatment with Erlotinib and LY364947 Abrogates the Enhanced Motility of PC-9ER Cells

We next examined combined blockade of the EGFR and TGF-β/Smad pathways. LY364947 reduced the motility of PC-9ER cells, whereas it had almost no effect on PC-9 parental cells (Fig. 5A). Erlotinib not only effectively abolished the motility of PC-9 cells, but also suppressed the motility of PC-9ER cells (Fig. 5A), suggesting that PC-9ER cells still retain partial sensitivity to erlotinib in terms of cell

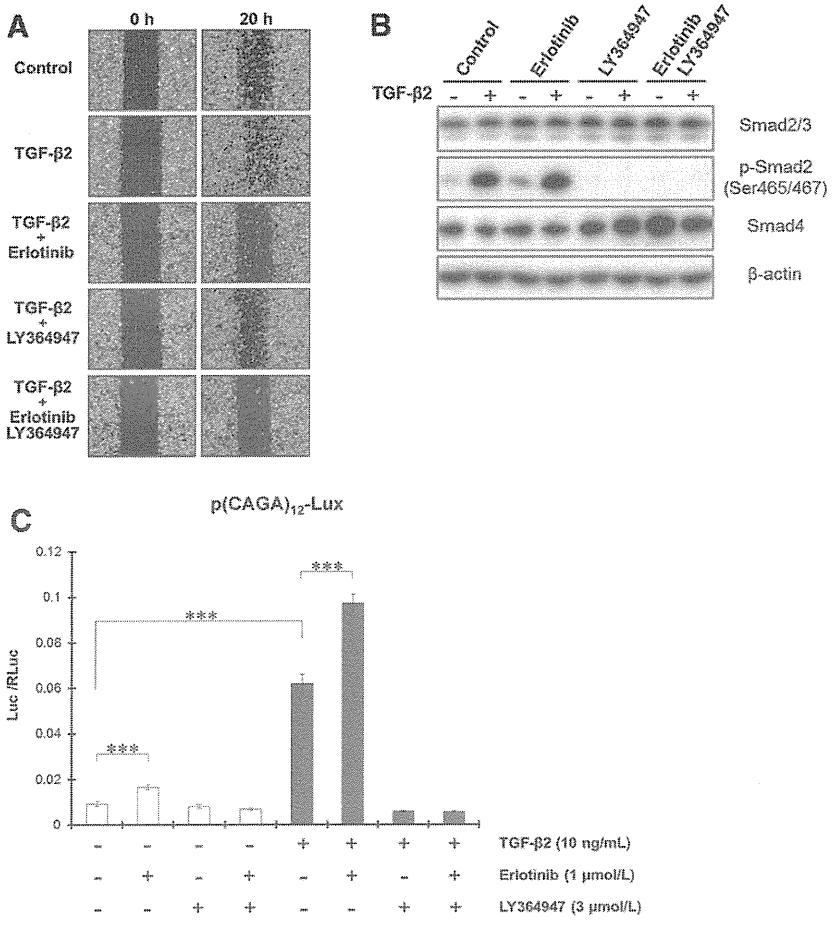

FIGURE 4. Exogenous TGF-β2 stimulation of PC-9 parental cells. *A*, PC-9 cells were grown on 6-well plates to 100% confluence, and then a wound was made with a 200-μl pipette tip, after which the cells were washed with FBS-free medium and incubated with FBS-free medium with or without TGF-β2 (10 ng/m;) in the absence or presence of erlotinib (1 μmol/l), LY364947 (3 μmol/l), or erlotinib and LY364947 in combination for 20 h. *B*, Cells were FBS-starved for 16 hours, pretreated with erlotinib (1 μmol/l), LY364947 (3 μmol/l), or erlotinib and LY364947 in combination for 3 hours, and stimulated for 1 hour with TGF-β2 (10 ng/ml). Cell lysates were immunoblotted to detect the indicated proteins. *C*, After transfecting the Smad-dependent reporter $p(CAGA)_{12}$-Lux into PC-9 cells, the cells were incubated for 16 hours in 10% FBS medium with or without TGF-β2 (10 ng/ml) in the absence or presence of erlotinib (1 μmol/l), LY364947 (3 μmol/l), or erlotinib and LY364947 in combination. The values were normalized relative to the *Renilla* luciferase activity of a cotransfected pRL-CMV plasmid. ***$p < 0.001$ (Student's *t* test). The error bars indicate SDs of the mean. TGF, transforming growth factor beta; FBS, fetal bovine serum.

motility, and that continuous treatment with erlotinib may be beneficial in preventing metastasis even after the failure of EGFR TKI monotherapy, especially in the absence of the EGFR T790M resistance mutation. Combined treatment with erlotinib and LY364947 suppressed the motility of PC-9ER cells more effectively than did each treatment alone (Fig. 5A). These findings were also confirmed by a transwell assay (Fig. 5B). LY364947 abrogated phospho-Smad2 expression and attenuated phospho-ERK1/2 expression in PC-9ER cells (Fig. 5C, Supplementary Fig. S3, Supplemental Digital Content 4, http://links.lww.com/JTO/A364). Erlotinib also significantly reduced phospho-ERK1/2 expression but did not affect phospho-Akt expression in PC-9ER cells (Fig. 5*C*). The combination of the two inhibitors completely suppressed the phosphorylation of both ERK1/2 and Smad2 in PC-9ER cells (Fig. 5*C*). These results suggest that the ERK1/2 pathway is prominently involved in the motility of PC-9ER cells. LY364947, either alone or in combination with erlotinib, did not suppress the persistent activation of the PI3K/Akt pathway, which may confer resistance to EGFR TKIs in PC-9ER cells (Fig. 5*C*), and did not affect the viability of PC-9ER cells or their sensitivity to EGFR TKIs (Fig. 5*D*). These results suggest that motility and growth are driven by different mechanisms in PC-9ER cells.

DISCUSSION

It is widely known that acquired resistance to EGFR TKIs eventually emerges in patients with EGFR-mutant lung tumors after treatment,[7–14] and it is not surprising when those tumors acquire additional biological features simultaneously, which may lead to a more aggressive/malignant phenotype. In this study, we demonstrated that PC-9ER cells acquired enhanced motility as an additional phenotypical and biological characteristic when they acquired resistance to EGFR TKIs such as erlotinib. Our findings shed light on the additional biological changes that occur when tumors become resistant to EGFR TKIs. Proposed mechanisms responsible for the acquired resistance to erlotinib and the enhanced motility of PC-9ER cells are indicated in Supplementary Fig. S5 (Supplemental Digital Content 5, http://links.lww.com/JTO/A365). We showed that PC-9ER cells acquired constitutive TGF-β/Smad pathway activation because of an increase in TGF-β2 mRNA expression and the subsequent increased secretion of TGF-β2 (Fig. 2, Supplementary Fig. S5A, Supplemental Digital Content 5, http://links.lww.com/JTO/A365), which resulted in enhanced cell motility (Fig. 1*C*, Supplementary Fig. S5A, Supplemental Digital Content 5, http://links.lww.com/JTO/A365). This phenomenon is consistent with the result that erlotinib treatment induced

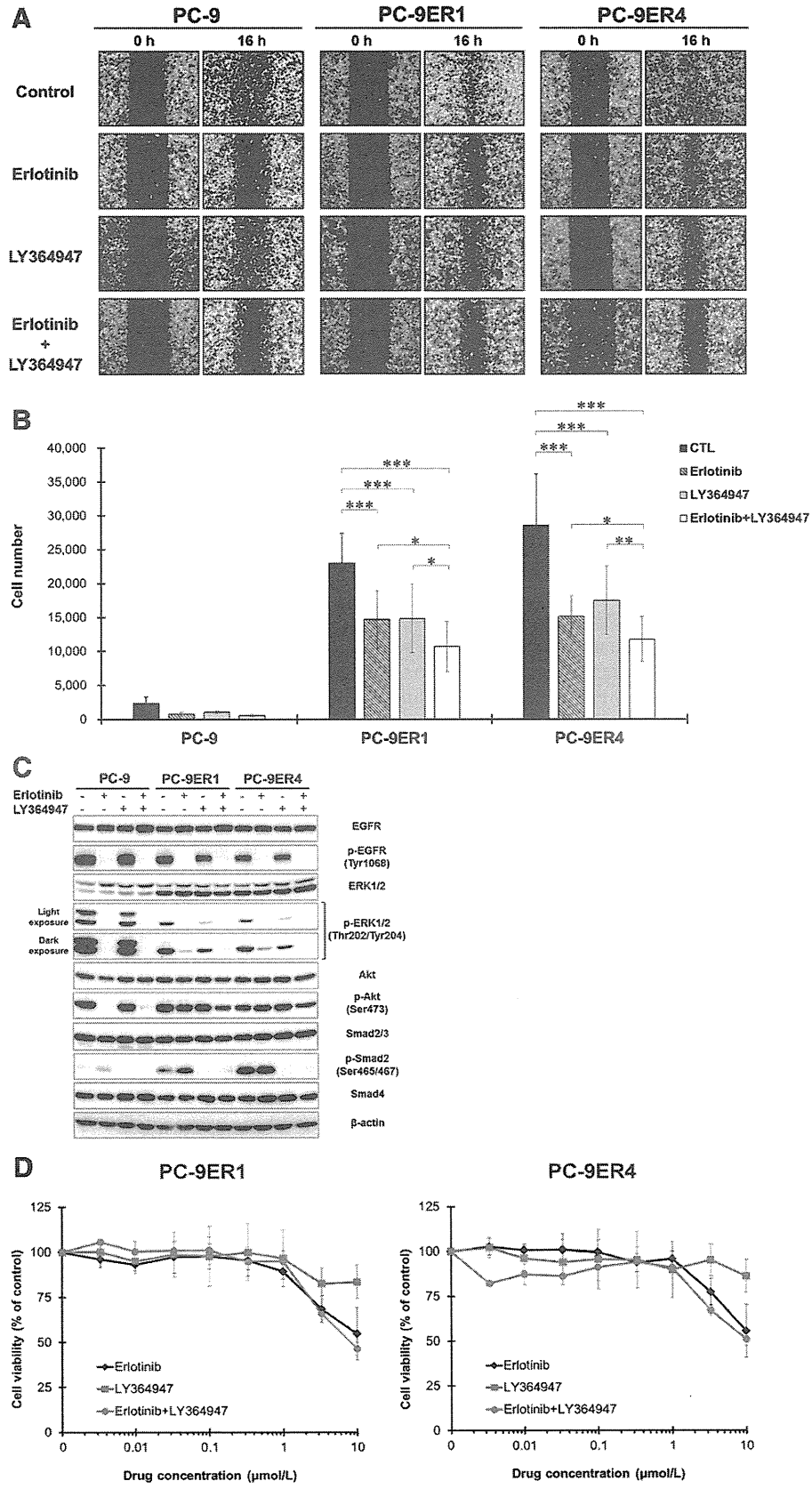

FIGURE 5. Combined blockade of the EGFR and TGF-β signaling pathways. *A*, Cells were seeded and grown to 100% confluence followed by scraping with a 200-μl pipette tip and incubation for 16 hours with 1% FBS medium with erlotinib (1 μmol/l), LY364947 (3 μmol/l), or erlotinib and LY364947 in combination. *B*, Cells were seeded into transwell chambers and incubated with 1% FBS medium in the absence or presence of erlotinib (1 μmol/l), LY364947 (3 μmol/l), or erlotinib and LY364947 in combination. The number of cells that migrated through the filter and attached to the bottom of the lower chamber was counted 24 hours after cell seeding. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ (Student's *t* test). The error bars indicate SDs of the mean. *C*, PC-9 and PC-9ER cells were treated with erlotinib (1 μmol/l), LY364947 (3 μmol/l), or erlotinib and LY364947 in combination for 12 hours. Cells were lysed, and the indicated proteins were detected using immunoblotting. *D*, PC-9ER cells were exposed to erlotinib, LY364947, or erlotinib and LY364947 in combination at the indicated concentrations. The viability of cells was measured after 72 hours of treatment by using the MTT assay. EGFR, epidermal growth factor receptor; TGF, transforming growth factor beta; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium.

Smad2 phosphorylation in parental PC-9 cells (Figs. 4*B* and 5*C*, Supplementary Fig. S5B, Supplemental Digital Content 5, http://links.lww.com/JTO/A365), suggesting that compensatory signaling and crosstalk occur between the EGFR and TGF-β/Smad pathways in these cells (Supplementary Fig. S5, Supplemental Digital Content 5, http://links.lww.com/JTO/A365). On the basis of the results of this study, we propose a strategy for the combined blockade of the EGFR and TGF-β/Smad signaling pathways and a putative biological explanation for this phenomenon (Supplementary Fig. S5D, Supplemental Digital Content 5, http://links.lww.com/JTO/A365). There have been several reports regarding the crosstalk between EGFR family members and the TGF-β pathway. TGF-β1 has been reported to activate EGF signaling in hepatocytes by promoting the shedding of EGF-like ligands and phosphorylation of Src.[26] It has also been reported that human epidermal growth factor receptor type 2 (HER2) overexpression in the nontumorigenic mammary epithelium is permissive of the ability of TGF-β to induce cell motility, and HER2 and TGF-β signaling cooperate in the induction of cellular events associated with tumor progression.[27] Linkage of ERK activation by TGF-β with angiogenesis was previously reported.[28] Our results indicate that Ras-mitogen-activated protein/extracellular signal-regulated (MEK)-ERK signaling is involved in both the EGFR and TGF-β pathways and that this signaling needs to be repressed to abrogate the enhanced motility of PC-9ER cells (Supplementary Fig. S5D, Supplemental Digital Content 5, http://links.lww.com/JTO/A365).

EMT is known to play an important role in cell motility, migration, and invasion, which potentially lead to metastasis.[25,29] Cells with a mesenchymal phenotype tend to have a greater motility, but this is not a universal finding; 4T1 breast cancer cells displayed high metastatic properties even though they retained an epithelial phenotype according to their EMT marker profile.[30] Meanwhile, 67NR breast cells exhibiting nonmetastatic properties displayed a mesenchymal phenotype based on their EMT marker profile.[30] These reports suggest that EMT is not the only mechanism that promotes cell motility; other mechanisms may also play pivotal roles in enhancing the metastatic potential of cancer cells.[30,31] In our study, PC-9ER cells exhibited a more epithelial-like phenotype according to their morphology and the presence of EMT markers such as vimentin and E-cadherin (Fig. 1*B*, Supplementary Fig. S1B, Supplemental Digital Content 2, http://links.lww.com/JTO/A362), and it is clear that the enhanced motility of PC-9ER cells is not caused by EMT, although TGF-β signaling activation is the driving force.

It was reported that TGF-β ligand-induced activation of the TGF-β pathway in erlotinib-resistant NSCLC cells (H1650-M3) led to both EMT, resulting in enhanced motility, and the development of resistance to erlotinib.[32] However, NCI-H1650 parental cells exhibit intrinsic resistance to EGFR TKIs because of C-terminal deletion of the *PTEN* gene,[12,33] and consequently, the biological nature of H1650-M3 cells is not necessarily comparable with that of PC-9ER cells, which originally harbor an EGFR-activating mutation. PC-9ER cells did not exhibit morphological and molecular evidence of EMT (Fig. 1*B*, Supplementary Fig. 1B, Supplemental Digital Content 2, http://links.lww.com/JTO/A362), and blockade of TGF-β signaling by LY364947 did not restore the sensitivity of PC-9ER cells to erlotinib (Fig. 5*D*). TGF-β/Smad signaling pathway activation in PC-9ER cells does not confer resistance to erlotinib, and this pathway is only involved in enhanced cell motility, unlike the H1650-M3 cells reported by Yao et al.[32] These two resistance models may represent two different possibilities accounting for acquired resistance to EGFR TKIs in the clinical setting.

It is noteworthy that TGF-β2 was up-regulated and responsible for the subsequent activation of TGF-β/Smad signaling in PC-9ER cells that resulted in enhanced cell motility instead of TGF-β1, which is the major TGF-β ligand and is well known to play a prominent role in TGF-β/Smad signaling (Supplementary Fig. S5A, Supplemental Digital Content 5, http://links.lww.com/JTO/A365).[17] Little has been reported on how TGF-β ligands such as TGF-β1, TGF-β2, or TGF-β3 play differential roles and their relevance to clinical outcome. Tsamandas et al.[34] reported that among all TGF-β ligands, TGF-β2 seemed to be most involved in tumor progression and related with a poorer prognosis in patients with advanced-stage colon cancer. There has been no report regarding the correlation between TGF-β2 and tumor progression and/or metastasis in NSCLC, and this is the first report on the involvement of TGF-β2 in the metastatic process in NSCLC. The differential roles of TGF-β ligands in lung cancer biology and their relationship with EGFR TKI resistance need to be further studied.

Reports suggest that continuous treatment with EGFR TKIs after disease progression based on Response Evaluation Criteria in Solid Tumors criteria is associated with better survival and that this treatment results in the stabilization and/or improvement of symptoms and a reduction in tumor size.[35,36] Moreover, the accelerated progression of disease after discontinuation of EGFR TKIs, termed a disease flare, during the washout period has been reported,[37] and the use of EGFR TKIs after disease progression is under clinical evaluation.[38–40] These clinical findings seem to support the hypothesis that tumors with acquired resistance and/or nonresistant residual tumors still rely on EGFR signaling and that blockade of EGFR signaling via continuous treatment with EGFR TKIs may be still beneficial. EGFR signaling is also known to play a role in cell motility.[41,42] In this study, we report an additional rationale for continuing the use of EGFR TKIs after disease progression in that resistant tumors may retain sensitivity to EGFR TKIs in terms of the inhibition of cell motility (Supplementary Fig. S5 B, Supplemental Digital Content 5, http://links.lww.com/JTO/A365). In addition, as demonstrated in this study, the enhanced motility of PC-9ER cells was effectively suppressed by the combination of erlotinib and LY364947 (Fig. 5*A*, *B*, Supplementary Fig. S5 D, Supplemental Digital Content 5, http://links.lww.com/JTO/A365).

Taken together, these data indicate that combined blockade of the EGFR and TGF-β pathways will be beneficial in preventing metastasis in patients with EGFR TKI–resistant NSCLC, although these results need to be further validated

using in vivo models and clinical specimens from patients with acquired resistance to EGFR TKIs.

ACKNOWLEDGMENTS

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ORIGINAL ARTICLE

Phase I and pharmacokinetic study of gefitinib and S-1 combination therapy for advanced adenocarcinoma of the lung

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Abstract
Background A phase I dose-escalation study was performed to investigate the safety and pharmacokinetics of the combination of S-1 and gefitinib in patients with pulmonary adenocarcinoma who had failed previous chemotherapy.
Methods Patients received gefitinib at a fixed daily oral dose of 250 mg, and S-1 was administered on days 1–14 every 21 days at doses starting at 60 mg/m^2 (level 1) and escalating to 80 mg/m^2 (level 2). The primary end point of the study was determination of the recommended dose for S-1 given in combination with a fixed dose of gefitinib.
Results Twenty patients were enrolled in the study. Two of the first six patients at dose level 2 experienced a dose-limiting toxicity (elevation of alkaline phosphatase of grade 3 in one patient; elevations of aspartate and alanine aminotransferases of grade 3 in the other). The recommended dose was thus determined as level 2, and an additional 11 patients were assigned to this level. All observed adverse events were well managed. The response rate was 50 % (10 of 20 patients), and the median progression-free survival (PFS) and overall survival times were 10.5 and 21.2 months, respectively. In *EGFR* mutation–positive patients ($n = 9$), seven patients achieved an objective response and the median PFS was 12.4 months, whereas none with wild-type *EGFR* ($n = 6$) responded. No pharmacokinetic interaction between S-1 and gefitinib was detected.
Conclusions The combination of S-1 and gefitinib is well tolerated and appears to possess activity against *EGFR* mutation–positive NSCLC.

Keywords Gefitinib · S-1 · Non-small-cell lung cancer · Epidermal growth factor receptor · Phase I study

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Introduction

Gefitinib was the first molecularly targeted agent to become clinically available for the treatment of non-small-cell lung cancer (NSCLC). Somatic activating mutations of *EGFR* have been identified as a major determinant of the clinical response to treatment with gefitinib, with achievement of a clinical benefit with this drug in NSCLC patients with wild-type *EGFR* having been problematic [1, 2]. Furthermore, despite the therapeutic efficacy of gefitinib for patients with *EGFR* mutation–positive NSCLC, most such patients ultimately develop resistance to the drug. The development of combination therapy with gefitinib and other chemotherapeutic agents is being pursued in an attempt to improve treatment efficacy.

S-1 is an oral fluorinated pyrimidine formulation that combines tegafur (FT), 5-chloro-2,4-dihydroxypyridine (CDHP), and oxonic acid (Oxo) in a molar ratio of 1:0.4:1 [3]. FT is a prodrug that generates 5-fluorouracil (5-FU) in blood largely as a result of its metabolism by cytochrome

P450 in the liver. CDHP increases the plasma concentration of 5-FU through competitive inhibition of dihydropyrimidine dehydrogenase, which catalyzes 5-FU catabolism. CDHP also attenuates the indirect cardiotoxic and neurotoxic effects of 5-FU by reducing the production of fluoro-β-alanine, the main catabolite of 5-FU. Oxo reduces the gastrointestinal toxicity of 5-FU. After its oral administration, Oxo becomes distributed selectively to the small and large intestine, where it inhibits the phosphorylation of 5-FU to fluoropyrimidine monophosphate catalyzed by orotate phosphoribosyltransferase within gastrointestinal mucosal cells, thereby reducing the incidence of diarrhea [4]. S-1 has shown promising antitumor activity as a single agent for the treatment of advanced NSCLC as well as a good safety profile with manageable toxicities [5]. Furthermore, we recently presented the results of a phase III trial showing that S-1 in combination with carboplatin is not less efficacious and is better tolerated than carboplatin–paclitaxel, a representative platinum-based doublet chemotherapy for first-line treatment of advanced NSCLC [6].

We have previously shown that the combination of S-1 and gefitinib has a synergistic antiproliferative effect on NSCLC cells regardless of the absence or presence of *EGFR* mutations and that this enhanced antitumor effect is mediated by gefitinib-induced down-regulation of thymidylate synthase, a major target of 5-FU [7]. The combination of S-1 and gefitinib also exerted a synergistic antitumor effect in gefitinib-resistant cells with *MET* amplification both in vitro and in vivo, suggesting that such combination therapy is a promising strategy to overcome gefitinib resistance [8]. On the basis of these preclinical data, we have performed a phase I trial to assess the safety–tolerability, pharmacokinetics, and antitumor efficacy of the combination of gefitinib and S-1 in patients with advanced adenocarcinoma of the lung.

Patients and methods

Patient selection

Eligible patients had a confirmed histological or cytological diagnosis of adenocarcinoma of the lung that was either recurrent or stage IIIB or IV; had failed at least one prior systemic anticancer regimen including one platinum-based regimen (up to two regimens allowed); had not previously received therapy with an EGFR-TKI or S-1; and had adequate organ function (hemoglobin level $\geq$9.0 g/dl, neutrophil count $\geq$1,500/mm^3, platelet count $\geq$100,000/mm^3, total bilirubin level $\leq$1.5 mg/dl, aspartate (AST) and alanine (ALT) aminotransferase levels of $\leq$100 IU/l, saturation of peripheral O_2 $\geq$90 %, serum creatinine concentration $\leq$1.2 mg/dl, and predicted creatinine clearance or 24-h creatinine clearance $\geq$60 ml/min as estimated by the Cockcroft and Gault formula [9]). The study protocol was approved by the institutional review board at each participating center, and the study was conducted in accordance with the guidelines of the Declaration of Helsinki. All patients provided written informed consent before study-related procedures were performed. This trial was registered at the UMIN Clinical Trials Registry (UMIN 000001594).

Study design

Patients received a fixed daily dose of gefitinib (250 mg) for an initial period of 14 days followed by continuous daily administration of gefitinib and the administration of S-1 for 14 consecutive days every 21 days until disease progression or development of intolerable toxicity. The dose level of S-1 was set at 40 mg/m^2 (level 0), 60 mg/m^2 (level 1), or 80 mg/m^2 (level 2), with the dose escalation following a traditional 3 + 3 phase I trial design. The dose escalation–reduction scheme was based on the occurrence of a drug-related dose-limiting toxicity (DLT) within the first treatment course. A DLT was defined as a toxicity occurring in cycle 1 that met one of the following criteria: neutropenia of grade 4 persisting for $\geq$7 days, febrile neutropenia, thrombocytopenia of grade 4, or a nonhematologic toxicity (with the exception of nausea, vomiting, or anorexia) of grade 3. A delay of >2 weeks in administering the second treatment cycle was also considered a DLT. The maximum tolerated dose (MTD) was defined as the highest dose level at which $\leq$33 % of the patients experienced a DLT during the first treatment cycle. After the MTD had been determined, the corresponding cohort was to be expanded to a maximum of 20 patients for a more complete assessment of the safety and tolerability of the dose level. At least 14 patients were to be treated at the recommended dose. The probability of adverse events (AEs) with an incidence of $\geq$20 % not being detected in any of the 14 patients was 4.4 %.

If a DLT was not observed in any of the first three patients in the first cohort (level 1), an escalated dose of S-1 (80 mg/m^2) was administered to the first three patients at level 2. If a DLT was observed in one or two of the first three patients, an additional three patients were enrolled to assess the tolerability of this dose level. If a DLT occurred in one or two of the six patients at level 1, the dose of S-1 was escalated (to 80 mg/m^2). If three or more of the six patients at level 1 experienced a DLT, additional patients were recruited at level 0. In addition to this dose escalation–reduction scheme, if the investigators and an independent data-monitoring committee agreed that additional patients were necessary to confirm the dose escalation–

reduction decision in cases in which two or more patients experienced DLTs that were not life-threatening and were reversible and manageable with or without medication, then the entry of additional patients at that dose level was allowed.

Pharmacokinetics

The plasma pharmacokinetics of single-agent and combination treatments were investigated in the dose-escalation phase of the study in order to assess the potential for interaction between gefitinib and S-1. The pharmacokinetics of gefitinib were evaluated for 2 days (day 14 of the run-in period of administration of gefitinib alone and day 1 of combination therapy with gefitinib and S-1), and those of S-1 were examined on the first day of combination therapy with gefitinib and S-1. The plasma concentration of gefitinib was measured by Shin Nippon Biomedical Laboratories (Wakayama, Japan). The plasma concentrations of S-1 components (FT, CDHP, and Oxo) and 5-FU were measured by FALCO Biosystems (Kyoto, Japan). All concentrations were determined with the use of liquid chromatography and tandem mass spectrometry [10].

Efficacy measures

All patients underwent a comprehensive baseline assessment including clinical laboratory tests and imaging studies. Toxicity evaluations were based on the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE) version 3.0. Computed tomography scans were obtained every 6 weeks for the first 3 months and every 2 months thereafter. Response was evaluated according to RECIST 1.0. Progression-free survival (PFS) was calculated from the first day of combination therapy with gefitinib and S-1 until the first occurrence of progression, death from any cause, or last follow-up. Overall survival (OS) was calculated from the first day of the combination therapy until death from any cause or the date of last contact. The probability of survival as a function of time was estimated with the Kaplan–Meier method.

Results

Patient characteristics

Between July 2008 and April 2010, twenty patients with advanced adenocarcinoma of the lung were enrolled in the study at the three participating centers. The characteristics of the 20 study patients are summarized in Table 1. The patients included 12 (60 %) women and 10 (50 %) never-smokers. All had adenocarcinoma, and 8 (40 %) had disease of stage IV. The median age was 61 years, with a range of 51–70 years. Thirteen (65 %) of the 20 patients had received one prior chemotherapy regimen, whereas 7 individuals (35 %) had been treated with two prior regimens. Samples from 15 patients were available for *EGFR* mutational analysis, with such mutations being detected in 9 patients [the L858R point mutation in 5 patients (56 %) and exon-19 deletions in 4 patients (44 %)].

Determination of recommended dose

No DLTs were apparent for the first three patients treated at dose level 1, and so three patients were entered at dose level 2 (Table 2). Two of these latter three patients experienced a DLT [alkaline phosphatase (ALP) increase in grade 3 in one patient; AST and ALT increases in grade 3 in the other] in the first cycle, and an additional three patients were therefore treated at dose level 2. None of these three additional patients experienced a DLT. According to the protocol definition, dose level 2 was determined as the recommended dose, and an additional 11 patients were assigned to this level. A total of 17 patients were therefore treated at dose level 2.

Table 1 Characteristics of the study patients ($n = 20$), the median age of whom was 61 years (range 51–70 years)

Characteristics	No. of patients
Sex	
Male	8 (40 %)
Female	12 (60 %)
Performance status (ECOG)	
0	4 (20 %)
1	16 (80 %)
Disease stage	
IIIB	8 (40 %)
IV	8 (40 %)
Postoperative recurrence	4 (20 %)
No. of previous chemotherapies	
1	13 (65 %)
2	7 (35 %)
EGFR mutation	
Positive (L858R, exon-19 deletion)	9 (45 %)
Negative	6 (30 %)
Unknown (not examined)	5 (25 %)
Smoking history (pack-years)	
0	10 (50 %)
1–19	4 (20 %)
≥20	6 (30 %)

ECOG Eastern Cooperative Oncology Group

Table 2 Dose-escalation scheme and dose-limiting toxicities (DLTs)

Level	Gefitinib (mg/body)	S-1 (mg/m^2)	No. of patients		Type of DLT
			Total	DLT in first course	
1	250	60	3	0	
2	250	80	6	2	ALP increase; AST/ALT increases

Safety

A total of 144 cycles of chemotherapy was administered, with a median of 6 treatment cycles per patient (range 1–19). The major AEs during the entire treatment period are shown in Table 3. The most frequent (≥50 %) AEs were anemia, rash, hyperpigmentation, nausea, anorexia, fatigue, diarrhea, stomatitis, AST elevation, ALT elevation, and hyperbilirubinemia, all of which were clinically manageable. At dose level 2 ($n = 17$), hematologic AEs of grade ≥3 were not observed, and nonhematologic toxicities of grade 3 included stomatitis, increased ALP, increased AST, and increased ALT (6 % each). Nonhematologic AEs of grade 4 were not apparent. Interstitial lung disease was not manifest in any patient, and there were no treatment-related deaths.

Pharmacokinetics

Eight patients (three at dose level 1 and five at dose level 2) in the dose-escalation phase of the study were evaluable for pharmacokinetics. The mean steady-state pharmacokinetic parameters for gefitinib (250 mg daily) administered alone or with S-1 are summarized in Table 4. There were no substantial differences in the mean values of the area under the plasma concentration–time curve over 24 h (AUC_{0-24}) or the maximal concentration (C_{max}) for gefitinib when this drug was administered with or without S-1, suggesting that S-1 at either dose did not affect the trough levels of gefitinib.

Pharmacokinetic analysis was also performed for the plasma concentrations of S-1 components (FT, CDHP, and Oxo) and the FT metabolite 5-FU on the first day of gefitinib and S-1 combination therapy. The increases in the mean values of AUC_{0-8} and C_{max} for FT, 5-FU, and CDHP at dose level 2 compared with those at dose level 1 were consistent with the increase in S-1 dose (Table 5), and the pharmacokinetic parameters obtained for S-1 at dose level 2 administered together with gefitinib in the present study did not appear to differ substantially from those obtained previously for S-1 administered alone at 80 mg/m^2 [4].

Efficacy

All 20 patients were evaluable for antitumor response. Three individuals showed a complete response and seven patients showed a partial response, yielding an overall response rate of 50 %. Five patients had stable disease, giving an overall disease control rate of 75 %. In *EGFR* mutation–positive patients ($n = 9$), seven patients achieved an objective response, whereas none with wild-type *EGFR* ($n = 6$) responded. The median PFS and OS for all treated patients were 10.5 months (95 % confidence interval 2.5–12.9 months) and 21.2 months (95 % confidence interval 13.1–26.0 months), respectively. The median PFS was 12.4 and 3.3 months for the *EGFR* mutation–positive patients ($n = 9$) and the patients with wild-type *EGFR* ($n = 6$), respectively.

Discussion

We have previously shown that combined treatment with S-1 and gefitinib has a synergistic antiproliferative effect on NSCLC cells [7]. On the basis of this finding and additional preclinical data, we undertook the present phase I trial to assess the safety–tolerability, pharmacokinetics, and antitumor efficacy of the combination of gefitinib and S-1 in previously treated patients with advanced adenocarcinoma of the lung. Our study has demonstrated that once-daily gefitinib (250 mg) combined with administration of S-1 (80 mg/m^2) for 14 consecutive days every 21 days has an acceptable tolerability profile in such patients, indicating that full single-agent doses of both drugs can be used in combination. Most toxicities were mild or moderate in extent and were similar in type to those observed in monotherapy studies of gefitinib or S-1 [5, 11]. AEs of grade 3 included stomatitis and etlevation of AST, ALT, and ALP levels. All toxicities of grade 3 were reversible and were manageable with symptomatic treatment and dose reduction or interruption. AEs of grade 4 were not observed. The incidence of AEs during combination therapy with gefitinib and S-1 was not higher than that previously determined for either single-agent therapy.

S-1 is an oral fluorinated pyrimidine formulation that combines FT, CDHP, and Oxo. Oxidation of FT (prodrug of 5-FU) is largely dependent on CYP2A6 [12], and 5-FU showed no inhibitory effect on CYP activity in human liver microsomes [13]. Urinary excretion is the primary elimination pathway for CDHP. Non-CYP enzymes, including xanthine oxidase, contribute to the degradation of Oxo. On the other hand, elimination of gefitinib is dependent largely on CYP3A4 and to a lesser extent on CYP2D6 [14, 15]. Given the differences in metabolism and elimination between gefitinib and S-1, no pharmacokinetic interaction

Table 3 Treatment-related adverse events according to treatment cohort and grade

	Level 1 (n = 3)			Level 2 (n = 17)		
	All grades (%)	Grade 3 (%)	Grade 4 (%)	All grades (%)	Grade 3 (%)	Grade 4 (%)
Hematologic						
Anemia	1 (33)	0	0	12 (71)	0	0
Thrombocytopenia	1 (33)	0	0	4 (24)	0	0
Leukopenia	0	0	0	0	0	0
Neutropenia	1 (33)	1 (33)	0	1 (6)	0	0
Nonhematologic						
Rash	3 (100)	0	0	13 (76)	0	0
Hyperpigmentation	1 (33)			10 (59)		
Vomiting	1 (33)	0	0	2 (12)	0	0
Nausea	2 (67)	0	0	4 (24)	0	0
Anorexia	1 (33)	0	0	11 (65)	0	0
Fatigue	2 (67)	0	0	8 (47)	0	0
Diarrhea	2 (67)	0	0	9 (53)	0	0
Stomatitis	2 (67)	0	0	8 (47)	1 (6)	0
ALP increase	1 (33)	0	0	3 (18)	1 (6)	0
AST increase	2 (67)	0	0	8 (47)	1 (6)	0
ALT increase	2 (67)	0	0	8 (47)	1 (6)	0
Hyperbilirubinemia	2 (67)	0	0	7 (41)	0	0

Table 4 Effect of S-1 on the pharmacokinetics of gefitinib

Parameter	Dose level 1 (n = 3)		Dose level 2 (n = 5)	
	Monotherapy	Combination	Monotherapy	Combination
C_{max} (ng/ml)	516.0 ± 100.5	524.1 ± 96.1	684.8 ± 246.9	741.0 ± 208.3
T_{max} (h)	4.8 ± 2.5	5.0 ± 2.0	4.8 ± 2.5	3.8 ± 1.1
$t_{1/2}$ (h)	20.2 ± 2.4	21.3 ± 6.5	21.5 ± 3.8	29.4 ± 9.3
AUC_{0-24} (ng h/ml)	8,567.2 ± 2,131.0	8,849.3 ± 822.8	12,612.7 ± 4,908.2	12,880.9 ± 4,108.6

Data are mean ± SEM

C_{max} maximal plasma concentration of gefitinib, T_{max} time to achieve C_{max}, $t_{1/2}$ plasma half-life of gefitinib, AUC_{0-24} area under the plasma gefitinib concentration–time curve for 0–24 h

between these two agents would be expected. We evaluated the pharmacokinetics of combination therapy with gefitinib and S-1 in the present study. The C_{max} and AUC values of gefitinib obtained here were similar to those determined in phase I trials in patients with solid malignant tumors who received continuous single-agent treatment with gefitinib [16, 17]. To investigate directly the possible effect of S-1 on the pharmacokinetics of gefitinib, we collected blood samples on day 14 during the run-in period of administration of gefitinib alone as well as on the first day of combination therapy with gefitinib and S-1. The plasma concentration profiles and pharmacokinetic parameters for gefitinib were not altered by coadministration of S-1. The pharmacokinetic parameters obtained for S-1 (80 mg/m^2) during gefitinib dosing did not appear to differ substantially from those previously obtained for S-1 administered as a single agent [4], suggesting that gefitinib affects neither the conversion of FT to 5-FU nor the biological behavior of CDHP or Oxo. Together, these data thus indicate that there was no substantial pharmacokinetic interaction between gefitinib and S-1.

Given that single-agent treatment with EGFR-TKIs is now an established first-line therapeutic option for *EGFR* mutation–positive NSCLC, on the basis of recent phase III trials comparing EGFR-TKIs with platinum-based chemotherapy [18–21], it seems reasonable to test EGFR-TKIs in combination with other chemotherapeutic agents in such patients. The promising safety profile and apparent lack of pharmacokinetic interaction observed for the combination of S-1 and gefitinib in our phase I study suggest that this drug combination is a new treatment option for *EGFR* mutation–positive patients with advanced NSCLC.

Table 5 Pharmacokinetic parameters for S-1 components and 5-FU at the two dose levels

Parameter	Dose level 1 ($n = 3$)				Dose level 2 ($n = 5$)			
	FT	5-FU	CDHP	Oxo	FT	5-FU	CDHP	Oxo
C_{max} (ng/ml)	1,445.0 ± 228.0	101.9 ± 42.9	130.7 ± 72.3	54.5 ± 49.8	1,798.0 ± 138.0	182.1 ± 63.8	251.8 ± 56.5	44.2 ± 21.5
T_{max} (h)	2.0 ± 1.0	4.0 ± 1.0	3.0 ± 0.0	3.0 ± 0.0	2.0 ± 1.0	3.0 ± 0.0	3.0 ± 1.0	3.0 ± 0.0
$t_{1/2}$ (h)	6.13 ± 0.96	1.73 ± 0.30	2.64 ± 0.54	2.95 ± 0.97	6.55 ± 1.37	1.56 ± 0.34	2.41 ± 0.40	2.57 ± 0.96
AUC_{0-8} (ng h/ml)	7,446.0 ± 1,546.0	454.0 ± 193.4	532.1 ± 242.2	208.9 ± 145.9	9,752.0 ± 956.0	794.6 ± 280.1	1,000.6 ± 246.9	190.7 ± 85.0

Data are mean ± SEM

A further clinical concern is that *EGFR* mutation–positive patients who initially respond to EGFR-TKIs eventually develop resistance to these agents. At present, no drug that is able to overcome such acquired resistance is available in clinical practice. We have previously shown that the combination of gefitinib and S-1 has a synergistic antiproliferative effect on EGFR mutation–positive NSCLC cells that have developed resistance to EGFR-TKIs [8]. The addition of S-1 to gefitinib may thus prove effective for the treatment of *EGFR* mutation–positive patients with acquired resistance to EGFR-TKIs.

In conclusion, combination therapy with gefitinib (250 mg/day) and S-1 (80 mg/m^2 for 14 days every 21 days) was well tolerated in previously treated patients with advanced pulmonary adenocarcinoma. Further studies are thus warranted to confirm the efficacy and safety of combination therapy with S-1 and gefitinib in comparison with gefitinib monotherapy.

Conflict of interest The authors declare no conflict of interest.

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Efficacy of Rechallenge Chemotherapy in Patients With Sensitive Relapsed Small Cell Lung Cancer

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Objectives: To evaluate the efficacy of rechallenge with current induction regimens for sensitive-relapse small cell lung cancer (SCLC) patients.

Methods: We defined sensitive relapse as treatment-free interval (TFI ≥ 90 d). Sensitive-relapse SCLC patients who received second-line chemotherapy were separated into those treated with rechallenge chemotherapy (rechallenge group) and those treated with other regimens (other group). The endpoints were overall survival (OS), progression-free survival, and toxicity.

Results: Sixty-five patients (19 rechallenge group and 46 other group) were assessable for efficacy and safety evaluation. No significant differences in age, sex, ECOG performance status at relapse, disease extent at diagnosis, or response to first-line treatment were found between the 2 groups, but TFI was significantly longer in the rechallenge group. Twenty-one patients of the other group received amrubicin. There was no significant difference in OS between the 2 groups [median survival time (MST): rechallenge group, 14.4 mo; other group, 13.1 mo; $P=0.51$]. In the patients treated with amrubicin, MST was 12.6 months. Comparing the rechallenge group with the patients treated with amrubicin, there was also no significant difference in OS ($P=0.38$). Both the rechallenge and other group included 11 patients with ex-sensitive relapse (TFI ≥ 180 d). There was no significant difference in OS between the 2 groups (MST 15.7 vs. 26.9 mo, $P=0.46$).

Conclusions: Rechallenge chemotherapy did not prove superior to other chemotherapies, suggesting that monotherapy, such as amrubicin, might be reasonable as second-line chemotherapy for sensitive-relapse SCLC patients.

Key Words: small cell lung cancer, rechallenge chemotherapy, second-line, sensitive relapse, amrubicin

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Lung cancer is the most common cause of cancer-related death. Small cell lung cancer (SCLC) accounts for approximately 12% of lung cancers.[1] SCLC has a very aggressive course, with approximately 60% to 70% of patients having disseminated disease at diagnosis. Although SCLC shows high sensitivity to chemotherapy and radiotherapy, about 80% of limited-disease patients and virtually all patients with extensive disease will develop disease relapse or progression.[2] The prognosis of relapsed SCLC patients is 2 to 4 months without treatment.[3]

Second-line chemotherapy may produce tumor regression, but the evidence of a clinical benefit is limited. In a phase III trial comparing oral topotecan with best supportive care, the median survival time (MST) was 25.9 weeks for patients receiving topotecan and 13.9 weeks for those receiving best supportive care (HR, 0.64; 95% CI, 0.45-0.90; $P=0.0104$).[4] Thus the efficacy of second-line chemotherapy for relapsed SCLC was demonstrated. However, selectable drugs are limited and topotecan is currently the only drug approved for the treatment of relapsed SCLC patients in the United States.[4–6]

Previous reports have shown that sensitive-relapse SCLC patients have a good chance of responding to the same induction chemotherapy (rechallenge chemotherapy).[7,8] Giaccone and colleagues reported the efficacy of rechallenge chemotherapy in 13 relapsed SCLC patients for whom the median treatment-free interval (TFI) was 30 weeks and the overall response rate (ORR) was 50%. Postmus and colleagues analyzed 37 relapsed SCLC patients and reported that the ORR of rechallenge chemotherapy was 62% (median TFI was 34 wk). Although these results suggest the effectiveness of rechallenge, the reported induction regimens were CAV (cyclophosphamide, doxorubicin, vincristine) or CDE (cyclophosphamide, doxorubicin, etoposide), which are not standard regimens at this time. It is unclear whether rechallenge with the currently standard regimens is effective. Therefore, to evaluate the efficacy of rechallenge with current induction regimens, we performed a retrospective analysis of second-line chemotherapy for sensitive-relapse SCLC patients.

MATERIALS AND METHODS

Patients

We collected data between September 2002 and May 2011 from the medical records of the Shizuoka Cancer Center. In this study, we defined TFI as the period from the date of completion of first-line treatment to the first relapse. When sequential radiotherapy or prophylactic cranial irradiation (PCI) was performed as first-line treatment, the date of completion of first-line treatment was defined as the last day of these treatments. We defined sensitive relapse as TFI ≥ 90 days, based on the definition in several previous trials.[9–11] Patients with TFI ≥ 180 days were considered as "ex-sensitive relapse," based on the NCCN guideline recommendation for rechallenge chemotherapy.

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We divided the sensitive-relapse SCLC patients into 2 groups according to the second-line chemotherapy regimen. The "rechallenge" group comprised patients who received rechallenge chemotherapy, which is defined in this study as retreatment with the same induction regimen. The "other" group comprised patients who received regimens other than rechallenge chemotherapy, including monotherapy such as amrubicin or irinotecan.

Evaluation and Statistical Analysis

We evaluated tumors according to the Response Evaluation Criteria in Solid Tumors by performing computed tomography of the chest and abdomen, and magnetic resonance imaging of the head and a bone scintiscan.[12] All patients were evaluated every 2 cycles or every 2 months. All categorical variables were analyzed by χ^2 test or the Fisher exact test, as appropriate. Clinical evaluation of progression-free survival (PFS) and overall survival (OS) after the start of second-line chemotherapy was conducted by the Kaplan-Meier method to assess the time of recurrence or death. The log-rank test was used to compare cumulative survival in each group. We assessed toxicity by National Cancer Institute Common Toxicity Criteria, version 2.0. All P values were reported as 2 sided, and values <0.05 were considered statistically significant. All statistical analyses were performed using JMP version 9.0 (SAS Institute Inc., Cary, NC).

The study protocol was approved by the Institutional Review Board of the Shizuoka Cancer Center.

TABLE 1. Sensitive-Relapse* SCLC Patient Characteristics for Rechallenge Group and Other Group

	Rechallenge Group (n = 19)	Other Group (n = 46)	P
Age at second-line chemotherapy (y)			0.24
Median	69	65.5	
Range	51-83	43-80	
Sex [n (%)]			0.14
Male	17 (89)	34 (74)	
Female	2 (11)	12 (26)	
PS at recurrence [n (%)]			0.33
0-1	18 (95)	40 (87)	
2-4	1 (5)	6 (13)	
Disease extent at diagnosis [n (%)]			0.20
LD	12 (63)	21 (46)	
ED	7 (37)	25 (54)	
Chemoradiation [n (%)]			0.77
Yes	9 (47)	20 (43)	
No	10 (53)	26 (57)	
Prophylactic cranial irradiation [n (%)]			0.09
Yes	7 (37)	8 (17)	
No	12 (63)	38 (83)	
Response to first-line therapy [n (%)]			0.88
CR/PR	18 (95)	44 (96)	
SD/PD	1 (5)	2 (4)	
Treatment-free interval (mo)			0.01
Median	7.1	4.8	
Range	3.1-39.2	3.0-8.7	

*Defined as TFI ≥ 90 days.

CR indicates complete response; ED, extended disease; LD, limited disease; PD, progressive disease; PR, partial response; PS, performance status; SCLC, small cell lung cancer; SD, stable disease; TFI, treatment-free interval.

RESULTS

Patient Characteristics

Of the 65 sensitive-relapse SCLC patients who received second-line chemotherapy, 19 were placed in the rechallenge group and 46 in the other group, including 21 patients treated with amrubicin. The sensitive-relapse patient characteristics are listed in Table 1. No significant differences in age, sex, ECOG performance status at relapse, disease extent at diagnosis, or response to first-line treatment were found between the 2 groups. PCI was more frequent in the rechallenge group. TFI was significantly longer in the rechallenge group than in the other group. In the rechallenge group, etoposide and platinum were used in 68% of the patients as second-line chemotherapy. In the other group, 46% of the patients were treated with amrubicin, and 11% were treated with topotecan (Table 2).

Both groups included 11 ex-sensitive-relapse patients; their characteristics are listed in Table 3. There were also no significant differences in patient characteristics and response to first-line treatment.

Response

Response to second-line chemotherapy in sensitive-relapse and ex-sensitive-relapse SCLC patients is shown in Table 4. In the sensitive-relapse patients, there was no significant difference in response between the rechallenge group and the other group (ORR: rechallenge group 37% vs. other group 44%, $P = 0.62$). ORR in patients treated with amrubicin was 38% and was not significantly different compared with the rechallenge group ($P = 0.93$). In the ex-sensitive-relapse patients, there was also no significant difference in ORR between the 2 groups (rechallenge group 46% vs. other group 55%, $P = 0.67$).

PFS and OS

In the sensitive-relapse patients, there was no significant difference in OS from the start of second-line chemotherapy between the 2 groups (MST: rechallenge group 14.4 mo vs.

TABLE 2. First-Line and Second-Line Chemotherapy of Sensitive-Relapse* SCLC Patients in Rechallenge Group and Other Group

	Rechallenge Group (n = 19)	Other Group (n = 46)
First-line chemotherapy [n (%)]		
Cisplatin and etoposide	7 (36)	20 (43)
Carboplatin and etoposide	6 (32)	10 (22)
Cisplatin and irinotecan	6 (32)	14 (30)
Other	0	2 (5)
Second-line chemotherapy [n (%)]		
Cisplatin and etoposide	7 (36)	1 (2)
Carboplatin and etoposide	6 (32)	2 (4)
Cisplatin and irinotecan	6 (32)	0 (0)
Amrubicin	0	21 (46)
Irinotecan	0	10 (22)
Topotecan	0	5 (11)
Other	0	7 (15)

*Defined as TFI ≥ 90 days.

SCLC indicates small cell lung cancer; TFI, treatment-free interval.

TABLE 3. Ex-Sensitive Relapse SCLC Patient Characteristics in Rechallenge Group and Other Group

	Rechallenge Group (n = 11)	Other Group (n = 11)	*P*
Age at second-line chemotherapy (y)			0.72
Median	69	69	
Range	52-79	48-79	
Sex [n (%)]			0.26
Male	10 (91)	8 (73)	
Female	1 (9)	4 (27)	
PS at recurrence [n (%)]			0.26
0-1	10 (91)	8 (73)	
2-4	1 (9)	3 (27)	
Disease extent at diagnosis [n (%)]			0.65
LD	8 (73)	7 (64)	
ED	3 (27)	4 (36)	
Chemoradiation [n (%)]			0.37
Yes	8 (73)	6 (55)	
No	3 (27)	5 (45)	
Prophylactic cranial irradiation [n (%)]			0.19
Yes	5 (45)	3 (27)	
No	6 (55)	8 (73)	
Response to first-line therapy [n (%)]			0.23
CR/PR	11 (100)	10 (91)	
SD/PD	0 (0)	1 (9)	
Treatment-free interval (mo)			0.02
Median	268	207	
Range	182-1176	6.0-262	

*Defined as TFI ≥ 180 days.

CR indicates complete response; ED, extended disease; LD, limited disease; PD, progressive disease; PR, partial response; PS, performance status; SCLC, small cell lung cancer; SD, stable disease; TFI, treatment-free interval.

other group 13.1 mo, $P=0.51$) (Fig. 1A). There was also no significant difference in PFS (median PFS 5.6 vs. 4.9 mo, $P=0.15$) (Fig. 1B). In the patients treated with amrubicin, MST was 12.6 months and median PFS was 4.6 months. Comparing the rechallenge group with the patients treated with amrubicin, there were also no significant differences in OS and PFS (Figs. 2A, B).

In the ex-sensitive-relapse patients, there was no significant difference in OS from the start of second-line chemotherapy between the 2 groups (MST 15.7 vs. 26.9 mo, $P=0.46$) (Fig. 3A). There was also no significant difference in PFS (median PFS 7.8 vs. 4.9 mo, $P=0.63$) (Fig. 3B).

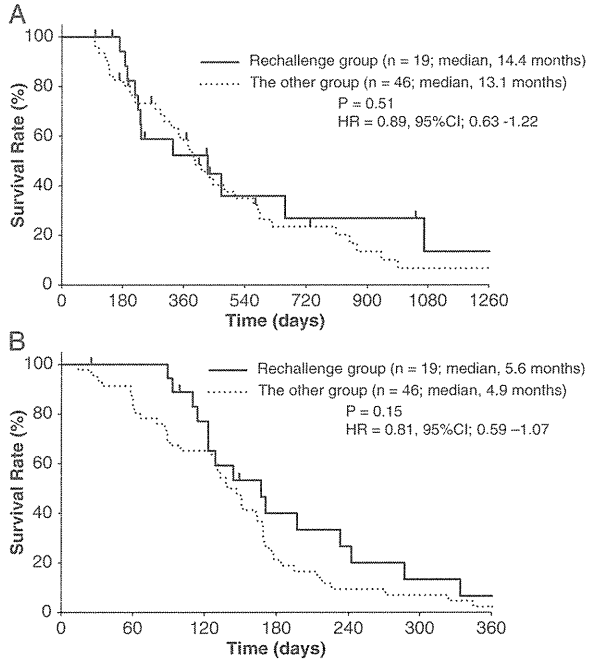

FIGURE 1. (A) Overall survival and (B) progression-free survival in sensitive-relapse SCLC patients in the rechallenge chemotherapy group and other regimen group. SCLC indicates small cell lung cancer.

Safety

Toxicity was evaluated in both group patients. The most common grade 3 or worse adverse events were hematologic toxicity and included neutropenia (rechallenge group 94% vs. other group 61%, $P=0.02$), thrombocytopenia (rechallenge group 26% vs. other group 22%, $P=0.76$), and anemia (rechallenge group 10% vs. other group 26%, $P=0.29$). Febrile neutropenia was noted in 3 rechallenge group patients (16%) and 2 other group patients (4%). No patients experienced nonhematologic toxicities worse than grade 3.

DISCUSSION

This study could not show the superiority of rechallenge chemotherapy over other regimens in sensitive-relapse SCLC patients. As TFI is a prognostic factor,[13,14] we analyzed treatment efficacy after adjusting the value. Although TFI was

TABLE 4. Response to Second-Line Chemotherapy in Sensitive-Relapse and Ex-Sensitive-Relapse SCLC Patients

	Sensitive Relapse (TFI ≥ 90 d) [n (%)]			Ex-Sensitive Relapse (TFI ≥ 180 d) [n (%)]	
	Rechallenge Group	Other group	Amrubicin	Rechallenge Group	Other Group
CR	1 (5)	0 (0)	0 (0)	1 (9)	0 (0)
PR	6 (32)	20 (44)	8 (38)	4 (37)	6 (55)
SD	9 (47)	17 (37)	7 (33)	3 (27)	3 (27)
PD	0 (0)	9 (19)	6 (29)	0 (0)	2 (18)
NE	3 (16)	0 (0)	0 (0)	3 (27)	0 (0)
ORR (%)	37	44	38	46	55
95% CI	19–59	30-57	20-59	21-72	28-78
P	—	0.62*	0.93*	—	0.67*

*Compared with the rechallenge group.

95% CI indicates 95% confidence interval; CR, complete response; NE, not evaluable; ORR, overall response rate; PD, progressive disease; PR, partial response; SCLC, small cell lung cancer; SD, stable disease; TFI, treatment-free interval.

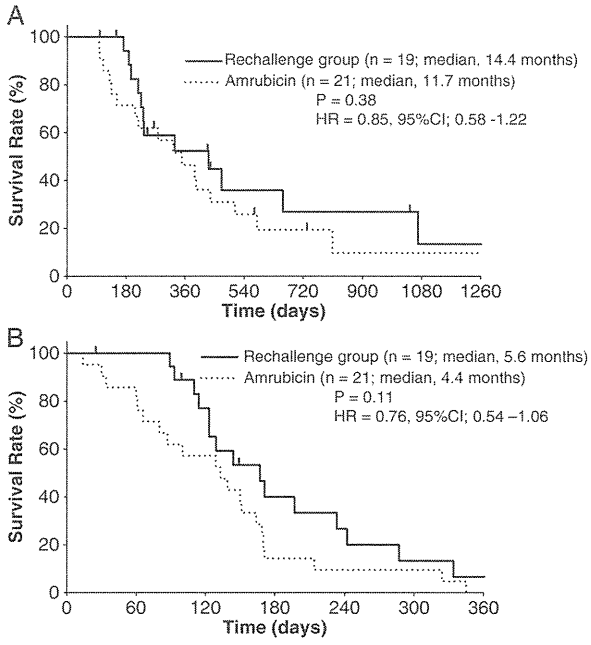

Figure 2. (A) Overall survival and (B) progression-free survival in sensitive-relapse SCLC patients in the rechallenge group and those taking amrubicin in the other group. SCLC indicates small cell lung cancer.

significantly longer in the rechallenge group than in the other group, rechallenge chemotherapy did not show significant differences in ORR, PFS, or OS compared with the other chemotherapies. In our study, neutropenia was more frequently observed in rechallenge group. Because a cure cannot be expected in relapsed SCLC, the purpose of second-line chemotherapy is improvement of prognosis and quality of life.[15] When quality of life and treatment results are taken into account, less toxic monotherapy may be reasonable.

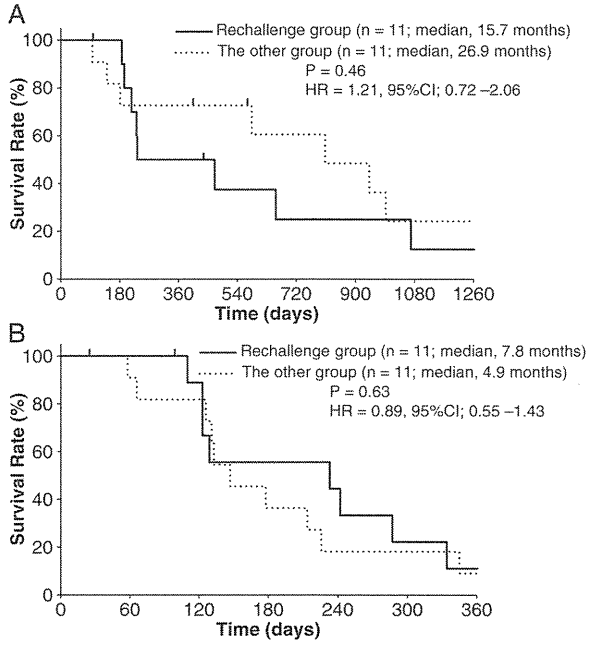

Figure 3. (A) Overall survival and (B) progression-free survival in ex-sensitive-relapse SCLC patients in the rechallenge group and other group. SCLC indicates small cell lung cancer.

Moreover, in comparing amrubicin with rechallenge chemotherapy, similar results were obtained. In the rechallenge group in this study, ORR was 37% whereas in previous reports it was 50% to 62%.[7,8] In this study, median TFI in rechallenge chemotherapy was 20 weeks, but in previous reports it was 30 to 34 weeks. These results suggest that the difference in TFI might have led to the difference in ORR.

At this time, clinical evidence of second-line chemotherapy for relapsed SCLC patients is limited. The number of randomized trials is small, and topotecan is the only established drug.[4–6] Amrubicin is a synthetic 9-amino-anthracycline, which showed response rates of 50% to 53% in 2 phase II trials.[16,17] In phase II trials comparing topotecan with amrubicin, the efficacy of amrubicin was promising.[9,10] On the basis of the results, a phase III trial was conducted.[11] However, this trial was unable to show the superiority of amrubicin over topotecan. MST with amrubicin was 9.2 months compared with 9.9 months with topotecan ($P=0.62$; HR, 0.88).

Although several guidelines recommend rechallenge chemotherapy for sensitive-relapse SCLC patients, the recommendation is not based on randomized trials. In addition, the reported induction chemotherapy regimens were not platinum based. Garassino et al[18] evaluated the clinical outcomes of SCLC patients who received second-line chemotherapy after platinum-etoposide chemotherapy. In their report, platinum-based rechallenge showed significant better results in ORR and OS than other chemotherapy regimens for sensitive-relapse and refractory-relapse SCLC patients. A platinum-containing regimen showed better results independently of the time to second-line therapy. However, there is a difference in subjects between our study and Garassino's report. We evaluated only sensitive-relapse SCLC patients. In addition, 46% of the patients received amrubicin in our study, whereas 44.8% of the patients received anthracycline-based regimens such as CAV in Garassino's report.

Our study had several limitations. First, the sample size was small and the timing of response assessment was decided by each physician, which might have resulted in variance of ORR and PFS. Second, we did not assess the influence of PCI, which is known to improve the prognosis.[19] Although the patients in the rechallenge group received more frequent PCI, there was no significant difference in ORR, PFS, or OS between the 2 groups. However, there are a few reports that evaluated the rechallenge chemotherapy for sensitive-relapse SCLC patients with the currently standard regimen.

In conclusion, superiority of rechallenge chemotherapy over other chemotherapies could not be demonstrated. The results suggest that monotherapy, such as amrubicin, may be reasonable as second-line chemotherapy for sensitive-relapse SCLC patients.

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