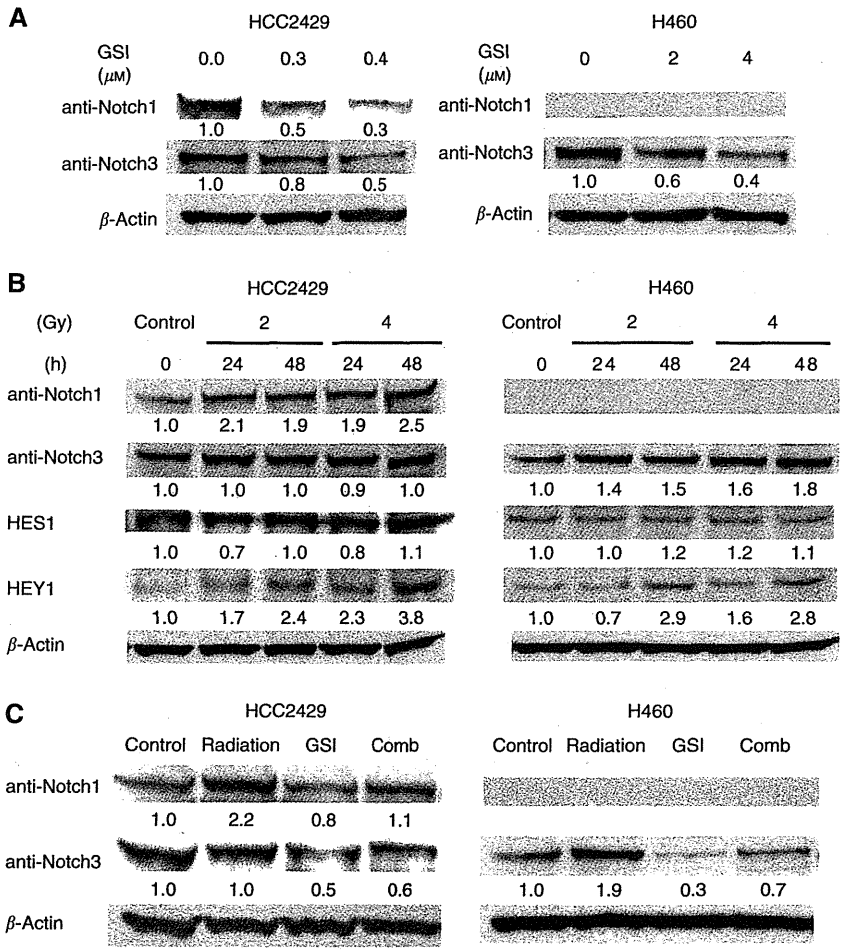

Figure 2 Combination and either GSI or radiation alone regulated the Notch pathway. (**A**) GSI suppressed Notch intracellular domain (NICD) expression in a dose-dependent manner. GSI I downregulated NICD1 and NICD3 in HCC2429, whereas NICD3 in H460. (**B**) Radiation upregulated NICD1, but not NICD3 in HCC2429, whereas NICD3 in H460 at 24 h after radiation (2 or 4 Gy). The downstream Notch target gene, HEY1, was also upregulated at 48 h after radiation. (**C**) Radiation-induced Notch upregulation was ameliorated by the combination. Standardisation was performed with actin measured in the same blots with anti-actin antibody. Quantifications were shown by the ratios of treated protein expression/untreated protein expression.

cytometry (Figure 3A). Either treatment induced apoptosis. However, the combination increased apoptotic cells two or three fold compared with either treatment alone (Figure 3B). When GSI XX was used, we also noted the higher induction of apoptosis in NSCLC cell lines (HCC2429, H460 and A549) with radiation compared with either treatment alone (Supplementary Figure 2).

Combination alters p-ERK, anti-apoptotic proteins, and pro-apoptotic proteins

As Notch has been shown to crosstalk with the epidermal growth factor receptor pathway (Haruki *et al*, 2005), we assessed the expression of phospo-ERK, a member of the MAPK family and phospho-AKT. The combination reduced the expression of p-ERK compared with either GSI I or radiation alone (Figure 4A). Although several studies have shown the crosstalk between Notch pathway and phosphatidylinositol 3-kinase (PI3K)/AKT pathway (Wang *et al*, 2007; Meng *et al*, 2009), we observed no effect on p-AKT level in any of the treatments (Figure 4A).

To confirm the effect of the combination on apoptosis, we examined the expression of the Bcl-2 family proteins. We found that the combination reduces levels of anti-apoptotic proteins, p-Bcl-2 and Bcl-xL, and increases pro-apoptotic protein Bim, compared with either GSI I or radiation alone in both cell lines. Furthermore, cleaved PARP was induced in single treatment and combination in both cell lines (Figure 4B).

Combination enhances antitumour activity *in vivo*

To determine the effect of combining GSI with radiation *in vivo*, we utilised a xenograft model. We previously have shown that GSI suppressed tumour growth of Notch expressing lung cancer cell lines, H460 and A549 *in vivo* (Konishi *et al*, 2007). The scheduling of radiation and GSI was outlined in Figure 5A based on our previous findings (Konishi *et al*, 2007; Tanaka *et al*, 2009). The radiation was given on days 1 and 8 at 8 Gy per dose, and GSI XX 200 $\mu g\,kg^{-1}$ was administered by i.p. injection on days 2–4 and 9–11. We noted a significant delay in growth of tumours in the combination group compared with either of the treatments alone (Figure 5B). Some tumours were resected on day 15 for molecular analysis. Consistent with our *in vitro* findings, expression of activated NICD3 was enhanced in tumours treated with radiation, and this activation was mitigated by the addition of GSI (Figure 5C). This observation supports our hypothesis that induction of Notch pathway by radiation is one mechanism of resistance to radiation and can be ameliorated by the addition of GSI. Intestinal toxicity has been major concern in using GSI owing to the loss of intestinal crypts cells and hyperplasia of intestinal goblet cells (Guilmeau *et al*, 2010). Others reported the observed gut toxicity from GSI was mitigated with intermittent dosing (Tamman *et al*, 2009). We stained intestine of treated mice with periodic acid schiff to examine the intestinal toxicity of GSI. No increase of intestinal goblet cells was seen among treated mice compared with controls (data not shown).

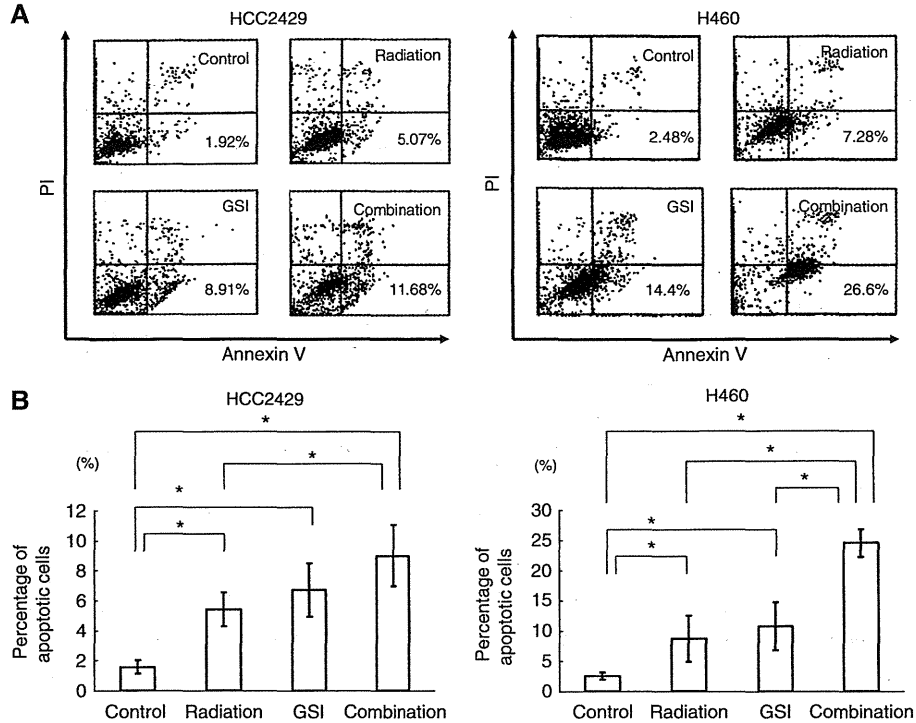

Figure 3 Combination treatment induced apoptosis of lung cancer. Cells were treated with GSI I (1 μM in HCC2429 and 9 μM in H460) at 24 h after 8 Gy of radiation. The percentage of apoptotic cells was measured using Annexin V and propidium iodide (PI) with flow cytometry. (**A**) Representative data of four independent experiments on HCC2429 and H460. (**B**) Mean percentage of apoptotic cells. Combination therapy induced significantly higher apoptosis in HCC2429 and H460 cells ($n=4$). *$P<0.05$.

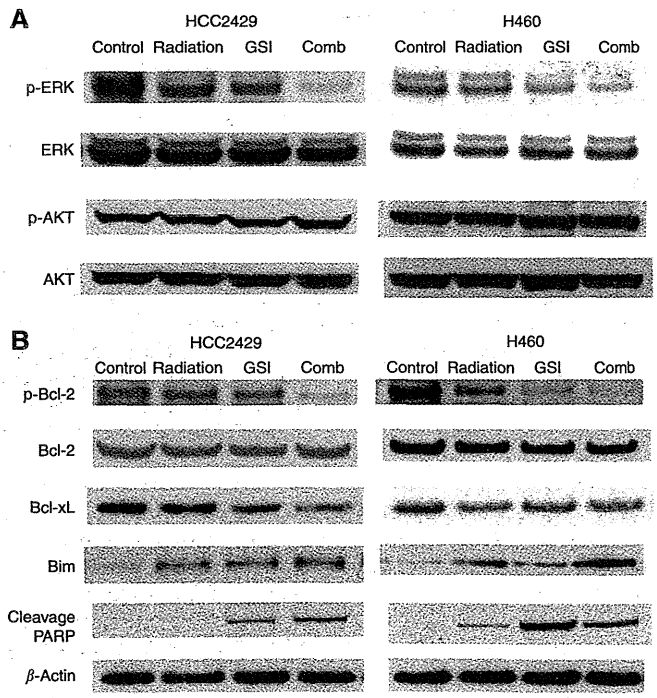

Figure 4 Apoptosis was induced through the MAPK pathway and Bcl-2 family proteins by the combination of GSI and radiation. Cells were treated with GSI I (1 μM in HCC2429 and 9 μM in H460) at 24 h after 8 Gy of radiation. (**A**) Combination therapy downregulated p-ERK, but had no effect on p-AKT in either HCC2429 or H460. (**B**) Combination therapy downregulated anti-apoptotic proteins, p-Bcl-2 and Bcl-xL, and upregulated apoptotic proteins, Bim and cleaved PARP, in both HCC2429 and H460.

Furthermore, no body weight loss was encountered (data not shown), indicating that all treatments, including the combination therapy was well tolerated.

DISCUSSION

Radiation therapy is the primary treatment for patients with locally advanced lung cancer. Although it is very effective in local control, majority of patients will die of their disease, suggesting that further studies are needed to better understand the mechanisms of radiation resistance and to develop new strategies to improve radiation-dependent tumour cytotoxicity.

The phenotypic outcome of Notch signalling is often context-dependent. In lung cancer, Notch1 was known to suppress tumour proliferation under normoxia, but in hypoxia, it had a converse role in tumour promotion (Chen *et al*, 2007). Moreover, the specific role of each individual Notch receptor in radiation is not fully understood, but our present observation that Notch1, but not Notch3, was enhanced by radiation in HCC2429 also suggests that the biological function of Notch depends on treatment context, suggesting that targeting specific Notch receptor may lead to better outcome by preventing unnecessary toxicity.

In breast cancer, Notch signalling is activated after radiation, suggesting that activation of this oncogenic pathway is a mechanism of radiation resistance (Phillips *et al*, 2006). We hypothesised that Notch activation by radiation has radioprotective role in lung cancer, this phenomenon can be prevented by the use of Notch inhibitors after radiation. In our study, we also showed that radiation-induced Notch activation, which was mitigated by the administration of GSIs, supporting our hypothesis and providing a rationale for the sequential treatment schedule.

Enhanced induction of PARP and reduction of pro-survival proteins, such as p-Bcl-2 and Bcl-xL, by the combination therapy

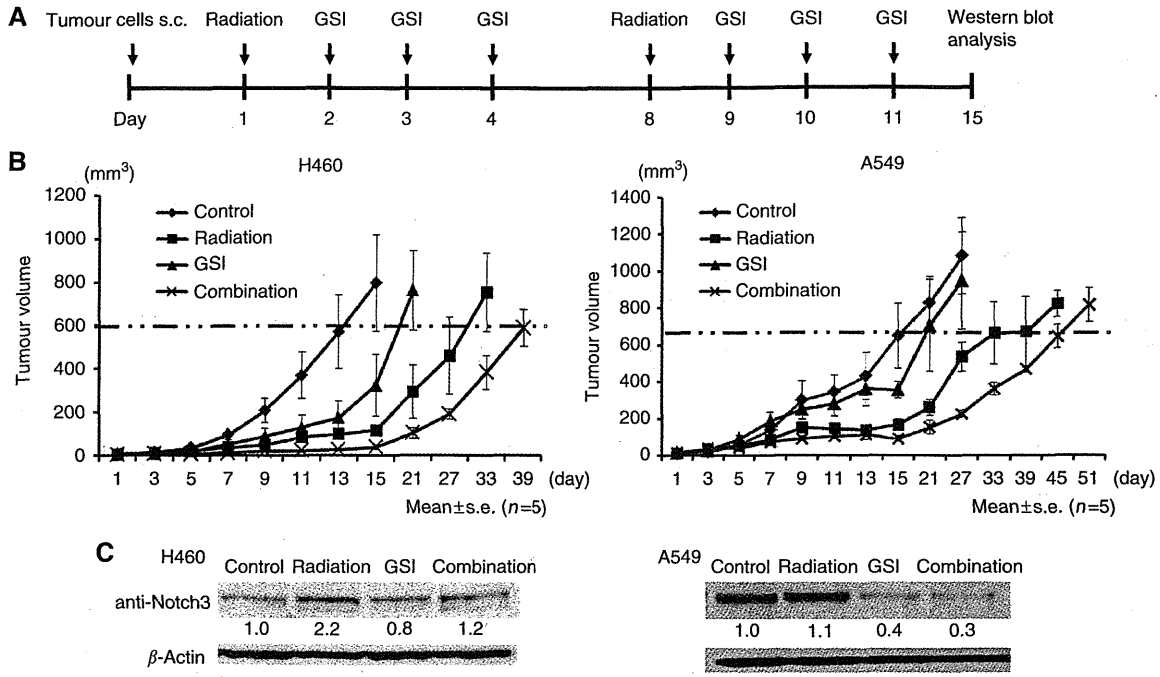

Figure 5 Combination therapy inhibited tumour growth of lung cancer *in vivo*. (**A**) Treatment schedule *in vivo*. H460 and A549 at 1.0×10^6 cells were inoculated subcutaneously into right posterior legs of nude mice. Treatment was initiated when tumours were palpable. Mice were treated with 200 $\mu g\,kg^{-1}$ GSI XX injected intraperitoneally 3 days per week after 8 Gy of radiation once a week. Tumour size was measured every 2 days. Some tumours were removed on day 15 and the expression of NICD3 was examined by western blot analysis. (**B**) Combination treatment showed the significant delay of tumour growth, compared with control or single treatment alone ($n = 5$). (**C**) NICD3 expression was upregulated in H460 and A549 tumour treated with radiation. In contrast, combination therapy reduced NICD3 expression.

suggested that the observed antitumour effect is mediated through induction of apoptosis. Our findings are compatible with the other reports that GSI or radiation activates the apoptosis pathway, including Bcl-2 family (Konishi *et al*, 2007; Han *et al*, 2009; Zhuang *et al*, 2009). Some studies have shown that Notch regulated the AKT pathway in several cancers (Liu *et al*, 2006; Meng *et al*, 2009; Efferson *et al*, 2010), but expression of p-AKT was unchanged in our study, suggesting that the crosstalks between Notch and other oncogenic pathways are context-dependent.

Cancer stem cells (CSCs) are a small population of cells that are responsible for tumour maintenance and spreading (Reya *et al*, 2001; Lobo *et al*, 2007). CSCs in lung cancer have been isolated and functions have been described in several reports (Ho *et al*, 2007; Eramo *et al*, 2008). CSCs are further reported to contribute to resistance of chemotherapy or radiation (Phillips *et al*, 2006; Ho *et al*, 2007; Eramo *et al*, 2008; Diehn *et al*, 2009; Rutella *et al*, 2009). Notch pathway was activated in several types of CSCs, including colon cancer, breast cancer and glioma (Dontu *et al*, 2003; Purow *et al*, 2005; Sikandar *et al*, 2010). For example, radiation appeared to enhance Notch pathway in both breast cancer ($CD24^{-/low}/CD44^{+}$) and glioma ($CD133^{+}$) stem-like cells. The activation of these cells was accompanied by radioresistance (Phillips *et al*, 2006; Wang *et al*, 2010). We did not examine how the combination therapy affects lung CSCs, as it is unclear that similar markers such as CD133 or CD44 can be used as markers for lung CSC. However, our study suggests GSI prevents the induction of CSCs by radiation, leading to the reduction of tumour growth.

In vivo, we observed a statistically significant delay of tumour growth in mice with combination therapy. GSI inhibited NICD both *in vitro* and *in vivo* and might get biochemical inhibition at much lower doses used *in vivo* than used *in vitro*. This suggests to us that the microenvironment contributes to the observable effect. For example, the Notch ligand DLL4 has a critical role in the angiogenesis (Ridgway *et al*, 2006; Scehnet *et al*, 2007) and GSI has been shown to inhibit tumour growth through preventing DLL4-dependent angiogenesis (Li *et al*, 2011). Furthermore, hypoxic tumour environment induces radiation resistance (Wilson and Hay, 2011). Notch 1 was activated under hypoxia in lung cancer cell lines and GSI-induced apoptosis of these cells (Chen *et al*, 2007; Eliasz *et al*, 2010). From these observations and our data, GSI may not only inhibit tumour cells but also tumour microenvironment, which promotes tumour survival.

In summary, our data provided the evidence that the addition of GSI enhanced the cytotoxicity of radiation in lung cancer both *in vitro* and *in vivo*. Because of the role of Notch signalling in tumour hypoxia and CSCs, the radiation-dependent Notch activation likely represents a mechanism of radioresistence. Further studies are needed to ascertain our hypothesis. Nevertheless, our study provides compelling evidence that combining GSI and radiation represents a rational strategy for the treatment of patients with NSCLC.

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Conflict of interest

The authors declare no conflict of interest.

Supplementary Information accompanies the paper on British Journal of Cancer website (http://www.nature.com/bjc)

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A phase II study of amrubicin as a third-line or fourth-line chemotherapy for patients with non-small cell lung cancer: Hokkaido Lung Cancer Clinical Study Group Trial (HOT) 0901

Running head: Third-line or fourth-line AMR in NSCLC

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Keywords: amrubicin, chemotherapy, fourth-line, non-small cell lung cancer, third-line

Abstract

Amrubicin, a third-generation synthetic anthracycline agent, has favorable clinical activity and acceptable toxicity for treatment of non-small cell lung cancer (NSCLC) and small cell lung cancer. We conducted this study to evaluate the efficacy and safety of amrubicin for advanced NSCLC patients as a third- or fourth-line therapy. Eligible patients had recurrent or refractory advanced NSCLC after second- or third-line therapy. Patients received amrubicin 35 mg/m^2 intravenously on days 1–3 every 3 weeks. The primary endpoint was disease control rate (DCR). Secondary endpoints were overall survival (OS), progression-free survival (PFS), response rate, and toxicity profile. Of the 41 patients enrolled, 26 received amrubicin as a third-line and 15 received it as a fourth-line therapy. The median number of treatment cycles was 2 (range 1–9). The objective responses were complete response (0), partial response (4), stable disease (21), progressive disease (15), and not evaluable (1), resulting in a DCR of 61.0% (95% confidence interval, 46.0–75.9%). Overall response rate was 9.8% (95% confidence interval, 0.6–18.8%). Median PFS was 3.0 months, median OS was 12.6 months, and the 1-year survival rate was 53.7%. Grade 3/4 hematological toxicities were neutropenia (68%), anemia (12%), thrombocytopenia (12%), and febrile neutropenia (17%). Nonhematological toxicities were mild and reversible. No treatment-related death was observed. Amrubicin shows significant clinical activity with manageable toxicities as a third- or fourth-line therapy for advanced NSCLC. This study provides relevant data for routine practice and future prospective trials evaluating third- or fourth-line treatment strategies for advanced NSCLC.

Introduction

Lung cancer is the leading cause of cancer-related death worldwide (1). First-line therapies, epidermal growth factor receptor (*EGFR*) tyrosine kinase inhibitors (TKIs) in *EGFR*-mutated patients as well as platinum-based chemotherapy in conjunction with third-generation antitumor agents, significantly improve survival and quality of life in patients with advanced non-small cell lung cancer (NSCLC) (2-5). Despite these favorable outcomes, most patients receiving first-line therapy experience disease progression and require salvage therapy. Second-line therapy also has beneficial effects on survival and quality of life (2, 6, 7).

Docetaxel, pemetrexed, gefitinib, and erlotinib are considered standard second-line therapies based on several randomized controlled trials (6-9). Because of the improved efficacy of first-line, second-line, and maintenance therapy in NSCLC, a high proportion of patients (26–38%) receive third-line therapy (10, 11). Thus, there is an urgent need for new third-line therapy options. To date, there is a paucity of studies that address the role of third-line therapy, and they are primarily retrospective analyses (12-14).

Amrubicin, a completely synthetic 9-amino-anthracycline, is a potent inhibitor of DNA topoisomerase II (15). Phase II study of amrubicin in both NSCLC and SCLC demonstrated the promising results and tolerable toxicity (16, 17). The clinical significance of amrubicin has recently focused on the treatment of recurring lung cancer. A phase I and a pharmacokinetic study of amrubicin in previously treated NSCLC and SCLC patients recommend an amrubicin dose of 35 mg/(m^2·day) on 3 consecutive days every 3 weeks (18). Amrubicin is a

promising third-line therapy agent because it has a different mechanism of action compared to other available anticancer agents.

There is no prospective study that specifically addresses the role of third-line therapy for NSCLC. We therefore conducted a multicenter prospective phase II trial of amrubicin 35 mg/m^2 in NSCLC patients as a third- or fourth-line therapy to confirm the efficacy and safety of the drug in the third-line or fourth-line therapy setting.

Patients and Methods

Patient Eligibility

Eligible patients met the following criteria: histologic or cytologic confirmation of NSCLC; recurrent or refractory disease after 2 or 3 previous treatment regimens; measurable disease; an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0–2; ≤75 years of age; adequate bone marrow function (leukocyte count of ≥3,000/mm^3, neutrophil count of ≥1,500/mm^3, platelet count of ≥100,000/mm^3, and hemoglobin content of ≥9.0 g/dL); adequate other organ function (total bilirubin concentration of ≤1.5 mg/dL, aspartate transaminase and alanine transaminase levels of ≤2.0 times the upper limit of normal, and creatinine clearance ≥50 mL/min); P_aO_2 ≥60 Torr or S_pO_2 ≥95%; a left ventricular ejection fraction of ≥60% on echocardiography; and a life expectancy of 3 or more months.

Patients with previous amrubicin therapy, exceeding critical dosage in prior anthracycline drug therapy, using corticosteroid or immunosuppressive drugs, with an active infectious disease with serious medical complications

(active peptic ulcer, heart disease, diabetes mellitus, or cerebrovascular disease), with radiographic signs of interstitial pneumonia or pulmonary fibrosis, with third-space fluid collection requiring drainage, who are lactating or pregnant, with symptomatic brain metastasis, or with active concomitant malignancy were deemed ineligible.

This study was performed in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice guidelines (19). The protocol was approved by the institutional review board of all participating institutions, and all patients provided written informed consent before treatment.

Treatment Plan

Amrubicin was dissolved in 20 mL of physiological saline and was administered intravenously for more than 5 min at a dose of 35 mg/m^2·per day on days 1 to 3 every 3 weeks. All patients received at least 2 cycles of treatment unless their disease progressed, unacceptable toxicity occurred, the patient refused further treatment, or the physician decided to discontinue the treatment.

Subsequent cycles of treatment were withheld until the following criteria were satisfied: the leukocyte count was ≥3,000/mm^3, the neutrophil count was ≥1500/mm^3, the platelet count was ≥100,000/mm^3, total bilirubin was ≤2.0 mg/dL, there was no infection, the ECOG PS was ≤2, and the grade of any nonhematologic toxicity was ≤2. If these criteria were not satisfied within 36 days after the onset of the last treatment, the patient was removed from the study. The dose of amrubicin was reduced to 30 mg/m^2 per day if leukopenia or neutropenia of grade 4 persisted for more than 4 days, thrombocytopenia of

grade 4 or requiring platelet transfusion, febrile neutropenia, or nonhematologic toxicity of grade ≥3 (except for anorexia, nausea, or alopecia) occurred during the previous course. If these toxicities occurred after reduction of the amrubicin dose to 30 mg/m^2 per day, the dose was reduced further to 25 mg/m^2 per day. A third reduction was not permitted and the protocol treatment was terminated. The use of the prophylactic antibiotics was not permitted.

Evaluation

Baseline assessment included a physical examination, complete blood counts (CBC) with differential, hepatic and renal function tests, urinalysis, 12-lead electrocardiogram, echocardiogram, and chest radiography. Visible and palpable tumors were measured in the baseline assessment by chest radiograph, computed tomography (CT) scans, or magnetic resonance imaging (MRI) scans (when clinically indicated). During the study, medical history and physical examination results, vital signs, ECOG PS, CBC, and blood chemistries were monitored weekly. Tumor responses were assessed using chest radiography, CT, or MRI (when clinically indicated) at every cycle until disease progression. Unidirectional measurements were adopted on the basis of the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.0 (20). A response of >4 weeks duration was considered a complete response (CR) or a partial response (PR) and a response of >6 weeks from the initiation of chemotherapy was considered stable disease (SD). Clinical response data were confirmed by central review.

Toxicities were assessed according to National Cancer Institute-Common Toxicity Criteria (NCI-CTC) version 3.0.

Progression-free survival (PFS) was defined as the time from the date of enrollment to the date of documented progression or death from any cause and was censored at the date of the last follow-up visit for surviving patients who had not progressed. Overall survival (OS) was defined as the time from the date of enrollment to the date of death or last follow-up. The data for patients without any events were censored on the last date with nonevent status.

Statistical Analysis

The primary end point was disease control rate (DCR), defined as the proportion of patients whose best response was CR, PR, or SD among all per-protocol patients. Sample size was determined according to one-arm binomial design devised by the South Western Oncology Group. Assuming that a DCR of 50% in eligible patients would indicate potential usefulness, whereas a DCR of 30% would be the lower limit of interest, with $\alpha = 0.05$ and $\beta = 0.20$, the estimated accrual number was 37 patients. Allowing for a patient ineligibility rate of 10%, we planned on enrolling 40 patients in the study. Secondary end points were OS, PFS, objective response rate (ORR), and toxicity profiles. Survival curves were estimated by the Kaplan–Meier method. Statistical analyses were performed using JMP 10 (SAS Institute Inc., Cary, NC, USA).

This study is registered with University Hospital Medical Information Network (UMIN), number UMIN C000002306.

Results

Patient Characteristics

From August 2009 to May 2011, 41 patients were enrolled from 10 participating institutions. Patient characteristics are summarized in Table 1. The median age was 66 years (range 43–74 years), of which 70.7% were male, and most patients (97.6%) had a good ECOG PS of 0–1. Histologic analysis revealed that 30 patients (73.2%) had adenocarcinoma and 8 patients (19.5%) had squamous cell carcinoma. Seven patients (17.1%) were positive and 26 patients (63.4%) were negative for the *EGFR* mutation. Twenty-six patients (63.4%) received amrubicin as a third-line therapy and 15 patients (36.6%) received the drug as a fourth-line therapy.

Seven patients (17.1%) received thoracic surgery and 9 patients (22.0%) received thoracic radiotherapy. Table 2 shows the content of prior therapeutic regimens. All patients had received a platinum-containing doublet regimen as a first- or second-line therapy. The regimens in the first-line therapy were as follows: platinum-containing doublets in 38 patients (92.7%); a single agent in 2 patients (4.9%); and gefitinib in 1 patient (2.4%). The regimens in the second-line therapy were as follows: a single agent in 21 patients (51.2%); platinum-containing doublets in 13 patients (31.7%); non-platinum doublets in 4 patients (9.8%); and gefitinib in 3 patients (7.3%). The regimens in the third-line therapy were as follows: platinum-containing doublets in 6 patients (14.6%); a single agent in 6 patients (14.6%); and non-platinum doublets in 3 patients (7.3%). Of the 7 patients harboring the *EGFR* mutation, 3 had not received

EGFR-TKIs before enrollment into this study due to the patient's refusal or later confirmation of the *EGFR* mutation.

Treatment Administered

The median number of treatment cycles was 2 (range 1–9 cycles). In all, 30 (73.2%) patients completed at least 2 cycles of treatment and a total of 109 treatment cycles were delivered overall. The mean relative dose intensity of amrubicin was 91.1%. A reduction of amrubicin dose was necessary, according to the study protocol, in 8 cycles (7.3% of total cycles). All patients received the first cycle of amrubucin in an inpatient setting for the check of the safety, and most of the patients received further cycle of amrubicin in an outpatient setting. Subsequent treatment delay was observed in 29 of 109 cycles (26.6%). The primary reasons for dose reduction were grade 4 neutropenia (4 of all cycles), febrile neutropenia (3 of all cycles), and grade 3 headache (1 of all cycles). Treatment was discontinued in 11 patients after the first cycle and in 10 patients after the second cycle; the reasons for discontinuation included progressive disease (24 patients), toxicity (6 patients), completing the scheduled treatment (4 patients), patient refusal (2 patients), and physician decision (2 patients).

Following the protocol treatment, 23 (56%) patients eventually received subsequent therapy: 9 (22%) received a single agent, 9 (22%) received EGFR-TKIs, 3 (7%) received platinum-containing doublets, and 2 (5%) received non-platinum doublets.

Response and Survival

Among the 41 assessable patients, there were 4 PR and no CR, for an overall response rate of 9.8% (95% confidence interval [CI], 0.6–18.8) (Table 3). Twenty-one patients (51.2%) had SD, yielding an overall DCR of 61.0% (95% CI, 46.0–75.9). Fifteen patients had PD as the best response and the response of 1 patient could not be confirmed due to receipt of subsequent chemotherapy before response evaluation. The lower end of the 95% CI was thus higher than the threshold DCR of 30%, and the primary endpoint was met. We found no significant difference in ORR or DCR between gender, age, tumor histology, *EGFR* mutation status, or treatment line, except for between DCR and *EGFR* mutation status; DCR of 100% in 7 *EGFR* mutated patients, 46.2% in 26 *EGFR*

wild-type patients, and 85.7% in 7 unknown patients (P = 0.012).

Of the 41 patients, 13 patients were alive as of May 2012 (>1 year after the last patient enrollment). With a median follow-up time of 12.6 months, median PFS and median survival time (MST) for all enrolled patients were 3.0 months (95% CI, 2.0–4.1) and 12.6 months (95% CI, 6.8–19.3), respectively (Fig. 1, 2). The 1-year survival rate was 53.7% (95% CI, 38.4–68.9).

Toxicity

All 41 treated patients were assessed for toxicity. Table 4 summarizes the hematological and nonhematological toxicities. With regard to the hematological toxicities, 68% of patients experienced grade 3 or 4 neutropenia and 17% developed febrile neutropenia. Nineteen patients (46%) were treated with granulocyte-colony stimulating factor (G-CSF) for 1–11 days during the first treatment cycle due to neutropenia. Although no serious hematologic events were observed, grade 3 or 4 thrombocytopenia was observed in 5 patients (12%; 1 received a platelet transfusion) and anemia was observed in 5 patients

(12%; 2 received a packed red blood cell transfusion). The most common nonhematologic toxicities of grade 3 or 4 were anorexia (12%), infection (10%), nausea/vomiting (10%), diarrhea (2%), stomatitis (2%), and pneumonitis (2%). Most nonhematologic toxicities were mild and reversible. Neither cardiac toxicity nor treatment-related deaths were observed in this study.

Discussion

This is the first prospective phase II study designed to evaluate the efficacy and safety of a cytotoxic agent as a third- or fourth-line chemotherapy for advanced NSCLC. Our study demonstrated the efficacy of amrubicin as shown by the ORR of 9.8%, DCR of 61.0%, median PFS of 3.0 months, median OS of 12.6 months, and 1-year survival rate of 53.7% in 41 patients. Although common, hematological toxicities were manageable and nonhematological toxicities were mild and reversible. Previous phase III trials for second- or third-line treatment of NSCLC have reported ORRs of 7.6–9.1%, median OSs of 6.7–8.3 months, and 1-year survival rates of 29.7–34% (6-9, 21). Amrubicin is a

potent inhibitor of topoisomerase II, with a different mechanism of action than currently available active cytotoxic agents for advanced NSCLC (15).

Several studies have evaluated the efficacy and safety of amrubicin for advanced NSCLC. With regard to the dose of amrubicin, favorable results with tolerable toxicity were demonstrated with 45 mg/m^2 per day on 3 consecutive days every 3 weeks for first-line therapy (17, 22), 40 mg/m^2 for second-line therapy (WJTOG0401) (23), 35 mg/m^2 for primarily second- and a small number of third-line therapy (24), and 35 or 40 mg/m^2 for third-line or subsequent line therapy (25). Based upon these results, we conducted a prospective phase II trial of amrubicin 35 mg/m^2 in NSCLC patients as a third- or fourth-line therapy.

In the present study, both hematological and nonhematological toxicities were well tolerated and manageable even in the third- or fourth-line setting. However, the incidence of febrile neutropenia was certainly higher in this study (17%, 7 of 41 patients). Five patients occurred in the first cycle, one patient in the second cycle, and one patient in the fourth cycle. Previous phase II study of amrubicin at 40mg/m^2 for the second-line therapy of NSCLC (WJTOG0401)

reported 29.5% of febrile neutropenia (23). Thus, possible reasons for the higher incidence of febrile neutropenia in this study are folowing; dose of amrubicn at 35mg/m^2 might be tough in the third-line or fourth-line setting; neither prophylactical use of G-CSF nor prophylactical use of antibiotics are allowed. An adverse event of particular concern related to amrubicin administration is cardiac toxicity, which was 3.2% in previous trials (17, 22). For safety reasons, this study allowed the enrollment of only patients with a left ventricular ejection fraction of 60% as determined by echocardiography. No cardiac toxicity was observed in our trial, and there were no treatment-related deaths in this study.

The number of patients who need third-line therapy is increasing, and third-line therapy represents a clinical problem in advanced NSCLC. However, there is no standard definition for third-line therapies because a population of patients who could be grouped as potential candidates is heterogeneous (13) and clinical trial information regarding this population is sparse. Retrospective analyses from 3 institutions reported that 20.3%, 28.2%, and 38.4% of patients received third-line chemotherapy in clinical practice (11, 14, 26). These analyses

showed that the ORR was 5.6-17.0%, the DCR was 34.4–44.4%, the median PFS was 2.4 months, and the median OS was 5.8–12.0 months. Patients who received the third-line therapy benefited compared with those who did not receive the therapy.

In the second-line or later setting, MST was not associated with the ORR, but was associated with the DCR (27). Because amrubicin has a different mechanism of action compared to other anticancer agents and has a high DCR with tolerable toxicity (15, 17), we conducted the present study in which the DCR was chosen as the primary end point. The DCR was 61.0% (95% CI, 46.0–75.9), which is promising. Based upon a DCR of 33-56% in previous phase III trials for second- or third-line therapy of NSCLC (7, 8, 28, 29) and the third-line or fourth-line setting of this study, we assumed that a DCR of 50% was a desirable target and a DCR of 30% was irrelevant. The lower end of the 95% CI in the present study was higher than the threshold DCR of 30%, and the primary endpoint was met. Single agent chemotherapy or EGFR-TKI therapy had an advantage over doublet chemotherapy in prolonging PFS and decreasing