new (RECIST) criteria, although measurement criteria would be different. The mean values and ranges of intercriteria reproducibility in the response rates between UD-MLS and BD were lower and narrower than those between UD and BD (Table 3). The introduction of MLS to UD improved the intercriteria reproducibility between WHO and RECIST.

As for intercriteria reproducibility, the mean values and ranges for intraobserver reproducibility were better than those for interobserver reproducibility (Table 3). Erasmus *et al.* have suggested that consistency can be improved if the same reader carries out serial measurements for any one patient.[15]

When MLS is included in the eligibility criteria, the number of patients with measurable lesions is less than that obtained with the previous WHO criteria because patients with only small lesions are excluded from measurement. In the present study, when MLS criteria were used the number of eligible cases decreased by 6.4% from 110 to 103 and the number of target lesions by 44.6% from 402 to 223. This reduction could affect the number of patients enrolled in clinical trials.

The present study had several limitations. First, the study cohort comprised NSCLC patients only and the application of the measurement modalities was limited to chest CT. Second, intraobserver variability between evaluations with different intervals was not investigated. Third, our reference was a 10 mm slice thickness and therefore the minimum lesion size was defined as 20 mm. However, RECIST guidelines allow for a minimum lesion size of 10 mm as a slice thickness of 5 mm measured by helical CT is used. Recently, multidetector CT, which creates a thinner slice thickness, has been developed and is being used in daily clinical practice. Therefore, the addition of the outcomes of patients ineligible for our study as a result of using a thinner slice thickness might change our results and should be evaluated in a further study.

In conclusion, the results of the present study suggest that UD yields poorer interobserver reproducibility of tumor response evaluation than BD; however, if MLS is applied to UD, interobserver reproducibility can improve and become the same as that obtained with BD. The introduction of MLS to UD could also improve intercriteria reproducibility between WHO and RECIST. It is therefore essential that investigators include MLS when using RECIST guidelines to ensure interobserver reproducibility comparable with the WHO criteria.

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Seminars in Oncology

Geographic Variation in the Second-Line Treatment of Non–Small Cell Lung Cancer

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Although there is broad agreement on management options for treating different stages of non–small cell lung cancer (ie, surgery for stage I and II disease, combined treatment modalities for stage III disease, and platinum-based chemotherapy as initial treatment for appropriate patients with stage IV disease), there is considerable geographic variation in practice patterns. These variations reflect a number of factors, including health care economics, the influence of national and regional regulatory bodies, the nature of physician and patient interaction, and probable biological differences between different populations in terms of drug metabolism and inherent susceptibility to both drug activity and toxicity. The approaches taken by three different geographic regions, the United States, European Union, and Japan, are evaluated. Clinically, the most striking differences in activity and toxicity between different regions have been seen with the epidermal growth factor receptor inhibitors gefitinib and erlotinib. Japanese patients experience significantly greater response and a greater degree of interstitial lung disease than patients in the European Union and North America (ie, US and Canada). Similar differences in efficacy and toxicity have also been noted with cytotoxic chemotherapy agents in the first-line setting. These geographic and ethnic differences in toxicity and efficacy will need to be considered in the design and comparison of future clinical trials.

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Lung cancer is the most lethal malignancy in the developed world, and was expected to account for over one million deaths worldwide in 2005.[1] Non–small cell lung cancer (NSCLC) accounts for approximately 85% of these cases.[2] The vast majority of cases are secondary to tobacco use. Other etiologies include asbestos and radon exposure as well as a genetic contribution.

Although standards of care have been established for different stages of the disease, there is considerable geographic variation in practice patterns. Three major geographic factors influence the choice of second- and third-line therapy. First is the influence of the regulatory agencies that govern the approval of antineoplastic agents. Second is the influence of the specific national healthcare system, including factors governing reimbursement to patients and physicians for treatment. Finally, and most significantly, is the emerging recognition that there are biological differences between different populations in terms of drug metabolism and inherent efficacy. This article will briefly review the approaches taken to second-line therapy in three different areas of the world: the United States, European Union (EU), and Japan.

Overview of Second-Line Therapy

Docetaxel

The first agent to show unequivocal activity in the second-line treatment of NSCLC was docetaxel. A National Cancer Institute of Canada trial compared docetaxel at 75 mg/m^2 or 100 mg/m^2 versus best supportive care. This trial found superior quality and length of life for patients treated with 75 mg/m^2 docetaxel.[3] An industry-sponsored study in the United States compared docetaxel at either 75 or 100 mg/m^2 versus a physician choice of either vinorelbine or ifosfamide. Again, quality of life and survival were superior for docetaxel 75 mg/m^2.[4] The concordant results of these two trials support

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the conclusion that docetaxel 75 mg/m² every 3 weeks has a clear role in this setting. Docetaxel has been approved for treatment of previously treated NSCLC in the United States, EU, and Japan.

Pemetrexed

Pemetrexed, a new antifolate agent that has shown activity in mesothelioma, has been tested in the second-line treatment of NSCLC. A phase III trial randomizing patients to either pemetrexed (500 mg/m² every 3 weeks with vitamin B_{12} and folate supplementation) or docetaxel (75 mg/m² every 3 weeks) showed a similar level of activity but superior tolerability.[5] There was considerably less myelotoxicity and alopecia in the pemetrexed arm, and significantly fewer patients required hospitalization after treatment than with docetaxel. Activity, in terms of response rate, median survival time, and 1-year survival rate, was superimposable for pemetrexed and docetaxel. Pemetrexed has been approved in the United States and EU for the second-line treatment of advanced NSCLC.

Gefitinib

Gefitinib was the first drug to receive approval for third-line therapy of NSCLC anywhere in the world (Japan). This approval was controversial as its basis was response rate rather than a more unequivocal outcome of patient benefit, such as survival rate.[6] The drug had previously failed to show benefit (in terms of response or survival) as a first-line treatment when combined with standard chemotherapy.[7,8]

Two large phase II trials of gefitinib monotherapy, the Iressa Dose Evaluation in Advanced Lung Cancer (IDEAL) 1 and IDEAL 2 studies, evaluated the agent in pretreated NSCLC. Both studies determined response and survival. The IDEAL 1 trial, conducted primarily in Japan and Europe, also evaluated the safety profile and symptom improvement, while the IDEAL 2 trial, conducted in North America, evaluated symptom improvement as an additional primary endpoint.[9,10] The response rates for dosages of 250 mg/day and 500 mg/day were 18.4% and 19% in IDEAL 1, and 12% and 9% in IDEAL 2, respectively. Many patients, even those with a poor performance status (ie, performance status 2–3) experienced symptom improvement (most notably in pulmonary symptoms of dyspnea and chest pain) within 2 weeks of starting gefitinib treatment. This improvement in quality-of-life scales, though questionable as there was no randomization against either best supportive care or another agent, was the major impetus for granting conditional approval to market the agent in the United States. Approval was granted under the provision that appropriate randomized trials be conducted. Gefitinib has not received approval in the EU, although it has been approved in Switzerland.

Subset analysis shows that female sex, adenocarcinoma (and, in particular, bronchioloalveolar histology), and nonsmoking status are predictors of response.[10,11] Female sex was a particularly strong predictor in both IDEAL trials. In the primarily North American IDEAL 2 study, 50% of women experienced symptomatic response versus 31% of men ($P = .006$). Radiographic regression was also seen in 19% of women versus only 3% of men ($P = .001$). Two groups in Boston, MA have recently reported that mutations in the aATP-binding pocket of the epidermal growth factor receptor (EGFR) tyrosine kinase (TK) domain predict for clinical benefit from gefitinib.[12,13] While others have confirmed the presence of mutations, the role of mutations versus other alterations in *EGFR* (copy number, expression as measured by fluorescence in situ hybridization) have also been proposed as predictors of response to EGFR TK inhibitors (TKIs). It remains unclear as to whether any of these molecular variables predict independently for outcome.[14]

The role of gefitinib has recently been questioned because of the results of the Iressa Survival Evaluation in Lung Cancer (ISEL) trial.[15] This trial, undertaken in countries in which gefitinib had not received approval (ie, countries other than the United States and Japan) randomized patients between gefitinib and placebo. The ISEL trial was conducted in cooperation with 210 institutes in 28 countries (not including Japan). An advantage was shown in terms of response rate.[15] However, a trend toward improved survival did not achieve statistical significance. The subset analysis in Asian and non-Asian patients showed that female sex and adenocarcinoma histology were more common characteristics in Asian patients (Table 1). The US Food and Drug Administration has recently restricted use of gefitinib to patients who are currently being treated with the agent and who demonstrate benefit, and those enrolled in clinical trials.

Erlotinib

Erlotinib is an agent very similar to gefitinib in terms of structure and activity. It too has been evaluated as a second-line drug in the treatment of NSCLC, showing 'promising results' in terms of response and survival in phase II trials.[16]

However, unlike gefitinib, a phase III trial was unequivocally positive. The National Cancer Institute of Canada led a study (JBR-21) comparing erlotinib with best supportive care in third-line therapy. This large study (more than 700 patients) provided definitive evidence of benefit in terms of survival for this agent.[17] Improvements in response (9% v >1%), median survival (6.7 v 4.7 months; $P < .001$), 1-year survival (31% v 21%), and symptomatology (cough, dyspnea, pain) were observed.[17] Erlotinib has been approved in the United States and EU for the second- and third-line therapy of advanced NSCLC.

Geographic Variations in Treatment

Variations in the efficacy and safety of second-line NSCLC therapies have been observed across geographic regions, and have had an impact on the choice of treatment options within the three key pharmaceutical markets of the United States, the EU, and Japan.

United States

As described above, three agents have been approved by the US Food and Drug Administration for use in the second-line

Table 1 A Comparison of Gefitinib Monotherapy Data Across Geographic Regions

Characteristics	Japanese[40]	Non-Japanese[40]	American[10]	Asian[15]	Non-Asian[15]
No. of patients by gefitinib dose					
250 mg/m²	51	53	102	235	894
500 mg/m²	51	55	114	0	0
Demographics					
Median age (yrs)	60	61	61	61	62
Age range (yrs)	28–77	38–85	30–84	NA	NA
Female (%)	37	22	43	40	31
PS 0–1 (%)	91	83	80	72	64
Stage IV (%)	80	81	89	NA	NA
Adenocarcinoma (%)	76	56	66	64	44
No. of prior chemotherapy regimens (%)					
1	53	59	1	54	48
2	47	41	41	46	52
3 or more	0	0	58	0	0
Treatment efficacy					
Response rate (%)	28	10	10	12	7
Median survival (mos)	12	9.9	6–7	9.5	5.2
1-year survival (%)	50	NA	24–27	44	21
Grade 3–4 toxicity (%)					
Diarrhea	4	3	3	NA	NA
Skin rash	3	5	2	NA	NA
ALT elevation	7	1	1	NA	NA
Interstitial lung disease	2	0	0	2	0.001

Abbreviations: ALT, alanine aminotransferase; NA, not applicable; PS, performance status.

setting: docetaxel, pemetrexed, and erlotinib. Erlotinib also has approval in the third-line setting. Gefitinib, which had been granted an accelerated approval based on the phase II data from the IDEAL studies,[18] has been re-labeled in light of data from the ISEL trial.[19] At present it may only be prescribed in a non-investigational setting for patients who are already receiving the agent and who have demonstrated benefit.

Agent Selection. Controversy exists over which of the three approved agents should be used in the second-line setting. Several factors enter into consideration in the United States. First, docetaxel has also received approval as a first-line agent and is frequently used in this setting with carboplatin or cisplatin. Therefore, a patient who has already received this agent and has progressed would not be a suitable candidate to receive the drug again in the second-line setting. Second, there are no trials comparing the value (in terms of patient benefit) of any of the second-line agents in this setting. As a result, clinical judgement and economic issues are relevant. Third, there appears to be an emerging trend for physicians to use erlotinib in patients who have demonstrated the greatest degree of benefit, ie, non-smokers, women, those patients with adenocarcinoma histology, and those with Asian ancestry. It is possible that selection of patients in the future will also be driven by objective biological markers, ie, the presence of *EGFR* gene mutations or increased *EGFR* copy number. Pemetrexed is therefore used in the remaining population. For most practitioners the superimposable results in terms of survival for pemetrexed and docetaxel, coupled with its superior toxicity profile, make pemetrexed the preferred agent when both drugs are considered for second-line therapy.

Economics. Economic issues are of considerable importance given the expense of the agents. Most insurance programs in the United States will cover the cost of administration of intravenous agents but vary considerably regarding the coverage for oral agents. The cost of gefitinib (USD $2,000 to $3,000/month) is considerable. An assistance program sponsored by the manufacturer is available.

European Union

It is difficult to separate any side effects or outcome differences between the EU countries and North America. Several of the trials described above, including JBR-21 and the randomized trial of pemetrexed versus docetaxel, were conducted with significant accrual from European countries. Approvals within Europe are granted by the European Medicines Agency; a separate Committee for Proprietary Medicinal Products provides clinical expertise for the review process. Pemetrexed, erlotinib, and docetaxel are the agents currently approved in the EU for use as second-line therapy.

Japan

Japan was the first country to approve gefitinib for use in the treatment of lung cancer. Drug approvals in Japan are granted by the Ministry of Health, Labor, and Welfare. The Japanese have a significant preference for oral medications, a factor that is likely to have contributed to the rapid approval of gefitinib.[20]

Approximately 50% of the patients enrolled into the IDEAL 1 trial were Japanese.[9] The remainder were from Europe, Australia, and South Africa, and were predominantly white. Significant differences emerged regarding both efficacy and toxicity; there was no comparison of survival. The response rate was clearly higher for the Japanese (27.5% v 10.4%; $P = .0023$). There were no pharmacokinetic differences to explain this response difference. However, in a multivariate analysis, ethnicity did not emerge as an independent factor for response. Baseline factors such as performance status, sex, and histology appear to explain the ethnic differences.

In the ISEL study, the response rate and median survival time were 12% and 9.5 months in Asian patients and 7% and 5.2 months in non-Asian patients, respectively (Table 1).[15] Mutations of the *EGFR* gene, recently identified in patients with gefitinib-responsive lung cancer,[12,13] correlated well with clinical response to gefitinib and patient survival in retrospective case series studies.[21,22] The relatively high frequency of the mutations in East Asian patients (27% to 34%), compared with 14% or less in American patients, may explain the geographical difference in the efficacy of gefitinib.[12,23] The frequencies of grade 3–4 common toxicities of gefitinib, including diarrhea, skin rash, and alanine transferase elevation, were the same among the study populations (Table 1).

Treatment-Associated Interstitial Lung Disease. Because of the limited number of patients evaluated in clinical trials, it is sometimes difficult to identify and analyze uncommon toxicity before marketing a drug. Interstitial lung disease (ILD) associated with administration of gefitinib came to light in October 2002, 4 months after approval of this agent in Japan.[24] In the IDEAL studies, two Japanese patients developed grade 3–4 ILD (2%), while no patients outside Japan experienced ILD. In the ISEL study, the incidence of grade 3–4 ILD was 2% in Asian patients and .001% in non-Asian patients. In a retrospective evaluation of 112 Japanese patients, the incidence of ILD was 5.4%. The primary risk factor was a prior history of pulmonary fibrosis.[24] Between July 2002 and December 2004, there were 86,800 patients with NSCLC who were estimated to have received gefitinib in Japan. According to the Ministry of Health, Labor, and Welfare 1,473 patients were suspected of having ILD associated with the use of gefitinib and 588 patients died of ILD.[25] A prospective survey of gefitinib toxicity in 3,354 NSCLC patients treated at 698 hospitals in Japan between June and December 2003 showed that the incidence of ILD was 5.8% and the mortality rate was 2.5%.[26] Risk factors for the development of ILD identified in the Japanese population were preceding pulmonary fibrosis, smoking history, poor performance status, and male sex.[24,26,27] ILD tends to appear rapidly after initiation of therapy.[28]

In an analysis by the US Food and Drug Administration comparing the incidence of ILD associated with gefitinib treatment in North America and Japan, there was an incidence of approximately 2% from a Japanese postmarketing experience and 0.3% in approximately 23,000 patients in the United States expanded-access program.[18]

It is interesting to note that ILD has been associated with weekly docetaxel therapy in Japanese patients. In a phase II study, docetaxel as a single agent was administered at a dose of 35 mg/m^2 on days 1, 8, and 15 every 4 weeks in 48 patients with advanced or recurrent NSCLC. Of these, 33 patients had had no prior chemotherapy and 15 had received one prior chemotherapy treatment. Patients who had previously undergone thoracic radiotherapy, who had preceding ILD or pulmonary fibrosis, or who had severe pulmonary emphysema were excluded from the study. Of the 48 patients in the study, five (10.4%) developed grade 3–4 ILD.[29] The incidence of ILD associated with weekly administration of docetaxel in other countries varies with reports: grade 3–4 pulmonary toxicity was noted in seven of 35 (20%) patients in a Spanish study,[30] one of 63 (1.6%) in a French study,[31] none of 110 patients in an Italian study, and none of 30 patients in an American study.[32,33] It is unclear from these data whether the development of ILD represents a toxicity to which Japanese patients are predisposed, or is a diagnosis that is made more frequently in Japan for other reasons.

Differences in Efficacy and Toxicity. The differences between Western populations and the Japanese (and other non-Western ethnicities) in both the efficacy and toxicity of an anticancer agent are an emerging issue. Two recent trials comparing carboplatin plus paclitaxel with other combinations for first-line therapy of NSCLC were conducted in the United States (by the Southwest Oncology Group) and Japan (Japan Cooperative Oncology Group, Four Arm Comparative Study).[34] The carboplatin plus paclitaxel arm was similar in both studies (differing only by a slightly lower dose of paclitaxel in the Japanese study), and criteria for entry, dose modifications, toxicity, and response assessment were identical. Considerable differences in toxicity and activity were noted between the two studies. The rate of febrile neutropenia was five-fold greater (16% v 3%; $P < .0001$) in the Japanese trial, while the rate of neuropathy was substantially lower (5% v 16%; $P = .001$). The response rates were similar, while the 1-year survival rate was better in the Japanese trial (51% v 37%; $P = .009$).

Distribution of Genetic Polymorphisms for Thymidylate Synthase

Another area of growing interest in this field is the observation that the activity of antifolate agents may be related to germline differences in the expression of the target enzyme, thymidylate synthase (TS). Pemetrexed, though a multitargeted antifolate, appears to have its primary activity at TS. TS expression is controlled in part by the *TS* enhancer region (*TSER*) within the 5′ untranslated region of the *TS* gene. Recent work has shown that the *TSER* is polymorphic with significant ethnic variation and relates to the activity of the agents. Tandem repeats of 28 base pairs have been identified,

Table 2 Geographic Differences in the Incidence of TSER*3 Polymorphism[35]

Population	Individuals Homozygous for *TSER*3* (%)
White	28
African-American	24
Southwest Asian	40
Chinese	67

and expression of the gene is increased with additional repeats. A triple tandem repeat (*TSER*3*) demonstrates 2.6-fold greater expression than the double repeat (*TSER*2*). There is considerable variation in this polymorphism both within and between ethnic groups (Table 2).[35]

Increased expression of this enzyme can alter both the activity and pharmacology of folate antagonist agents. For example, the activity of 5-fluorouracil activity in colon cancer is influenced by the *TSER* polymorphism.[36] Patients homozygous for *TSER*3* show increased intratumoral levels of TS protein. Higher levels of TS are associated with poorer response rates and survival. In lung cancer, there is evidence from Japanese studies that elevated TS levels correlate with increased proliferation and decreased sensitivity to antifolate agents (specifically 5-fluorouracil).[37,38] Preliminary data indicate that *TS* gene polymorphisms are prognostic for patients treated with platinum-based chemotherapy.[39] Studies are currently in preparation to determine whether *TS* gene polymorphisms are a predictive or prognostic factor (or both) for treatment with pemetrexed in NSCLC.

Conclusion

Second- and third-line treatments have now emerged as a standard of care throughout the world. Regulatory agencies in the United States and EU have approved docetaxel, pemetrexed, and erlotinib for second-line use. Japan was the first country to approve an EGFR TKI (gefitinib) for second-line use. There appears to be a substantially greater response to both gefitinib and erlotinib in Japan, but also a significant risk of life-threatening pneumonitis. Moreover, this variation in efficacy and side-effect profile appears to be present in other Asian populations. These ethnic differences may be surrogates for differences in genetic aspects of drug metabolism or potential differences in tumor susceptibility. The findings of a recent 'common arm' study performed in the United States and Japan in first-line therapy, as well as the studies of the two EGF TKIs, clearly demonstrate that the benefits and risks of anticancer agents may differ between populations. It is clear that the benefits and risks of anticancer agents differ between populations.

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Review Article

Recent trends in the treatment of advanced lung cancer

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Lung cancer is one of the major causes of death in many countries because of high rates of smoking, especially in Asian countries. Lung cancer is divided into two major categories based on their biological characteristics and the selection of treatment methods: non-small cell lung cancer (NSCLC; 85%) and small cell lung cancer (15%). Early detection and complete resection are very important in NSCLC, but the cure rate is not very high, except in stage 1A disease. It is extremely important to understand the biology of lung cancer and to introduce more effective treatments in order to improve the survival of NSCLC patients. Numerous clinical trials involving lung cancer patients have led to 'state-of-the-art' treatments for each stage of the disease. Progress in chemotherapy and molecular target based therapy have altered the standard therapy for NSCLC. (*Cancer sci* 2006; 97: 448–452)

Chemotherapy for advanced non-small cell lung cancer

Platinum-based doublets are considered to be the standard treatments for stage IV non-small cell lung cancer (NSCLC).[1,2] Although the majority of regimens contain cisplatin, carboplatin can be used in combination with paclitaxel because numerous phase III data exist on this combination. The question remains, however, as to whether or not we can treat advanced NSCLC patients with a non-platinum-based regimen. To date, the answer would appear to be that platinum-based therapy is superior, although platinum drugs and/or non-platinum doublets could be used to treat elderly and/or frail patients because of their low renal toxicity. Kosmidis, the chairman of the Hellenic Cooperative Oncology Group, reported the results of their randomized controlled trials looking at the combination of paclitaxel/gemcitabine versus carboplatin + gemcitabine in advanced NSCLC. More than 500 patients were accrued, of which 445 were evaluative. There was no difference in response rate, time to progression or median survival. There was slightly more hematological toxicity with carboplatin and gemcitabine, although it was relatively mild with only 28% having grade 3 and 4 neutropenia. There was slightly more neurotoxicity in the paclitaxel and gemcitabine arm, and the difference was statistically significant. Kosmidis concluded that this was enough evidence to show that non-platinum-based chemotherapy is as good as platinum-based chemotherapy.[3] However, no studies have demonstrated the superiority of a non-platinum doublet over a platinum-based doublet.

Several doublets that include new drugs improve survival, but no one regimen is clearly superior to the others.[1,2] We have conducted a four-arm cooperative study (FACS) in advanced NSCLC. The study was designed to demonstrate non-inferiority of three experimental arms: paclitaxel + carboplatin; gemcitabine + cisplatin; and navelbine + cisplatin in comparison with cisplatin + CPT-11 (control arm). One-year survival (59%) was higher than expected in cisplatin + CPT-11. No statistically significant differences in response rate, time to progression (TTP) or overall survival were observed between the reference and experimental regimens. Non-inferiority of the three experimental arms was not demonstrated. The response duration in the cisplatin + CPT-11 arm was relatively longer than in the other three arms. No statistical test was conducted because these data were obtained from selected populations based on response, such that there is no statistical basis for comparison (Ohe Y *et al.*, unpublished data, 2006). In conclusion, all four platinum-based doublets have similar efficacy for advanced NSCLC but with different toxicity profiles. Cisplatin + CPT-11 is still regarded as the reference regimen in Japan.

The chemotherapy outcomes were compared in Japanese and American NSCLC patients accrued to the FACS trial and the SWOG 0003 trial,[4] respectively. The two trials had similar eligibility and evaluation criteria, although the dose of paclitaxel was 200 mg/m^2 in the Japanese trial and 225 mg/m^2 in the SWOG trial. The purpose of the analysis was to demonstrate similarities and differences in patient characteristics and outcomes between the Japanese and USA trials for advanced stage NSCLC treated by the same regimen, to provide a basis for standardization of the study design/process to facilitate interpretation of future trials, and to take the first step toward possible joint NCI-sponsored studies in lung cancer between Japanese and American investigators. This analysis using carboplatin and paclitaxel as the common arm shows great similarities in patient characteristics between the FACS trial and the SWOG 0003 trial. Frequencies of neutropenia and febrile neutropenia were significantly higher in FACS trials although the paclitaxel dose was lower

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in this group. There may be some differences in population-based pharmacogenomics. Grade 3/4 neuropathy, conversely, was more frequent in the SWOG 0003 trial due to differences in the cumulative paclitaxel dose because of the higher absolute dose and higher median numbers of treatment courses. The response rates were exactly the same, but 1 year survival was better in the FACS trial. These results suggest that future joint Japan–USA clinical trials should consider possible pharmacogenomic differences in drug disposition between Japanese and American populations.[5]

Molecular target-based drugs in advanced recurrent NSCLC

Numerous molecular target-based drugs have been introduced for the treatment of NSCLC, but can they replace current therapy? Can they be used as an adjuvant to current therapy? Can they be combined with other chemotherapeutic agents, radiotherapy and/or surgery?

We hypothesize that incorporation of novel molecular target-based therapies into current treatment paradigms will improve outcomes. However, carefully designed clinical trials and translational science will be required to identify subsets of patients who will benefit.

If we are to use them, we must first answer the following critical questions. Is the target required for a response? Whether or not we know a real and correct molecular target is still questionable. Is the presence of the target sufficient for a response, and can we measure the target in a biologically relevant and/or technologically valid way? Does the agent inhibit the proposed target at the dose and schedule used? Is the target a critical driving force for cell growth in the tumor type in question? The answers to these questions are crucial to treatment with molecular target-based drugs.

Various molecular target-based drugs for advanced NSCLC have been evaluated in randomized controlled trials, but the majority, including a matrix metalloprotease inhibitor, a protein kinase C inhibitor and trastsuzumab, have yielded negative results.[6–8] Gefitinib is an orally available selective epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor that exhibits antitumor activity in patients with previously treated advanced NSCLC.

Clinical trial of gefitinib and erlotinib

Four open-label multicenter phase I studies have identified diarrhea, skin rash/acne and nausea as common adverse events.[9,10] Two large-scale, multicenter randomized controlled phase II trials, IDEAL 1 and 2, have demonstrated clinically significant antitumor activity of gefitinib monotherapy, and erlotinib has also shown promising antitumor activity.[11] Neither drug showed any additive and/or synergistic effect when combined with platinum-based chemotherapy as a first-line treatment for NSCLC.[12,13]

On December 17, 2004, AstraZeneca announced the preliminary results of their Iressa Survival Evaluation in Lung Cancer (ISEL) study. The study had accrued 1692 patients with advanced recurrent/refractory NSCLC. Unfortunately, Iressa failed to significantly prolong survival compared with a placebo (HR = 0.89, $P = 0.087$) in the overall patient population or among patients with adenocarcinoma (HR = 0.83, $P = 0.089$), although a tendency toward a survival benefit was observed in the gefitinib group.[14] The less than 10% response rate did not result in an overall prolongation of survival. A retrospective analysis of patients treated with gefitinib in clinical practice showed that tumor response predictors included 'adenocarcinoma', 'no history of smoking', 'women', and 'Japanese'. Survival in the gefitinib group in the ISEL study was significantly higher for non-smokers ($P < 0.01$) and Asians ($P < 0.01$) than in the placebo group. The survival curves of the two treatment groups were the same for non-Asians. The data obtained from the ISEL study were not surprising, although most observers had expected positive overall results.

The results of similar randomized trials of erlotinib (BR21) were presented at the American Society Clinical Oncology (ASCO) meeting in 2004. Erlotinib significantly prolonged survival in patients with advanced, previously treated refractory/recurrent NSCLC.[15] The two studies referred to above differed in several respects. Sample size was larger in the ISEL study than in the BR21 study, and 10% of the patients in the latter study had a performance status (PS) of 3, whereas only PS-2 patients were accrued by the ISEL study. The follow-up period of the ISEL study was also relatively short (4 months). The overall percentage of patients with adenocarcinoma and the percentage of non-smokers was 50% and 20%, respectively, in both studies. Data stratification into Asians and non-Asians was only performed in the ISEL study. The stratified survival data for Asians in the BR21, submitted to the US FDA, showed a tendency that was similar to the stratified data in the ISEL study. The survival of non-smokers in the erlotinib group in the BR21 study was extremely good and contributed to the improvement in overall survival in the erlotinib group. How can we explain the discrepancy of the result from the ISEL and BR21 studies? Part of the explanation is that the dose of gefitinib in the ISEL study was low, while the BR21 study used nearly the maximum tolerated dose. Another hypothesis is that patient populations in the ISEL study were inappropriately selected, for example, subjects with poor prognostic factors. The shapes of the survival curves for the Intact 1 and 2, TALENT and TRIBUTE studies and for the non-Asians in the ISEL study suggest that EGFR-TKI does not prolong the survival of non-Asian patients with NSCLC, with or without prior chemotherapy.[12,13,16,17] The stratified survival data of the Asians in the Intact 1 and 2, TALENT and TRIBUTE studies should be analyzed.

In the SWOG 0023 trial, patients with stage III NSCLC received chemoradiation therapy then three cycles of a single agent, docetaxel, followed by either a placebo or gefitinib as maintenance. This trial was projected to have 80% of the patients receiving either placebo or gefitinib with a drop off of 20% during this part of the therapy. The drop off rate before randomization was a bit larger than the expected rate because of progressive disease or death. Investigators asked the Data Safety Monitoring Committee to look at the data to see if they should actually continue the trial because the results of the ISEL study were negative. This early unplanned analysis showed there was no difference in time to progression in either arm and the P-value for difference was 0.54. Similarly, there was no statistically significant difference in

survival and the *P*-value was 0.09, favoring the placebo group. It was surprising and disappointing that the gefitinib-treated patients were actually experiencing worse survival than the placebo patients. This trial had the power to show a 0.33% advantage for gefitinib and the data were sufficient to state that the likelihood of showing a 33% survival improvement was 0.0015.[18] These data suggested that there is no rationale for using gefitinib in locally advanced NSCLC in the adjuvant setting.

Molecular marker predicting clinical outcome of EGFR-TKI

The activities of epidermal growth factor receptor (EGFR) inhibitors, gefitinib and erlotinib in lung cancer and the correlation of responses to somatic mutations are the focus of translational research performed in 2004 and 2005. This answers the major question; which patients respond and why? We have demonstrated that PC-9 cells with a 15 bp deletion in exon 19 of the EGFR gene are extremely sensitive to EGFR-TKI.[19] In April and May 2004, Paez and Lynch reported that activating mutations in EGFR are present in a subset of NSCLC tumors and that the tumors are highly sensitive to gefitinib and erlotinib.[20,21] EGFR expression levels are not a predictor of response and EGFR amplification may have an impact, but EGFR-TK mutations seem to be better predictors of responsiveness to gefitinib and erlotinib.[22–24] Mutant EGFR are more sensitive to ligand stimulation and are dramatically more sensitive to EGFR-TKIs.[19–21] The incidence of EGFR mutations is reportedly higher in Asians, including Japanese,[25,26] and Mitsudomi has reported cumulative percentages of those with EGFR mutation-positive status in 1104 patients with NSCLC to be 34% among Asians and 8% among non-Asians.[27] Eighty percent of the patients who responded to EGFR-TKI carried an EGFR mutation (non-Asians, 79% [30/35]; Japanese, 81%: [39/48]). Among non-responders, 0% of non-Asians and 21% of Japanese patients carried an EGFR mutation. These data suggest that the presence of an EGFR mutation is a strong predictor of a favorable response to EGFR-TKI. Mutations have been reported to be significantly more frequent in women, in patients with adenocarcinoma, and in never smokers, and these findings are consistent with the clinical predictors of tumor response in patients treated with EGFR-TKI. Mitsudomi recently reported that the del 746–750 mutation might be superior to the L858R mutation for predicting the gefitinib response and those patients with EGFR mutations survived longer after the initiation of gefitinib treatment than those without mutations.

Recently it has been demonstrated that an additional mutation at codon 790 induced resistance to originally sensitive mutant cells.[28,29]

A variety of results were presented at the ASCO 2005 meeting in Orland with regards to molecular analysis of the EGFR gene and protein expression in patients accrued to pivotal studies of EGFR-TKIs.[30] Lynch reported the results of an analytical study using resected specimens and biopsy samples obtained during IDEAL and INTACT studies of gefitinib.[31] Patients with either an EGFR mutation or amplification represented distinct populations. Among cases with mutations, large numbers were female, non-smokers, had adenocarcinoma or bronchioloalveolar carcinoma, were Eastern-Asian and often showed dramatic response rates to gefitinib. Because the number of cases for this analysis was not sufficient, it was impossible to draw any conclusions about the impact of mutation and amplification on survival.

Tsao tried to identify certain relations among the response rate and survival and molecular biological features such as the mutation, protein expression and gene copy numbers in the BR21 study conducted by NCI-Canada clinical trial group, which demonstrated that erlotinib does significantly prolong survival as compared with a placebo. Response rates were higher in patients with EGFR mutations, immunohistochemistry (IHC)-positive tumors and high gene copy numbers, but a statistically significant difference was observed for copy numbers only. Survival benefit was greater in patients who were IHC positive and had high gene copy numbers. However, mutation positive patients did not benefit more than mutation negative patients. From these data, Tsao concluded that mutation analysis is not required for the selection of patients who will receive erlotinib.[32]

There are some controversial data on the relationship between biomarkers and clinical outcome.[33–37] One of the reasons for discrepant data is the validity of techniques including the quality of the samples analyzed. Giaccone conducted a cross validation analysis of EGFR mutations in samples obtained from the Free University (the Netherlands) and the Dana Faber Cancer Institute.[38] The results were discrepant in some samples because of poor quality. Another reason is patient selection because it was impossible to obtain samples from all patients with advanced lung cancer. In the retrospective studies reported to date, only a small proportion of patients have had tumor samples evaluable for each biomarker, making patient selection problematic and prone to the introduction of selection bias. It is therefore extremely important that samples be obtained from all patients in studies evaluating the relationships between clinical outcome and biomarkers such as EGFR expression, amplification and mutation. Of course, the techniques for evaluable biomarkers should be valid. In this regard, the report of Takano is most reliable because they analyzed all the samples from all patients using three techniques: IHC, gene copy number and mutation. There were no problems with patient selection. Because they used surgically resected specimens they were able to obtain adequate specimen amounts. It could be concluded that if the analyses were conducted accurately, EGFR mutational status would be the major predictor of outcome and increased EGFR copy number associated with gefitinib sensitivity would significantly depend on the presence of EGFR mutations.[39] Technical innovations are essential for the reproducible and reliable analysis of samples from advanced disease patients because only small amounts of the specimen could be obtained from inoperable lung cancer patients.

EGFR-TKI seems to be a very promising drug for the treatment of East-Asian patients with NSCLC with and without a history of prior chemotherapy. The response rate has ranged from 20% to 33% clinically, and it was 30% in a prospective phase II trial on 100 previously untreated NSCLC patients. The median survival time of the Japanese population in the IDEAL 1 trial was 13.8 months.[11] To date, no survival

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data from a phase III study of gefitinib and erlotinib in East Asia are available because no phase III study has been conducted. However, a randomized controlled trial comparing gefitinib and docetaxel as a second-line treatment is in progress in Japan. The trial has a non-inferiority design and a definitive conclusion will be difficult to obtain. An erlotinib phase II evaluation has just finished the accrual of patients in Japan, but government approval will require more time.

The frequency of EGFR mutations and response rate are higher in East-Asian populations than in Western countries. A global randomized controlled trial is scheduled for comparison of first-line standard platinum-based chemotherapy versus gefitinib in East Asians, non-smokers versus light smokers, and patients with adenocarcinoma.

Bevacizumab

Vascular endothelial growth factor (VEGF) was originally described as vascular permeability factor. VEGF is involved in the regulation of new vessel growth, promotion of the survival of immature vasculature and binding to one of two receptors such as FLT-1 or KDR.[40]

Bevacizumab is a monoclonal antibody against VEGF. It is 93% human, it recognizes all isoforms of VEGF-A and has a prolonged half life which makes it very convenient to administer on an every 2- or 3-week basis.

The preliminary randomized phase II trial of ECOG using 7.5 mg/kg or 15 mg/kg of bevacizumab every 3 weeks did meet its primary objective of improvement in time to progression on the high dose arm; 7.4 versus 4.2 months. Also, response and survival were numerically better. Problems with hemoptysis or pulmonary hemorrhage occurred in six patients (four squamous cell and two adeno), four of which actually proved to be fatal.[41] Based on these experiences, the ECOG 4599 trial was designed. The primary objective was to compare survival and secondary objectives were to look at the response rate, time to progression and toxicity.

Eligibility criteria included non-squamous cell carcinoma, no history of major hemoptysis and of neither thrombotic nor hemorrhagic disorders, and no central nervous system metastasis. Patients received standard dose carboplatin and paclitaxel with or without high dose bevacizumab 15 mg/kg every 3 weeks. The sample size was calculated to be over 842, providing the investigators with 80% power to detect a 25% improvement in median survival time from the usual 8–10 months. ECOG had two planned interim analyses at 286 and 455 deaths. The study was closed after the second interim analysis. Response rate was significantly higher in the bevacizumab arm (27%) versus the control arm (10%). Progression free survival also favored the bevacizmab arm. Overall survival was highly statistically significant; 12.5 months in the bevacizumab arm and 10.2 months in the control arm. The hazard ratio was 0.77.[42] Hemorrhage was more common in the bevacizumab arm with a 45% incidence compared to less than 1% in the control arm. There were eight treatment-related deaths in the bevacizumab arm and two in the control arm. These data lead to the conclusion that bevacizumab improves survival compared to platinum and paclitaxel in patients with non-squamous NSCLC, although a small increase in severe bleeding can be expected. ECOG considers paclitaxel, carboplatin with bevacizumab to be a standard for the treatment of this NSCLC subgroup. The study group suggested some future plans for combining bevacizumab with chemotherapy, radiotherapy and other targeted agents in neoadjuvant or adjuvant settings. In Europe, a clinical trial of bevacizumab combined with cisplatin + gemcitabine is ongoing. The critical question is whether or not they can obtain reproducible positive data even if the chemotherapy regimen is changed from pacltiaxel + carboplatin to cisplatin + gemcitabine. In Japan, a combination phase I/II study of bevacizumab with 5FU + LV or FOLFOX recently completed the accrual of patients. Combination treatment using bevacizumab with paclitaxel + carboplatin is scheduled. How to manage severe bleeding, even in selected populations, and the extremely high cost of bevacizumab will be major issues.

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Effects of different combinations of gefitinib and irinotecan in lung cancer cell lines expressing wild or deletional EGFR

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Summary EGFR mutations are a major determinant of lung tumor response to gefitinib, an EGFR-specific tyrosine kinase inhibitor. Obtaining a response from lung tumors expressing wild-type EGFR is a major obstacle. The combination of gefitinib and cytotoxic drugs is one strategy against lung cancers expressing wild-type EGFR. The DNA topoisomerase inhibitor irinotecan sulfate (CPT-11) is active against lung cancer. We examined the sensitivity of lung cancers expressing wild- or mutant-type EGFR to the combination of gefitinib and CPT-11. The in vitro effect of gefitinib and SN-38 (the active metabolite of CPT-11) was examined in seven lung cancer cell lines using the dye formation assay with a combination index. When administered concurrently, gefitinib and SN-38 had a synergistic effect in five of the seven cell lines expressing wild-type EGFR, whereas the combination was antagonistic in PC-9 cells and a PC-9 subline resistant to gefitinib and expressing deletional mutant EGFR (PC-9/ZD). When administered sequentially, treatment with SN-38 followed by gefitinib had remarkable synergistic effects in the PC-9 and PC-9/ZD cells. In an in vivo tumor-bearing model, this combination had a schedule-dependent synergistic effect in the PC-9 and PC-9/ZD cells. An immunohistochemical analysis of the tumors in mice treated with CPT-11 and gefitinib demonstrated that the number of Ki-67 positive tumor cells induced by CPT-11 treatment was decreased when CPT-11 was administered in combination with gefitinib. In conclusion, the sequential combination of CPT-11 and gefitinib is considered to be active against lung cancer.

1. Introduction

Lung cancer is one of the leading causes of cancer-related death, despite the use of conventional chemotherapy regi-

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mens. The epidermal growth factor receptor (EGFR) is frequently expressed in non-small cell lung cancer (NSCLC) and is correlated with a poor prognosis. Gefitinib ('Iressa') is an orally active, selective EGFR-tyrosine kinase inhibitor that blocks signal transduction pathways. Its clinical efficacy has been show in refractory NCSLC patients, but the survival benefit of this agent remains unclear. EGFR mutations have been identified in NSCLC, and lung cancers carrying the EGFR mutation have been reported to be hyperresponsive to gefitinib [1,2]. Mutant EGFR is a major determinant of lung tumor response to gefitinib, but the hyperresponsiveness of tumors expressing mutant EGFR has been observed in a small population. Now, obtaining a clinical benefit in lung tumors expressing wild-ype EGFR is a major obstacle. The combination of gefitinib and cytotoxic drugs is one strategy against lung cancers expressing wild-type EGFR. The DNA topoisomerase I inhibitor irinotecan (CPT-11) is a key drug in the treatment of patients with lung cancer and has been shown to prolong survival. SN-38 is the active metabolite of CPT-11 in vitro. The objective of this study was to determine the potential therapeutic utility of gefitinib when combined with CPT-11 therapy to lung cancer cell according to the treatment schedule and EGFR status.

Acquired resistance to gefitinib is also of clinical interest. Recently, Kobayashi et al. [3] reported that an EGFR mutation was related to the development of acquired resistance to gefitinib. We have established subclone PC-9/ZD cells that are resistant to gefitinib [4]. Our results suggested that another mechanism of resistance was active in PC-9/ZD cells. The effect of the combination of gefitinib and SN-38 in these PC-9/ZD cells was also examined.

2. Materials and methods

2.1. Drugs and chemicals

Gefitinib (*N*-(3-chloro-4-fluorophenyl)-7-methoxy-6-[3-(morpholin-4-yl)propoxy]quinazolin-4-amine) was provided by AstraZeneca (Cheshire, UK). Gefitinib was dissolved in dimethyl sulfoxide (DMSO) for the in vitro study. CPT-11 and SN-38 were obtained from Yakult Honsha (Tokyo, Japan) and were dissolved in dimethyl sulfoxide (DMSO) for both of the in vitro studies.

2.2. Cells and cultures

Human NSCLC cell lines PC-9, PC-7, and PC-14 derived from untreated patients with pulmonary adenocarcinoma were provided by Professor Y. Hayata, Tokyo Medical College. A small cell lung cancer cell line, H69, was established at the National Cancer Institute (Bethesda, MD, USA). The gefitinib-resistant subline, PC-9/ZD, was established from intrinsic hypersensitive cell PC-9 [5] in our laboratory [4]. A small cell lung cancer cell line, SBC-3, and an adenocarcinoma cell line, A549, were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). All cell lines were maintained in RPMI1640 (Nikken Bio Med. Lab., Kyoto, Japan) supplemented with 10% heat-inactivated fetal calf serum, 100 μg/ml streptomycin, and 100 units/ml penicillin in an incubator at 37 °C and 100% humidity in 5% CO_2 and air, as described previously [6].

2.3. RT-PCR

Specific primers designed for EGFR CDS were used to detect the EGFR mRNA, as described elsewhere [1]. Sixteen first-strand cDNAs were synthesized from the cells' RNA using an RNA PCR Kit (TaKaRa Biomedicals, Ohtsu, Japan). After the reverse transcription of 1 μg of total RNA with Oligo(dT)-M4 adaptor primer, the whole mixture was used for PCR with two oligonucleotide primers (5′-AATGTGAGCAGAGGCAGGGA-3′ and 5′-GGCTTGGTTTGGAGCTTCTC-3). PCR was performed with an initial denaturation at 94 °C for 2 min and 25 cycles of amplification (denaturation at 94 °C for 30 s, annealing at 55 °C for 60 s, and extension at 72 °C for 105 s).

2.4. Western blot analysis

The cultured cells were washed twice with ice-cold phosphate buffered saline (PBS), lysate in EBC buffer (50 mM Tris–HCl, pH 8.0; 120 mM NaCl; 0.5% Nonidet P-40; 100 mM NaF; 200 mM Na orthovanadate; and 10 mg/ml each of leupeptin, aprotinin and phenylmethylsulfonyl fluoride). The lysate was cleared by centrifugation at 20,000 × *g* for 5 min, and the protein concentration of the supernatant was measured using a BCA protein assay (Pierce, Rockford, IL, USA). For immunoblotting, 20 μg samples of protein were electrophoretically separated on a 7.5% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The membrane was then probed with rabbit polyclonal antibodies against EGFR, HER2/neu, Her3 and Her4 (Santa Cruz Biotech, Santa Cruz, CA, USA) and phospho-EGFR specific for Tyr 845, Tyr 1045, and Tyr 1068 (numbers 2231, 2235 and 2234; Cell Signaling, Beverly, MA, USA).

2.5. Growth-inhibition assay

We used the tetrazolium dye (3,(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, MTT) assay to evaluate the cytotoxicity of various drug concentrations. After incubation for 72 h at 37 °C, 20 μl of MTT solution (5 mg/ml in PBS) was added to each well; the plates were then incubated for a further 4 h at 37 °C. After centrifuging the plates at 200 × *g* for 5 min, the medium was aspirated from each well and 180 μl of dimethylsulfoxide was added to each well to dissolve the formazan. Optical density was measured at 562 and 630 nm using a Delta Soft ELISA analysis program interfaced with a Bio-Tek Microplate Reader (EL-340; Bio-Metallics, Princeton, NJ, USA). Each experiment was performed in six replicate wells for each drug concentration and was independently performed three or four times. The IC_{50} value was defined as the concentration needed for a 50% reduction in the absorbance, as calculated based on the survival curves. Percent survival was calculated as follows:

$$\frac{\text{Mean absorbance of six replicate wells containing drugs} - \text{mean absorbance of six replicate background wells}}{\text{mean absorbance of six replicate drug-free wells} - \text{mean absorbance of six replicate background wells}} \times 100.$$

2.6. Combined effect of gefitinib and SN-38 in vitro

After 24 h of incubation, gefitinib and SN-38 were added to each cell line according to one of the two combination schedules. For the concurrent schedule, gefitinib and SN-38 were added concurrently and were then incubated under the same conditions for 72 h. For the sequential schedule, gefitinib or SN-38 were added sequentially and were then incubated under the same conditions for 72 h. The combined effect of gefitinib and SN-38 on lung cancer cell growth was evaluated using a combination index (CI) [7]. The CI was produced using CalcuSym software (Biosoft, NY, USA). For any given drug combination, the CI represents the degree of synergy, additivity, or antagonism. CI was expressed in terms of fraction-affected (F_a) values, which represents the percentage of cells killed or inhibited by the drug. Using mutually exclusive ($\alpha = 0$) or mutually non-exclusive ($\alpha = 1$) isobologram equations, the F_a/CI plots for each cell line were constructed by computer analysis of the data generated from the median effect analysis. The CI values were interpreted as follows: <1.0 = synergism; 1.0 = additive; >1.0 = antagonism.

2.7. In vivo growth-inhibition assay

Experiments were performed in accordance with the United Kingdom Coordinating Committee on Cancer Research Guidelines for the welfare of animals with experimental neoplasia (second edition). Fig. 2A shows the treatment schedule. For the in vivo experiments, the combined therapeutic effect of orally or intraperitoneally administered gefitinib and intravenously injected CPT-11 was evaluated according to a predetermined schedule. The dose of each drug was set based on the results of a preliminary experiment involving the administration of each drug alone. Ten days before administration, PC-9 and PC-9/ZD cells were injected subcutaneously into the backs of the mice. Six mice per group were injected with tumor cells. Tumor-bearing mice were given either gefitinib (40 mg/kg/day, p.o.) on days 2–6, CPT-11 (50 mg/kg/day, i.v.) on day 1, both, or a placebo (5%(w/v) glucose solution). Alternatively, tumor-bearing mice were given gefitinib on days 2–6 and CPT-1 on days 2. The diameters of the tumors were measured using calipers on days 1, 5, 8, 12, 15 and 20 to evaluate the effects of treatment, and tumor volume was determined using the following equation: tumor volume $ab^2/2$ (mm^3) (where a is the largest diameter of the tumor and b is the shortest diameter). Day 20 denotes the day on which the effects of the drugs were estimated, and day "0" denotes the first day of treatment. All mice were sacrificed on day 20 after their tumors had been measured.

2.8. Immunohistochemistry

The tumors were harvested from the mice at the time of sacrifice. For hematoxylin-eosin (HE) and anti-CD31 and Ki-67 staining, the resected tumors were fixed in zinc-buffered formalin (Shandon Lipshaw, Pittsburgh, PA) overnight at 4 °C. After paraffin embedding and sectioning at 6 μm, formalin-fixed sections were stained with Mayer's H&E (Richard Allen, Kalamazoo, MI, USA). For anti-Ki-67 and anti-CD31 immunohistochemistry, the slides were heated in a water bath at 95–99 °C in Target Retrieval Solution (DAKO, Carpinteria, CA, USA) for 20 min, followed by a 20-min cool-down period at room temperature. After heat retrieval, the sections were rinsed well in PBS and stained with rabbit antihuman Ki-67 antigen (DAKO N-series, ready to use) or rat antimouse CD-31 antibody (BD PharMingen, Tokyo, Japan) according to the manufacturer's instructions and then were lightly counterstained with Mayer's hematoxylin. The sections were finally stained with an in situ Death Detection POD Kit (Roche Diagnostic GmbH, Mannheim, Germany), according to the manufacturer's instructions.

TUNEL staining was performed using the Apoptosis Detection System, Fluorescein (Promega, Madison, WI, USA). Briefly, 6-μm cryostat sections were fixed in 4% paraformaldehyde for 10 min at room temperature and rinsed in PBS with 0.1% Triton X-100. The sections were then incubated in Equilibration Buffer for 5 min at room temperature followed by incubation in TUNEL Mix, prepared according to the manufacturer's instructions, for 1 h at 37 °C. After successive washes in PBS, the sections were coverslipped using an antifade reagent.

Microvessel density was determined by calculating the proportion of CD31-positive cells. The Proliferation Index was determined by Ki-67 immunostaining and calculating the population of Ki-67-positive cells in five fields at 200×. The Apoptosis Index, determined by TUNEL staining, was calculated from the population of TUNEL-positive cells in five fields at 200×. The apoptosis:proliferation ratio equals the apoptosis index/proliferation index × 100. At least 1000 tumor cell nuclei from the most evenly and distinctly labeled areas were examined in each examination.

At least 1000 cancer cells were counted and scored per slide. Both the percentage of specifically stained cells and the intensity of immunostaining were recorded. Blood vessels were detected with an anti-von Willebrand factor (vWF) antibody (Chemicon). Microvessel density was determined by calculating the proportion of vWF-positive cells.

3. Results

3.1. Expression of Her-receptors and cellular sensitivity to gefitinib or SN-38 in lung cancer cell lines

The expression levels of EGFR in seven lung cancer cell lines were examined using RT-PCR with a primer set for exon 20 in EGFR. PC-14, SBC-3, H69, PC-7, and A549 cells showed a 570-bp-long PCR amplified product exhibiting wild-type EGFR mRNA (data not shown). On the other hand, a smaller PCR product was also detected in the PC-9 and PC-9/ZD cells, and this band was confirmed to be an in-frame 15-base deletion of exon 20 (E746_A750del).

We examined the protein levels of EGFR, Her2, Her3, and Her4 in the lung cell lines using immunoblotting. The quantitative data obtained by densitometorical analysis is summarized in Table 1. The protein levels of EGFR, Her2, and Her3 in the PC-9 cells were one- to four-fold higher than those in the other cell lines (PC-7, H69, PC-14, A549, and SBC-3).

Table 1 Comparison of Her family protein levels and gefitinib- and SN-38-induced growth inhibition

Cell lines	Relative expression[a]				Growth inhibition[b], $IC_{50} \pm S.D.$	
	EGFR	Her2	Her3	Her4	Gefitinib (μM)	SN-38 (nM)
PC-9	2.8[c]	3.2	3.7	ND	0.047 ± 0.061	8.09 ± 1.9
PC-9/ZD	1.6[c]	2.6	3.8	ND	7.7 ± 0.5	38.9 ± 7.0
PC-14	1.5	2.8	1.1	ND	17.1 ± 0.8	42.1 ± 2.6
SBC-3	2.4	2.6	1.0	ND	19.9 ± 5.4	1.07 ± 0.1
A549	2.3	2.3	1.4	ND	30.2 ± 2.2	293 ± 64.5
H69	1.3	1.3	2.0	ND	56.5 ± 3.2	27.2 ± 4.1
PC-7	1.0	1.0	1.2	ND	68.8 ± 14.8	20.5 ± 8.2

The IC_{50} value (μM) of each drug was measured by MTT assay, as described in Section 2. Each value is the mean ± S.D. of three or four independent experiments.

[a] Protein expression levels were analyzed by Western blotting.

[b] Drug concentration responsible for 50% growth inhibition in MTT assay at 72 h, calculated data for at least three dependent experiments.

[c] 15-base deletion EGFR, ND: not determined.

3.2. Cellular sensitivity of lung cancer cells to gefitinib and SN-38

The growth inhibitory effect of gefitinib and SN-38 on lung cancer cells was examined using an MTT assay. The IC_{50} values of gefitinib for the cell lines ranged from 46 nM (PC-9 cells) to 68 μM (PC-7 cells). The PC-9/ZD cells were ~200-fold resistant to gefitinib, compared with the parental PC-9 cells. Cellular sensitivity to gefitinib and the expression levels of EGFR and Her2 were negatively correlated with the IC_{50} values of gefitinib (Table 1). The IC_{50} values of SN-38 for these cell lines ranged from 1 nM (SBC-3) to 300 nM (A549). The range of sensitivity to gefitinib was wider than that to SN-38. No correlation in cellular sensitivity to gefitinib and SN-38 was seen.

3.3. In vitro combined effect of gefitinib and SN-38 on lung cancer cell lines

To evaluate the potential combined effect of gefitinib and SN-38, the combination index was determined using an MTT assay. The combined effects of gefitinib and SN-38 under the concurrent schedule are shown in Fig. 1. CI values of <1, >1, and 1 indicate a supra-additive effect (synergism), an antagonistic effect, and an additive effect, respectively. An additive to supra-additive growth-inhibitory effect was observed for all doses of gefitinib and SN-38 tested in cell lines expressing wild-type EGFR. On the other hand, a high CI index was observed in PC-9 cells and PC-9/ZD cells expressing mutant EGFR over a wide range of inhibition levels. These results suggest that gefitinib and SN-38 are synergistic in lung cancer cells expressing wild-type EGFR but not in cell lines expressing mutant EGFR in vitro.

3.4. Schedule-dependent synergy of gefitinib and SN-38 in lung cancer cells

Next, we examined the schedule dependency of the combined effects of gefitinib and SN-38 in the cell lines. The five cell lines expressing wild-type EGFR showed synergistic (PC-14, H69, and A549 cells) or additive effects (SBC-3 and PC-7 cells) for all three schedules: concurrent administration, SN-38 followed by gefitinib administration, and gefitinib followed by SN-38 administration (Fig. 1A). In the PC-9 cells, concurrent administration and gefitinib followed by SN-38 administration were antagonistic, but SN-38 followed by gefitinib administration was synergistic (Fig. 1B). In the PC-9/ZD cells, concurrent administration was antagonistic, but sequential administration was synergistic. These schedule-dependent combined effects were observed in the cells expressing mutant EGFR.

3.5. Combined effects of gefitinib and SN-38 in vivo

To estimate the schedule-dependent effects in vivo, nude mice bearing tumors were treated with gefitinib and CPT-11 according to sequential or concurrent schedules (Fig. 2A). Mice bearing PC-14 tumors were treated with gefitinib and CPT-11 according to sequential or concurrent schedules. CPT-11 (50 mg/kg) alone potentially reduced the tumor size, and the combination of gefitinib and CPT-11 was synergistic. In particular, the administration of CPT-11 followed by gefitinib cured the mice bearing PC-14 cells (Fig. 2B).

Mice bearing PC-9 or PC-9/ZD tumors were treated with gefitinib and CPT-11 according to sequential or concurrent schedules. Gefitinib (40 mg/kg) alone potentially reduced the PC-9 tumors, and CPT-11 (50 mg/kg) followed by gefitinib administration reduced the tumor size of PC-9 xenografts more dramatically (gefitinib alone: $P = 0.012$, sequential combination: $P = 0.005$) (Fig. 2B). On the other hand, the concurrent schedule produced an antagonistic effect. Body weight loss was not observed in any of the mice treated according to the above schedules (Fig. 2C). CPT-11 followed by gefitinib administration is a potentially beneficial schedule against PC-9 and PC-9/ZD cells expressing mutational EGFR. The results of these in vivo experiments were consistent with those of the in vitro studies.

To elucidate the synergistic mechanisms of CPT-11 and gefitinib in vivo, tumor samples of the PC-9 and PC-9/ZD

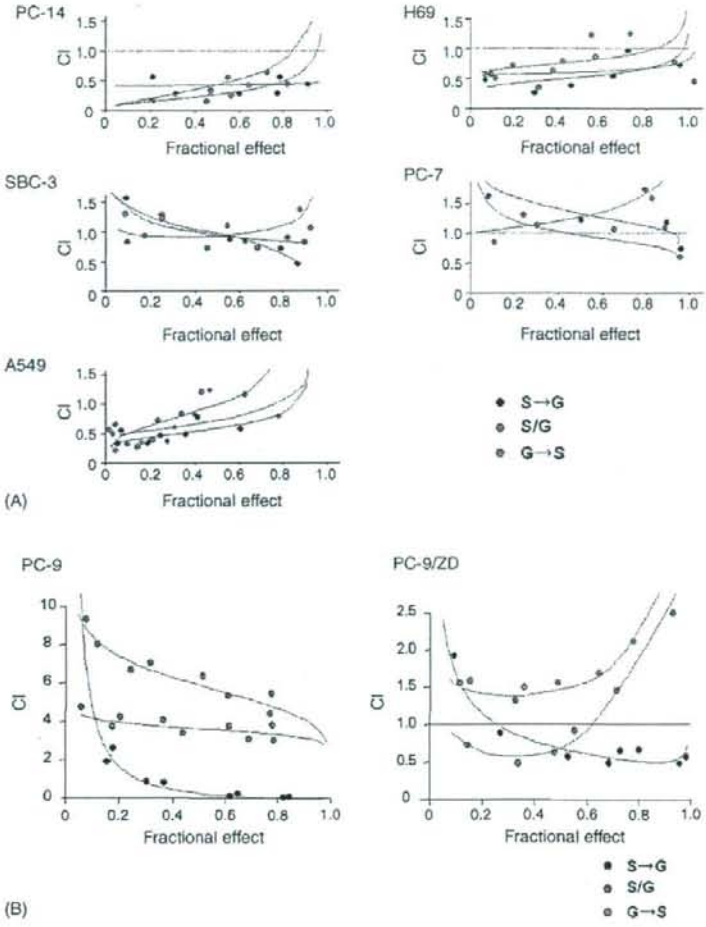

Fig. 1 Combination index (CI) plots of interactions between gefitinib and SN-38 in lung cancer cell lines. Each cell line was treated with gefitinib and SN-38, either alone or in combination at a fixed molar ratio. (A) (PC-14) gefitinib: SN-38 = 425:1; (SBC-3) 20000:1; (A549) 100:1; (H69) 2000:1; (PC-7) 3500:1. (B) (PC-9) gefitinib: SN-38 = 6:1; (PC-9ZD) 175:1. Treatment schedule: (1) SN-38 was applied first and gefitinib was applied 12 h later, followed by incubation in medium for 72 h (blue). (2) SN-8 and gefitinib were applied concurrently, followed by incubation in medium for 72 h (red). (3) Gefitinib was applied first and SN-38 was applied 12 h later, followed by incubation in medium for 72 h (green). S → G: sequential combination (SN-38 followed by gefitinib); C/G: concurrent combination; G → S: sequential combination (gefitinib followed by SN-38).

cells were stained with anti-Ki-67, anti-CD31 and the TUNEL assay (Fig. 3A and B). A reduction in tumor cell proliferation (Ki-67 staining), a reduction in tumor vasculature (CD31 staining), and an increase in tumor apoptosis (TUNEL staining) were observed in tumors treated with gefitinib alone or gefitinib and CPT-11. The administration of CPT-11 alone increased the number of Ki-67 positive tumor cells. In the PC-9 tumors, sequential treatment resulted in a 2.7-fold increase in tumor cell apoptosis and a 1.9-fold decrease in vessel staining, compared with the results obtained in tumors treated concurrently. The ratio of apoptosis:proliferation increased 1.7-fold in sequentially treated tumors compared with tumors treated with both drugs concurrently. Quantitative analysis of tumor cell proliferation and apoptosis showed a significant difference between the effects of the concurrent and sequential schedules ($P < 0.001$), but not between concurrent and gefitinib-alone ($P > 0.01$ for all comparisons, Fig. 3C). No significant difference in CD31-positive cells was observed between the control and gefitinib-alone treatments, suggesting that gefitinib exerts no remarkable anti-angiogenetic effects ($P > 0.01$, Fig. 3C). Similar findings were observed in PC-9/ZD tumors. These findings suggest that the antitumor activity of sequential treatment using gefitinib and CPT-11 is mediated by an increase in tumor cell apoptosis, compared with concurrent treatment.

4. Discussion

The EGFR-targeting drug gefitinib has been approved in many countries for the treatment of NSCLC patients who have previously received chemotherapy. Previous preclinical models have demonstrated the synergistic effects of gefitinib and platinums or taxanes [8,9]. However, no significant difference in survival was demonstrated in two randomized placebo-controlled phase II trials examining over 2000 previously untreated patients with NSCLC. In these trials, gefitinib was given in combination with paclitaxel and carboplatin or with gemcitabine and cisplatin [10,11]. Different administration schedules for gefitinib and cytotoxic agents may be necessary for select populations.

EGFR gene mutations have been demonstrated in NSCLC, and patients with lung cancers expressing mutant EGFR are strongly suspected to be hypersensitive to gefitinib alone. An in-frame short deletion in exon 19 of EGFR is strongly related to hyperresponsiveness to gefitinib and other tyrosine kinase inhibitors [12,13]. Cells expressing this deletional EGFR mutation are hypersenstivie to EGFR-targeted tyrosine kinase inhibitors [5]. On the other hand, the treat-

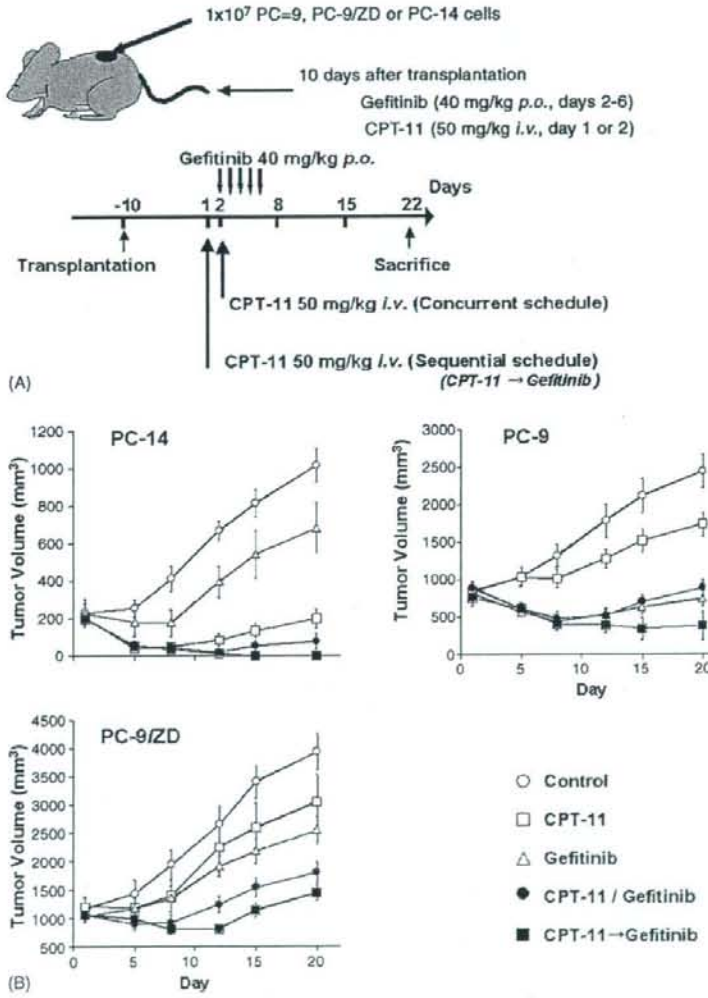

Fig. 2 Dose-dependent effects of combination therapy in PC9 and PC9/ZD cells in vivo. (A) Treatment schedule; (B) significant tumor growth-inhibition was observed in mice treated with the combination of gefitinib and CPT-11. Mice were allocated to five groups (6 mice/group) (○: 5% (w/v) glucose solution; □: CPT-11 50 mg/kg; △: gefitinib 40 mg/kg; ■: ZD1839 40 mg/kg + CPT-11 50 mg/kg concurrently; ●: CPT-11 50 mg/kg followed by ZD1839 40 mg/kg). (C) Treatment-related body weight loss in mice treated with gefitinib and/or SN-38. (○: 5% (w/v) glucose solution; □: CPT-11 50 mg/kg; △: ZD1839 40 mg/kg; ■: ZD1839 40 mg/kg + CPT-11 50 mg/kg concurrently; ●: CPT-11 50 mg/kg followed by ZD1839 40 mg/kg). Bars: ±S.D.

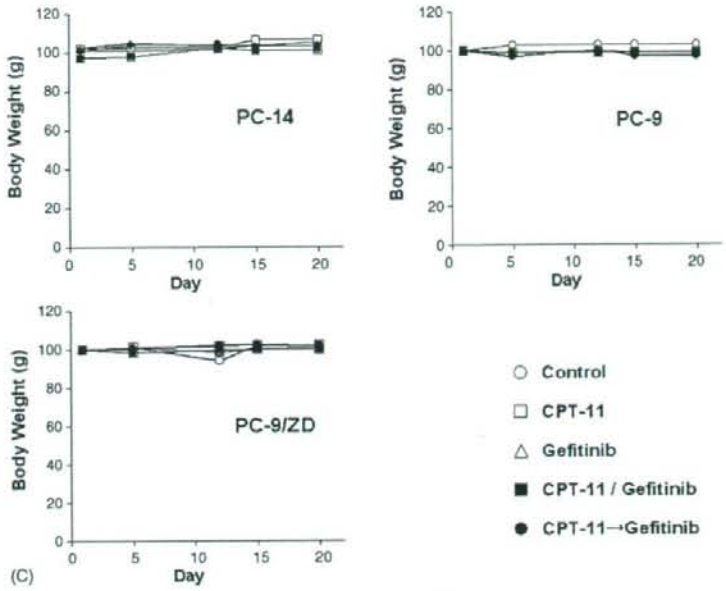

Fig. 2 (*Continued*).

ment of lung cancers expressing wild-type EGFR is a major obstacle. Combined therapies are still considered to be a major strategy against lung cancer expressing wild-type EGFR. Our previous preclinical study demonstrated that gefitinib and CPT-11 have synergistic effects in colorectal cancer cell lines [14]. Here, we reevaluated the combined effects of gefitinib and cytotoxic agents based on the status of EGFR mutations in lung cancer.

We demonstrated that gefitinib and SN-38, the active form of CPT-11, have synergistic or additive effects in lung cancer cells expressing wild-type EGFR. The combination of gefitinib and CPT-11 may be useful against lung cancers expressing wild-type EGFR. On the other hand, this combination had antagonistic effects in PC-9 cells expressing mutant EGFR, even though PC-9 cells are basically hypersensitive to gefitinib alone.

The concurrent administration of gefitinib and SN-38 also had an antagonistic effect in the PC-9/ZD cells. The PC-9/ZD cells developed an acquired resistance to gefitinib after exposure to gefitinib in vitro. New treatment strategies for patients who are refractory to gefitinib treatment are clinically needed. We demonstrated that the sequential administration of SN-38 (CPT-11) and gefitinib improved the combined effects in PC-9/ZD cells both in vitro and in vivo.

The above results led us to propose a combined gefitinib and CPT-11 treatment strategy based on the EGFR mutation status of lung cancers: (1) combined treatment according to any schedule for lung cancers expressing wild-type EGFR, (2) gefitinib treatment alone for lung cancers expressing mutant EGFR, and (3) the sequential administration of gefitinib and CPT-11 for patients who are refractory to gefitinib treatment. Based on the above preclinical evidence, we are preparing to begin a clinical phase II trial for combined gefitinib and CPT-11 treatment in Japan.

We previously demonstrated that CPT-11 and gefitinib have a synergistic effect against colorectal cancer [14]. EGFR mutations are rarely observed in colorectal cancer cells [15]. Therefore, the combined effects of these agents against colorectal cancers were consistent with those against the lung cancers expressing wild-type EGFR in this study.

Different combined effects were observed for the concurrent and sequential schedules in vitro and in vivo. While the mechanisms responsible for the combined effects remain unclear, cell cycle distributions might explain some of the differences. In cells treated according to the sequential gefitinib followed by SN-38 (CPT-11) treatment schedule, treatment with gefitinib resulted in an increase in the G0–G1 phase and a decrease in the S phase populations (data not shown). The decreased S phase population was not sensitive to CPT-11 [16]. Thus, the antagonistic effects of the sequential administration of gefitinib followed by CPT-11 (SN-38) could be explained by this mechanism. On the other hand, in cells treated according to the sequential SN-38 followed by gefitinib treatment schedule, SN-38 treatment induced an increase in the S phase population. If the S phase population is sensitive to gefitinib, this might explain the synergistic effects of this sequential schedule [17]. An increase in EGFR phosphorylation induced by CPT-11 is another previously reported possible mechanism responsible for this synergistic action [14].

In conclusion, we demonstrated the different effect on lung cancer cell expressing mutant EGFR according to the

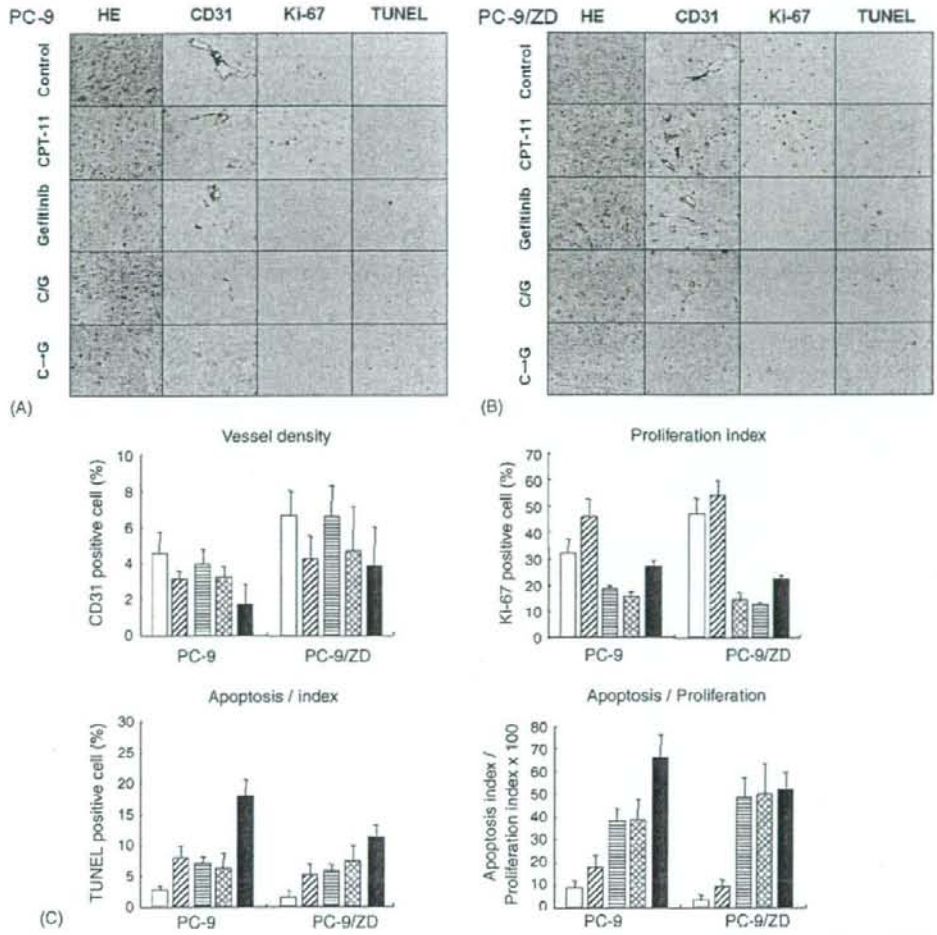

Fig. 3 (A) Historical examination of PC-9 tumor xenografts (day 22) stained with H&E, anti-CD31 vessel staining, TUNEL staining (magnification: 400×) and anti-Ki-67 nuclear antigen (magnification: 200×). The number of Ki-67-positive cells increased with the administration of CPT-11. The number of Ki-67-positive cells decreased with the gefitinib-alone and combination treatments. C/G: concurrent combination, C→G: sequential combination. (B) Historical examination of PC-9ZD tumor xenografts (day 22) stained with H&E, anti-CD31 vessel staining, TUNEL staining (magnification: 400×) and anti-Ki-67 nuclear antigen (magnification: 200×). The number of Ki-67-positive cells increased with the administration of CPT-11. The number of Ki-67-positive cells decreased with the gefitinib-alone and combination treatments. C→G: sequential combination; C/G: concurrent combination. (C) Quantitation of CD31 vessel staining, Ki-67 proliferation index, apoptosis index, and apoptosis: proliferation ratio. The columns represent the mean population of positive cells in five fields. Bars: ±S.D. Tumors from mice treated with vehicle (white), CPT-11 (diagonal hatched), Gefitinib (horizontal hatched), concurrent combination of CPT-11 plus Gefitinib (cross-hatched), or sequential combination of CPT-11 plus Gefitinib (cross-hatched).

combination schedule of gefitinib and CPT-11. The sequential combined treatment also active against lung cancer cell expressing wild-type EGFR.

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