Table 2 Disagreement between FDG-PET and conventional staging procedures (regional lymph node metastases)

Patient no.	Age (years)	Gender	CT N	PET N	PET M	Interval between CT scan of the chest and FDG-PET (days)	Comments
1	63	Male	3	3	0	8	Contralateral supraclavicular lymph node metastasis (PET)
5	64	Female	1	2	0	34	Subcarinal lymph node metastasis (PET)
16	71	Male	3	3	0	7	Contralateral supraclavicular lymph node metastasis (PET)
20	69	Male	3	3	0	20	Ipsilateral supraclavicular lymph node metastasis (PET)
25	60	Male	3	3	0	27	Ipsilateral supraclavicular lymph node metastasis (PET)
30	66	Male	2	2	0	7	Pretracheal lymph node metastasis (PET)
33	72	Male	3	3	0	13	Ipsilateral supraclavicular lymph node metastasis (PET)
41	49	Female	3	3	0	19	Contralateral supraclavicular lymph node metastasis (PET)
43	73	Male	2	0	0	34	False-positive pretracheal lymph node metastasis (CT)
56	48	Female	3	3	0	11	Ipsilateral supraclavicular lymph node metastasis (PET)
61	74	Male	2	0	0	27	False-positive superior mediastinal and subcarinal lymph nodes (CT).

FDG, fluorodeoxyglucose; PET, positron emission tomography; CT, computed tomography; N, node; M, metastasis.

We employed no special strategies to reduce the bias of PET readers. PET readers might have reported in such a way as to reduce or increase the impact of PET. One-third of patients received FDG-PET after commencement of chemotherapy. However, the median interval between commencement of chemotherapy and FDG-PET was 4 days (range: 1–11 days). We considered the chemotherapy to have had no effects on the findings of FDG-PET in such a short time after the initiation of chemotherapy.

FDG-PET is expected to have the potentially to both up- and downstage patients with SCLC as well as NSCLC. A previous study demonstrated that FDG-PET correctly downstaged ED to LD in three of 120 patients with SCLC [10]. These three patients had adrenal swelling on CT scan, but these lesions were negative on FDG-PET. On the other hand, FDG-PET correctly upstaged LD to ED in 10 of 120 patients with SCLC. It seems that SCLC seldom has a solitary distant metastasis because of its aggressive clinical behaviour. Most ED-SCLC has multiple, not solitary, or obvious distant metastasis. Furthermore, the health insurance system does not allow patients who obviously have metastatic lung cancer to receive FDG-PET in Japan. Therefore, we did not include patients with ED-SCLC in our analysis. Needless to say, FDG-PET is considered to be useful in patients with possible, but not evident, distant metastasis on other imaging tests, such as a solitary adrenal swelling.

According to the VALSG system, LD-SCLC is defined as a tumour confined to one hemithorax and regional lymph nodes [1]. Contralateral hilar or contralateral supraclavicular nodal involvement was classified as ED. According to the International Association for the Study of Lung Cancer (IASLC) consensus report, the classification of LD-SCLC includes bilateral hilar and/or supraclavicular nodal involvement, and ipsilateral pleural effusion [18]. A previous retrospective study demonstrated that the IASLC staging criteria for SCLC patients had a higher prognostic impact than VALSG criteria [19]. Therefore, we adopted the IASLC staging criteria for SCLC in our study.

In conclusion, FDG-PET scans detected unsuspected distant metastases in five of 63 patients with LD-SCLC (95% CI: 3–18%) and these findings resulted in a change of therapeutic strategies in these five patients. FDG-PET scans also detected contralateral supraclavicular lymph node metastases that had been negative on CT scans in three other

patients. These additional findings facilitated setting appropriate irradiation fields. FDG-PET scan is recommended as an initial staging tool in patients with apparent LD-SCLC.

Conflict of interest

The authors certify that there are no potential conflicts of interest.

Acknowledgments

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Pertuzumab, a novel HER dimerization inhibitor, inhibits the growth of human lung cancer cells mediated by the HER3 signaling pathway

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A humanized anti-HER2 monoclonal antibody pertuzumab (Omnitarg, 2C4), binding to a different HER2 epitope than trastuzumab, is known as an inhibitor of heterodimerization of the HER receptors. Potent antitumor activity against HER2-expressing breast and prostate cancer cell lines has been clarified, but this potential is not clear against lung cancers. The authors investigated the *in vitro* anti-tumor activity of pertuzumab against eight non-small cell lung cancer cells expressing various members of the HER receptors. A lung cancer 11_18 cell line expressed a large amount of HER2 and HER3, and its cell growth was stimulated by an HER3 ligand, heregulin (HRG)-α. Pertuzumab significantly inhibited the HRG-α-stimulated cellular growth of the 11_18 cells. Pertuzumab blocked HRG-α-stimulated phosphorylation of HER3, mitogen-activated protein kinase (MAPK), and Akt. In contrast, pertuzumab failed to block epidermal growth factor (EGF)-stimulated phosphorylation of EGF receptor (EGFR) and MAPK. Immunoprecipitation showed that pertuzumab inhibited HRG-α-stimulated HER2/HER3 heterodimer formation. HRG-α-stimulated HER3 phosphorylation was also observed in the PC-9 cells co-overexpressing EGFR, HER2, and HER3, but the cell growth was neither stimulated by HRG-α nor inhibited by pertuzumab. The present results suggest that pertuzumab is effective against HRG-α-dependent cell growth in lung cancer cells through inhibition of HRG-α-stimulated HER2/HER3 signaling. (*Cancer Sci* 2007; 98: 1498–1503)

The HER family of receptor tyrosine kinases consists of four members: EGFR (also termed HER1/ErbB-1), HER2/ErbB-2/Neu, HER3/ErbB-3, and HER4/ErbB-4.[1] Binding of ligands leads to the homo- and heterodimer formation of the receptor tyrosine kinase.[2] There are numerous HER-specific ligands that generate signaling diversity within the cell.[3] EGF, amphiregulin, and TGF-α are known as a specific ligand of EGFR. HB-EGF, β-cellulin, and epiregulin have dual specificity for binding to EGFR and HER4. HRG-α binds HER3 and HER4.[4] No direct ligand for HER2 has been discovered. Dimerization consequently stimulates the intrinsic tyrosine kinase activity of receptors, and activates the downstream-signaling molecules such as MAPK, Akt, JAK, and STAT.[5,6]

Pertuzumab is a humanized monoclonal antibody and binds to the dimerization domain of HER2 distinct from the domain that trastuzumab binds to.[7] Therefore, pertuzumab is known as a dimerization inhibiter between HER2 and the other HER family receptors. A phase I trial of pertuzumab has been performed for advanced tumors,[8] and phase II studies of pertuzumab are underway. Two members of the HER family, HER2 and HER3, act as key oncogenes in breast cancer cells.[9,10] *In vitro* and *in vivo* anti-tumor activities of pertuzumab have been reported in breast tumors through the inhibition of the HER2/HER3 heterodimer formation.[11,12] In lung cancer cells, EGFR plays a crucial role in their biological behavior, but it is unclear whether pertuzumab inhibits the growth of the lung cancer cells mediated by HER family receptors.

The authors have focused on the growth inhibitory effect of pertuzumab against NSCLC cells expressing different types of HER receptors, and analyzed the mechanism of action of pertuzumab in response to the HER receptor ligand.

Materials and Methods

Reagents. Pertuzumab (Omnitarg, 2C4) was provided in sterile water at 25 mg/mL by Genentech, Inc. (South San Francisco, CA, USA) before use. All chemicals and reagents were purchased from Sigma (St Louis, MO, USA) unless noted otherwise.

Cell lines. The human NSCLC cell lines PC-7, PC-9, and PC-14 (Tokyo Medical University, Tokyo, Japan),[13,14] A549 (American Type Culture Collection, Manassas, VA, USA), and PC-3, Ma-1, Ma-24, and 11_18,[15] were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS (Life Technologies, Rockville, MD, USA).

Cell stimulation and lysis. Cells were starved in serum free RPMI 1640 medium for 24 h and treated with EGF, TGF-α, HB-EGF, and HRG-α at 100 ng/mL for 10 min. Cells were washed twice with ice-cold PBS, and lysed with lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 5 mM sodium pyrophosphate, 50 mM NaF, 1 mM sodium vanadate, 4 mg/mL leupeptin, 4 mg/mL apoprotein, 1 mM PMSF). Protein concentration of the supernatants was determined by the BCA protein assay (Pierce, Rockford, IL, USA).

Immunoprecipitation. Cell lysates (1000 μg) were incubated with the anti-HER2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. Protein G magnetic beads (New England BioLabs, Beverly, MA, USA) were added for 2 h. Beads were washed three times with lysis buffer, resuspended in SDS sample buffer with 2% β-mercaptoethanol, boiled, and separated using SDS-PAGE.

Western blotting. Cell lysates were electrophoretically separated on SDS-PAGE and transferred to a polyvinylidene difluoride

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Abbreviations: BCA, bicinchoninic acid; ECL, electrochemiluminescence; EDTA, ethylene diamine tetra-acetic acid; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; HB-EGF, heparin-binding epidermal growth factor; HRG-α, heregulin-α; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; NSCLC, non-small cell lung cancer; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; RPMI, Roswell Park Memorial Institute; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; STAT, signal transducer and activator of transcription; TGF-α, transforming growth factor-α.

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membrane (Millipore, Bedford, MA, USA). The membrane was probed with each antibody against EGFR and HER2 (Transduction Laboratory, San Diego, CA, USA), HER3 (Santa Cruz Biotechnology), phospho-EGFR (Tyr1068), phospho-HER3 (Tyr1289), MAPK, phospho-MAPK (Thr202/204), Akt, phospho-Akt (Ser473) (Cell Signaling, Beverly, MA, USA), phosphotyrosine (PY-20, Transduction Laboratory), and β-actin (Sigma) as the first antibody, followed by detection using a horseradish peroxidase-conjugated secondary antibody. The bands were visualized with ECL (Amersham, Piscataway, NJ, USA), and images of blotted patterns were analyzed with NIH image software (National Institutes of Health, Bethesda, MD, USA).

Growth inhibition assay. A 100-μL volume of cell suspension (5000 cells/well) in serum-free RPMI 1640 medium was seeded into a 96-well plate and 50 μL of each drug at various concentrations and 50 μL of EGF, TGF-α, HB-EGF, and HRG-α, at 100 ng/mL was added. Human IgG1 (Calbiochem, Cambridge, MA, USA) was used as isotype control. After incubation for 72 h at 37°C, 20 μL of MTS solution (Promega, Madison, WI, USA) was added to each well and the plates were incubated for a further 2 h at 37°C. The absorbance readings for each well were determined at 490 nm with a Delta-soft on a Macintosh computer (Apple, Cupertino, CA, USA) interfaced to a Bio-Tek Microplate Reader EL-340 (BioMetallics, Princeton, NJ, USA). For ligand-stimulated growth of cells, the experiment was performed in six replicate wells for each ligand and carried out independently three times. For growth inhibition of pertuzumab, the experiment was performed in three replicate wells for each drug concentration and carried out independently three times as described elsewhere.[16]

Results

HRG-α dependent cell growth in lung cancer cells. Ligand-dependent cell growth of lung cancer cells was examined (Fig. 1). The addition of EGF, TGF-α, and HB-EGF increased the cell growth of the PC-3, 11_18, and A549 cells, but not that of the PC-7, PC-9, PC-14, Ma-1, and Ma-24 cells. HRG-α addition significantly increased the growth of the 11_18 cells (390% of control, $P < 0.01$ by t-test) and Ma-24 cells (204% of control, $P < 0.01$ by t-test), but did not influence the growth of any other cells. These findings suggest that the growth of the 11_18 and Ma-24 cells is depending upon HRG-α.

Pertuzumab inhibits HRG-α-dependent cell growth of the 11_18 and Ma-24 cells. Pertuzumab inhibited cell growth stimulated by HRG-α ($IC_{50} = 0.12$ μg/mL) but not stimulated by EGF, TGF-α, and HB-EGF in the 11_18 cells ($IC_{50} > 100$ μg/mL; Fig. 2). Pertuzumab also inhibited HRG-α dependent cell growth in the Ma-24 cells ($IC_{50} = 39.8$ μg/mL). Isotype control human IgG1 had no effect on ligand-dependent growth in the 11_18 and Ma-24 cells (data not shown). The growth of the other cells was not affected by exposure to pertuzumab (data not shown). This finding suggests that pertuzumab selectively inhibits HRG-α-dependent cell growth.

Ligand-stimulated phosphorylation of HER receptors. The expression levels of the HER receptors in the pertuzumab-sensitive (11_18 and Ma-24 cells) and pertuzumab-resistant cell (PC-9 cells) lines were determined using western blotting (Fig. 3a). Comparison of the protein expression levels of EGFR revealed high to moderate expression in the PC-9 and Ma-24 cells. EGFR was also detected in the 11_18 cells, although the expression in this

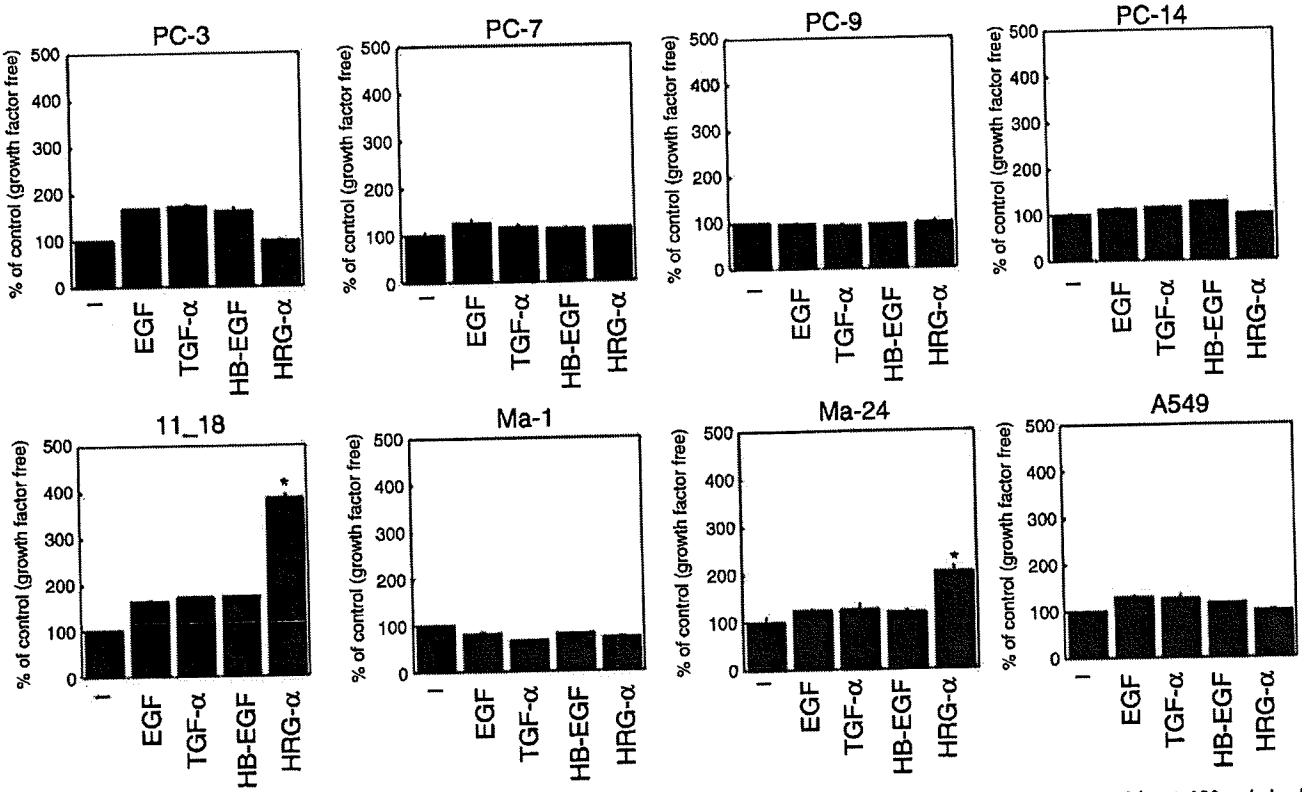

Fig. 1. Ligand-dependent cell growth in the lung cancer cells. Non-small cell lung cancer cells were stimulated with or without 100 ng/mL of epidermal growth factor (EGF), transforming growth factor (TGF)-α, heparin-binding epidermal growth factor (HB-EGF), and heregulin (HRG)-α. After incubation for 72 h, cell growth was determined using the MTS assay. The growth of cells was presented as the percentage of absorbance compared with ligand-untreated cells. Error bars represent SE. *Significant difference ($P < 0.01$; t-test) compared to the ligand-non-stimulated cells. Data shown are representative of at least three independent experiments with similar results.

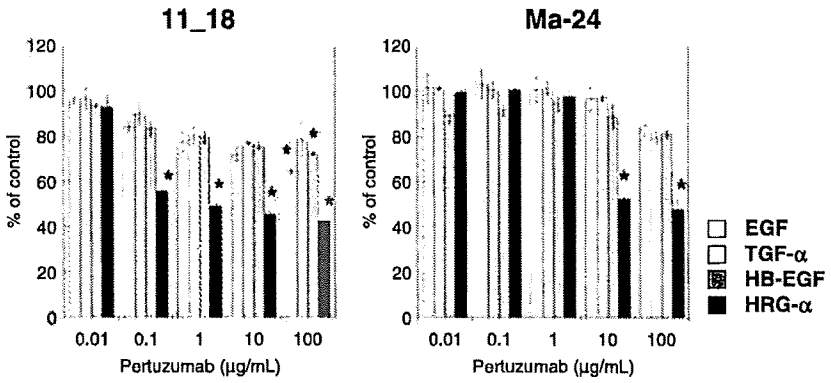

Fig. 2. Growth inhibitory effect of pertuzumab in the lung cancer cells. The lung cells were exposed to pertuzumab (0.01–100 μg/mL) for 72 h in serum free medium with or without 100 ng/mL of epidermal growth factor (EGF), transforming growth factor (TGF)-α, heparin-binding epidermal growth factor (HB-EGF), or heregulin (HRG)-α. The viability was determined using the MTS assay. Result are presented as the percentage of absorbance compared with pertuzumab-untreated cells. Error bars represent SE. *Significant difference ($P < 0.01$; *t*-test) compared to pertuzumab-untreated cells. Data shown are representative of at least three independent experiments with similar results.

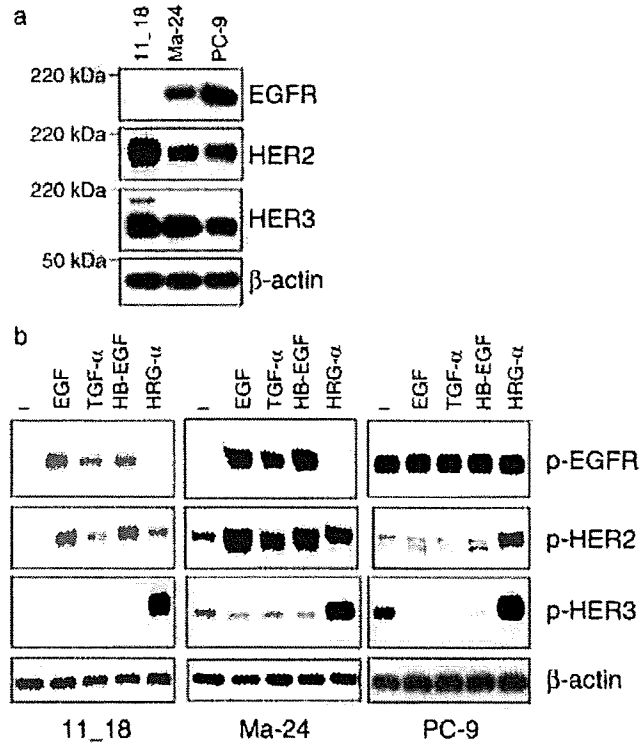

Fig. 3. Expression and phosphorylation of HER receptors in non-small cell lung cancer cells. (a) Expression of epidermal growth factor receptor (EGFR), HER2, and HER3 was detected using western blot analysis. Each lane contained 20 μg protein. β-Actin was used as a loading control. (b) The cells were stimulated with or without 100 ng/mL of epidermal growth factor (EGF), transforming growth factor (TGF)-α, heparin-binding epidermal growth factor (HB-EGF), and heregulin (HRG)-α for 10 min. Phosphorylation of EGFR and HER3 was detected using western blot analysis. Phosphorylation of HER2 was detected using immunoprecipitation followed by western blotting. β-Actin was used as a loading control. Data shown are representative of at least two independent experiments with similar results.

cell line was weak. The expression levels of HER2 were higher in the PC-9 and 11_18 cells than in the Ma-24 cells, which only expressed moderate levels of this receptor. All three cell lines showed strong expression of HER3. HER4 could not be detected in any of the three cell lines (data not shown). In contrast, these lung cancer cell lines expressed different types of EGFR mutations; the PC-9 cells had a 15-base deletion mutant (delE746-A750, exon 19), the 11_18 cells had a L858R point mutation (exon 21) of EGFR, and the Ma-24 cells had a E709G point mutation (exon 18) of EGFR. No mutations were detected in exons 19–21 of HER2 (data not shown).

Next, the ligand-stimulated phosphorylation of the HER receptors in the lung cancer cells after serum starvation was examined (Fig. 3b). While the ligands for EGFR (EGF, TGF-α, and HB-EGF) phosphorylated cellular EGFR in the 11_18 and Ma-24 cells, the EGFR in the PC-9 cells was hyperphosphorylated even under the non-stimulated condition, because PC-9 cells express an active mutant of EGFR. These results suggest that the EGF/TGF-α or HB-EGF–EGFR signals are active in lung cancer cells. The ligands for HER3 (HRG-α) specifically phosphorylated HER3 in the 11_18, Ma-24, and PC-9 cells. Phosphorylation of HER2 was analyzed by immunoprecipitation using an anti-HER2 antibody followed by western blotting for phosphotyrosine. The ligands for EGFR and HER3 phosphorylated HER2 in the 11_18 and Ma-24 cells, whereas only HRG-α but not the other ligands specifically phosphorylated HER2 in the PC-9 cells. These findings also suggest that the HRG-α–HER3 signal is active in lung cancer cells.

Pertuzumab blocks HRG-α but not EGF-stimulated signals. An inhibitory effect of pertuzumab on HRG-α-dependent cell growth in the 11_18 cells was demonstrated. To examine the effect of pertuzumab on signal transduction of both EGFR and HER3 in this cell line, the 11_18 cells were exposed to pertuzumab (0.2–200 μg/mL for 6 h) (Fig. 4a,b). HRG-α-stimulated phosphorylation of HER3 was dose-dependently inhibited by exposure to pertuzumab in the 11_18 cells, whereas EGFR phosphorylation was not stimulated by HRG-α stimulation (data not shown). MAPK and Akt were phosphorylated by HRG-α stimulation and these were inhibited by pertuzumab dose-dependently in the 11_18 cells. In contrast, EGF-stimulated phosphorylation of EGFR and MAPK was not inhibited by pertuzumab in the 11_18 cells. Phosphorylation of Akt was not detected by addition of EGF in the 11_18 cells. EGF did not phosphorylate HER3 and pertuzumab did not affect it (data not shown). Taken together, these results showed that pertuzumab inhibited HRG-α-stimulated phosphorylation of HER3, MAPK, and Akt, but not EGF-stimulated EGFR phosphorylation signaling.

HER3 is phosphorylated in response to HRG-α in the PC-9 cells as observed in the 11_18 cells, but the growth of the PC-9 cells was not increased by HRG-α (Figs 1,3b). To clarify the phosphorylation-inhibitory potential of pertuzumab, the effect of pertuzumab on signal transduction of the PC-9 cells was examined (Fig. 4c). When the PC-9 cells were stimulated by the addition of HRG-α, HER3 was phosphorylated in the PC-9 cells, but phosphorylation of HER3 was not inhibited by pertuzumab (20 and 200 μg/mL for 6 h). EGFR expressed in the PC-9 cells is constitutively active and pertuzumab failed to affect

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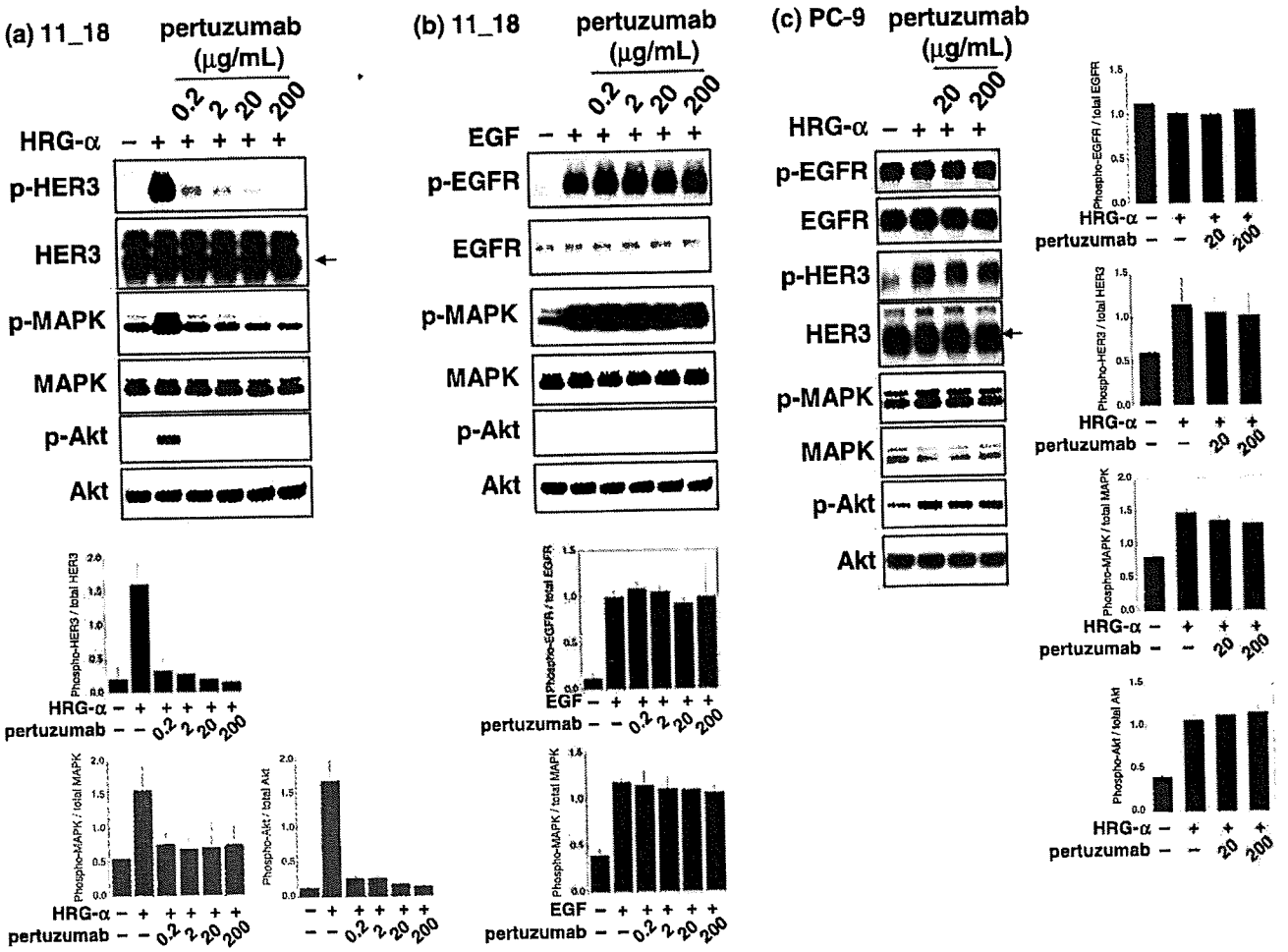

Fig. 4. Effect of pertuzumab on epidermal growth factor receptor (EGFR) and HER3 phosphorylation and their downstream signaling pathways. The 11_18 and PC-9 cells were exposed to pertuzumab for 6 h and stimulated with either heregulin (HRG)-α or epidermal growth factor (EGF) for 10 min. Cell lysate were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotted for indicated antibodies. The intensities of bands were quantified by densitometer. (a) HRG-α-stimulated 11_18 cells. (b) EGF-stimulated 11_18 cells. (c) HRG-α-stimulated PC-9 cells. Data shown are representative of at least two independent experiments with similar results. MAPK, mitogen-activated protein kinase.

the phosphorylation level of the EGFR. Phosphorylation of MAPK and Akt was detected by the addition of HRG-α, but these were not inhibited by pertuzumab. These results suggest that pertuzumab is unable to affect HRG-α-stimulated phosphorylation of HER3 in the PC-9 cells.

To clarify the effect of pertuzumab on HER2 phosphorylation and HER2/HER3 heterodimer formation, cell lysates were immunoprecipitated with anti-HER2 antibody (Fig. 5a,b). HRG-α stimulation increased HER2/HER3 heterodimer formation in the 11_18 cells, and pertuzumab decreased HRG-α-stimulated heterodimer formation. EGFR/HER2 heterodimer formation could be barely detected by HRG-α stimulation because of slight expression of EGFR in the 11_18 cells. In the case of EGF stimulation, HER2/HER3 heterodimer was not increased in the 11_18 cells. These findings suggest that pertuzumab inhibits HER2/HER3 heterodimerization by HRG-α stimulation. The HRG-α-stimulated phosphorylation of HER2 was inhibited by pertuzumab in the 11_18 cells. In contrast, the EGF-stimulated phosphorylation of HER2 was not inhibited. These data suggest that pertuzumab inhibits HRG-α stimulated phosphorylation in 11_18 cells. In the PC-9 cells, HRG-α stimulated HER2/HER3 heterodimer formation could be detected without any ligand stimulation, and pertuzumab diminished HRG-α-stimulated heterodimer formation (Fig. 5c). Phosphorylation of HER2 was increased by HRG-α stimulation, but not inhibited by pertuzumab in PC-9 cells. EGFR/HER2 heterodimer formation could be detected without any ligand stimulation, but pertuzumab did not affect it. Based on these results, it is speculated that the cell growth of the PC-9 cells is predominantly dependent on active EGFR signaling, and phosphorylation of HER3 is maintained by active mutant EGFR.

Discussion

Overexpression of HER3 was observed in the lung cancer cell lines and the HER3 was phosphorylated by the HER3 ligand in these cells. These results suggest that HER3 signaling is active in some types of lung cancer cells. Recently it was reported that high HER3 expression was associated with decreased survival.[17] A relationship between lung cancer metastasis and the expression of HER3 as well as EGFR and HER2 has been reported.[18] These bodies of evidence suggest that HER2/HER3 signaling is activated in a subpopulation of lung cancers and that HER2 and HER3 play an important role in the biological behavior of these lung cancers. Both HER2 and HER3 are therefore considered as a possible important target in the therapeutic strategy against lung cancer, just as they are in breast cancers.

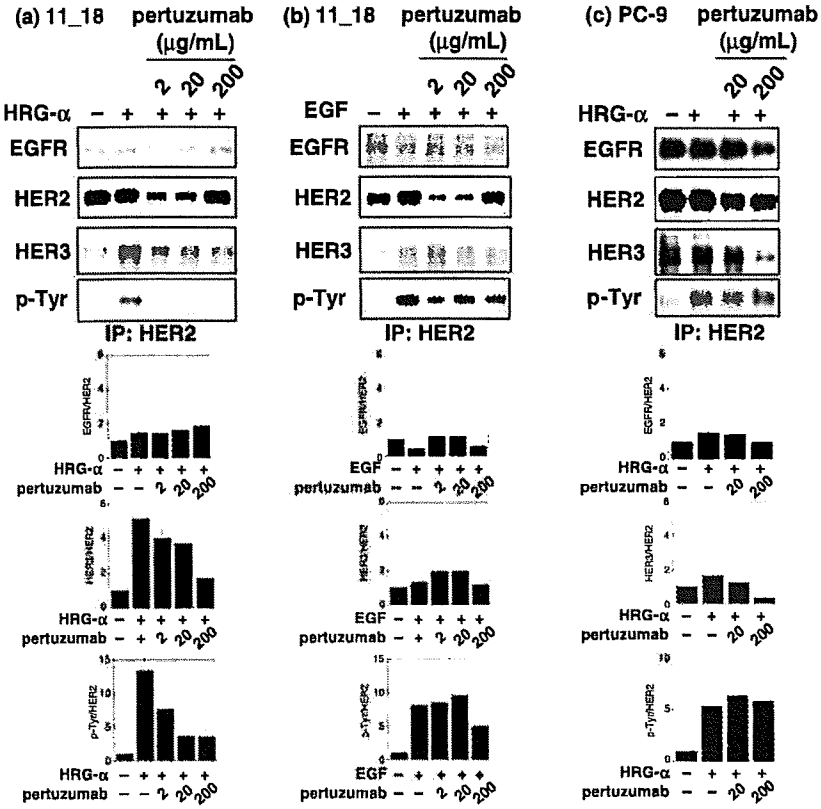

Fig. 5. Effect of pertuzumab on heterodimer formation. The 11_18 and PC-9 cells were exposed to pertuzumab for 6 h and stimulated with either heregulin (HRG)-α or epidermal growth factor (EGF) for 10 min. Cell lysates were immunoprecipitated with anti-HER2 antibody, separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and blotted for indicated antibodies. The intensities of bands were quantified by densitometer. (a) HRG-α-stimulated 11_18 cells. (b) EGF-stimulated 11_18 cells. (c) HRG-α-stimulated PC-9 cells. Data shown are representative of at least two independent experiments with similar results.

HER3 lacks kinase activity because of several base substitutions in motifs that are essential to tyrosine kinase and heterodimerization with HER2 or EGFR is essential for its signal transduction. Therefore co-expression of HER3 and its partners are determinants for the cellular sensitivity against pertuzumab in cancer cells. The present results showed that HER2/HER3 heterodimers are detected by HRG-α stimulation and these data are consistent with previous reports.[19] In contrast, the authors monitored the downstream phosphorylation signal, and demonstrated that HRG-α, but not EGF, phosphorylated Akt in the 11_18 cells. This finding allows us to speculate that HRG-α stimulation leads to Akt phosphorylation through HER2/HER3 heterodimerization.[20–22]

Recently, EGFR mutations have been reported in lung cancers and it was of great interest to clarify the relationship between the EGFR mutation and sensitivity to EGFR-targeted tyrosine kinase inhibitors.[23–25] The PC-9 cells express the deletional mutant EGFR (delE746-A750 in exon 19 of EGFR),[16,23,26,27] and their EGFR was constitutively phosphorylated under non-stimulated conditions (Fig. 3a). The authors speculate that the cell growth of the PC-9 cells is predominantly dependent on active EGFR signaling. In Fig. 3b, treatment with EGF and TGF-α seemed to decrease the phosphorylation of HER3 in PC-9 cells. Unfortunately, we could not conclusively explain this phenomenon. PC-9 cells express deletional EGFR and form EGFR homodimers in the absence of ligand stimulation. At the same time, phospho-HER3 was also detected under these conditions, suggesting that heterodimers of EGFR–HER3 were also formed. Ligand stimulation may alter the balance between homodimers and heterodimers, causing a reduction in HER3 phosphorylation, although there is not any evidence to support this hypothesis. In contrast, the phosphorylation of EGFR in the 11_18 cells that express a different type of mutant EGFR (L858R in exon 21 of EGFR),[26] was not constitutive. This finding may be explained by the differences between deletion mutant EGFR and L858R; constitutive active in the deletion mutant versus hyper-response to ligand stimulation in L858R.[28] Engelman *et al.* suggested that the mutant EGFR is used to couple HER3 in gefitinib-sensitive NSCLC cell lines.[29] The expression level of EGFR in the 11_18 cells was much lower than in the PC-9 cells, and a similar extent of HER3 expression was observed in these cell lines (Fig. 3a). The authors have demonstrated the differential inhibitory effect of pertuzumab against 11_18 and the PC-9 cells. Pertuzumab inhibited HER2/HER3 heterodimer formation and phosphorylation in the 11_18 cells, considering that mutant EGFR do not influence HER3 signals in the 11_18 cells. HER3 phosphorylation in the PC-9 cells was also increased by HRG-α stimulation. Although pertuzumab decreased HER2/HER3 heterodimer formation, it failed to inhibit HRG-α-stimulated HER3 phosphorylation, speculating that an active mutant EGFR transactivates HER3 in the PC-9 cells.

Several EGFR-targeted small inhibitors and antibodies have been under clinical evaluation in the treatment of lung cancer. An EGFR-targeted tyrosine kinase inhibitor, erlotinib, has been clinically applied as a second or third-line single agent therapy in NSCLC patients who have failed standard chemotherapy.[30] Anti-EGFR monoclonal antibodies such as cetuximab and ABX-EGF have been examined in a clinical study.[31] In addition to EGFR, HER2 and HER3 are also considered as important targeting molecules in lung cancers. The present results indicated that pertuzumab effectively inhibited signaling within HER2 and HER3, and may thus be effective in lung cancers expressing HER2 and HER3. To confirm the pertuzumab-sensitive population of lung cancer cells, experiments using small interfering RNA for mutant EGFR will be necessary in future studies.

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In conclusion, the authors have demonstrated that pertuzumab inhibits HRG-α-stimulated cell growth in lung cancer cells through the inhibition of HRG-α-stimulated HER3 signaling. It was further demonstrated that pertuzumab exerts an antiproliferative activity against lung cancer cells expressing HER2 and HER3. The next step will be to examine the clinical relevance of the occurrence of heterodimer formation between HER2 and the other HER receptors in lung cancer.

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Review

Problems with Registration-Directed Clinical Trials for Lung Cancer in Japan

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SEKINE, I., NOKIHARA, H., YAMAMOTO, N., KUNITOH, H., OHE, Y., SAIJO, N. and TAMURA, T. *Problems with Registration-Directed Clinical Trials for Lung Cancer in Japan.* Tohoku J. Exp. Med., 2007, **213** (1), 17-23 —— New anticancer agents against lung cancer are needed because efficacy of chemotherapy is limited. The long time required, low quality, and considerable costs of registration-directed clinical trials in Japan ("Chiken") have been pointed out. The quality of 24 phase I and 41 phase II trials of an anticancer drug for lung cancer were analyzed according to the approval year of the drug. The human resources and infrastructure to support oncology clinical practice and clinical trials were compared between Japan and the USA. A maximum tolerated dose was not defined in any of seven phase I trials before 1989, and was determined in two of six trials between 1989 and 1996 and in seven of 10 trials thereafter. Before 1989, 29 (20%) of 142 patients registered in two trials were ineligible, and the number of ineligible patients was not reported in the five trials. Sample size calculations were not performed in any of seven phase II trials before 1989 and were performed in only four of 10 trials between 1989 and 1996 and in all 23 trials conducted thereafter. The shortage of human resources, including medical oncologists, oncology nurse practitioners and clinical research coordinators, is serious and acute. The infrastructure to support clinical trials also remains insufficient in Japan. In conclusion, registration-directed clinical trials of anticancer agents have advanced significantly during last three decades but remain unsatisfactory. The development of infrastructure and human resources is an urgent task to ensure high-quality clinical trials without unnecessary delays. ——— clinical trials; medical oncologists; nurse practitioners; lung cancer; anticancer agents

Lung cancer is one of the most common malignancies and the leading cause of cancer-related deaths in many countries. In the year 2000, the annual number of deaths from lung cancer was estimated to be 1.1 million worldwide, and global lung caner incidence is increasing at a rate of 0.5% per year (Schottenfeld and Searle 2005). About 80% of patients with lung cancer have already developed distant metastases or pleural effusion, either by the time of the initial

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diagnosis or by the time recurrence is detected after surgery for local disease. These patients can be treated with systemic chemotherapy, but the efficacy of currently available anticancer agents is limited to the extent that patients with advanced disease rarely live long. Therefore, new chemotherapeutic agents continue to be developed against lung cancer (Sekine and Saijo 2000).

The Japanese Pharmaceutical Affairs Law (PAL) was enacted in 1948, and was first amended in 1960 to provide for regulations to ensure the maintenance of the quality, efficacy, and safety of drugs and medical devices, and to promote research and development of these medical and pharmaceutical products. Good Clinical Practice (GCP) was enforced by the Bureau Notification of the Ministry of Health and Welfare of Japan ("Kyokuchou-Tsuuchi") in 1989 (the former GCP). In 1996, the PAL and its related laws were amended to strengthen GCP (the new GCP), Good Laboratory Practice, Good Post-Marketing Surveillance Practice, and standard compliance reviews, conforming to the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use. In contrast to the laws prevailing in the US and EU, marketing approval for anticancer agents in Japan has been granted based on reports of the anti-tumor effects of the new agents in phase II trials (Fujiwara and Kobayashi 2002).

Under this Japanese drug approval system regulated by the PAL, 23 anticancer drugs have been approved for use against lung cancer during the last five decades (Fig. 1). Of these, 9 drugs are original to Japan, some of which are routinely used all over the world. Several problems, however, have been pointed out in registration-directed clinical trials in Japan ("Chiken"), including the long time required, low quality, and considerable cost (The Ministry of Health, Labour and Welfare of Japan 2002; The Ministry of Education, Science and Culture and the Ministry of Health, Labour and Welfare 2003). As a result, Japanese cancer patients must wait for a long time

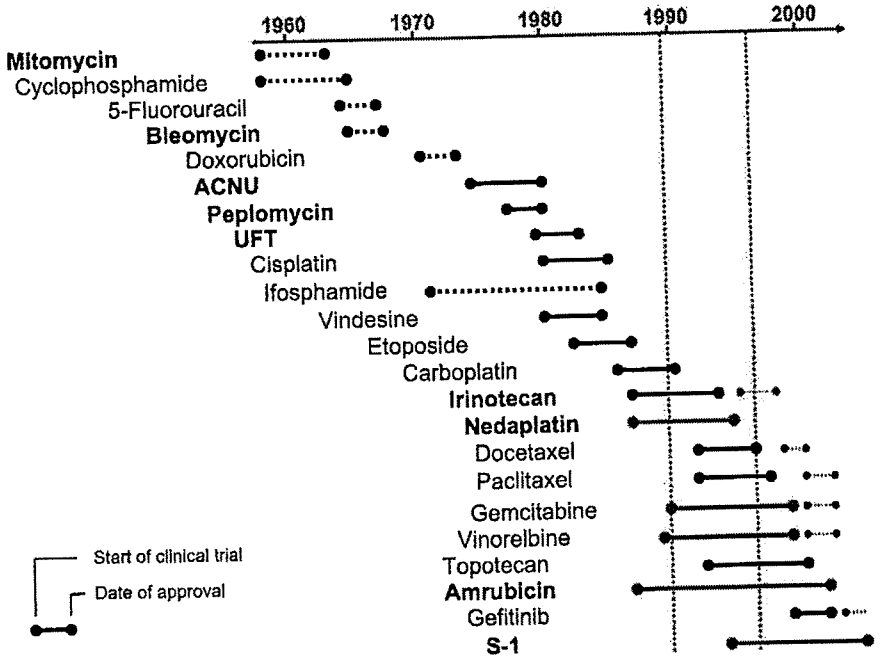

Fig. 1. Anticancer drugs approved for lung cancer in Japan.
Bold: original to Japan. Dotted line: case series studies, solid thick line: investigational new drug phase I-II trials for approval, and dotted thin line: post-marketing sponsored phase III trials. Vertical dotted lines indicate the year when the former and new GCP were issued.

until they receive new anticancer drugs which have been approved long before in other countries (The Ministry of Health, Labour and Welfare of Japan 2005). We discuss the aspects and issues of registration-directed trials in Japan by reviewing such trials for the 23 anticancer drugs.

Review of registration-directed clinical trials for the 23 anticancer drugs

A total of 65 phase I and II trials of an anticancer drug for approval were reviewed in terms of definition of eligibility criteria, maximum tolerated dose (MTD), sample size, response criteria, and extramural review for tumor responses. The MTD is the dose associated with seriouis but reversible toxicities in a sizeable proportion of patients and the one that offers the best chance for a favorable therapeutic ratio (Piantadosi 1997). The number of patients accrued in a trial, percentage of ineligible patients, number of participant hospitals in a trial, and the study period defined as the months between the first and last patient accrual were also analyzed. They were obtained from a published paper for 53 trials, from a meeting abstract and in-company resource for one trial, and from in-company resource alone for the remaining 11 trials. The clinical developmental period of an anticancer drug was defined as years between the start month of the first phase I trial and the month of the approval for lung cancer. These parameters are compared according to the approval year of the drug. We categorized three periods of approval: 1) before 1989, 2) between 1989 and 1996, and 3) between 1997 and 2004, because the former GCP was enforced in 1989, and the new GCP in 1997 (Fujiwara et al. 2002).

Of the 23 anticancer drugs, six drugs whose clinical development started before 1974 were approved on the basis of the clinical experience of the use of the drug without clinical trials (Fig. 1). A total of 24 phase I trials were identified (Table 1). The MTD was not defined in the protocol of any trials before 1989, but was defined in 33% of trials between 1989 and 1996, and in 70% of trials after 1996. Instead of the MTD, maximum acceptable dose, defined as the dose associated with grade 2 or severer toxicity in two thirds or more patients, was used in a trial after 1996. About twice more patients were registered in a trial before 1989 than thereafter, but 20% of the registered patients before 1989 were ineligible. The study period of a phase I trial got longer as the number of participant hospitals decreased, from 7 months and 11 hospitals before 1989 to 13 months and 4 hospitals after 1996, respectively.

In this review, 41 phase II trials for approval were analyzed (Table 2). Calculation of the sample size was not made in any trials before 1989, was seen in 40% of trials between 1989 and 1996, and in all trials thereafter. Response criteria were

TABLE 1. Investigational new drug phase I trials for approval.

	Before 1989	1989-1996	1997 or thereafter
Total number of trials	7	6	11
Defined, number (%) of trials			
Eligibility criteria	4 (57)	6 (100)	11 (100)
Maximum tolerated dose*	0 (0)	2 (33)	7 (70) ‡
Results of trials, median (range)			
Number of patients**	61 (32-170)	24 (18-54)	29 (9-43)
% of ineligible patients	20 (20-21) †	8 (0-33)	6 (0-22)
Number of hospitals	11 (1-21)	9 (1-18)	4 (1-17)
Study period in months	7 (5-30)	10 (5-11)	13 (8-24)

*Statistically significant difference obtained ($p = 0.014$ by the chi-square test); **Statistically significant difference obtained ($p < 0.01$ by the Kruskal Wallis test); †Data were available in 2 trials only; ‡Data were available in 10 trials only.

TABLE 2. Investigational new drug phase II trials for approval.

	Before 1989	1989-1996	1997 or thereafter
Total number of trials	7	11	23
Defined, number (%) of trials			
Eligibility criteria	4 (57)	11 (100)	23 (100)
Sample size calculation*	0 (0)	4 (40) ‡	23 (100)
Response criteria	6 (86)	11 (100)	23 (100)
Extramural review	3 (43)	9 (82)	23 (100)
Results of trials, median (range)			
Number of patients	71 (10-127)	68 (18-153)	61 (11-102)
% of ineligible patients	18 (0-29) †	3 (0-22)	3 (0-12)
Number of hospitals	27 (3-103)	17 (1-30)	20 (5-46)
Study period in months	18 (12-36)	12 (6-34)	26 (4-48) §

*Statistically significant difference obtained ($p < 0.01$ by the chi-square test); †Data were available in 5 trials only; ‡Data were available in 10 trials only; §Data were available in 22 trials only.

defined in almost all studies, but an extramural review was conducted only after 1989. The median number of registered patients in a trial was constant through the three periods, but the percentage of ineligible patients was high in trials conducted before 1989. The number of patients in a trial, and the number of hospitals in a trial were similar regardless of the year. The median study period in recent trials was 26 months.

The clinical development period was evaluated in the 23 drugs. Cisplatin was approved for germ cell tumors in 1983 and additionally approved for non-small cell lung cancer (NSCLC) in 1986. S-1 was firstly approved for gastric cancer in 1999, and additionally approved for NSCLC in 2004. The other drugs were approved for lung cancer for the first time. The median (range) clinical development period was 5.2 (3.2-14.5) years before 1989, 6.0 (4.8-9.1) years between 1989 and 1996, and 9.0 (3.9-15.4) years in 1997 or thereafter.

Development and recent problems of phase I and phase II trials in Japan

The concept of the "clinical trial" was not widely followed in Japan until 1974, when a phase I trial of nimustine hydrochloride (ACNU) was launched as one part of the United States-Japan Cooperation Cancer Research Program on the basis of the agreement between the National Cancer Institute and Japan Society for the Promotion of Science (Sugano 1982; Niitani 1999). Phase I trials before 1989 required the accrual of many patients, because 1) the maximum tolerated dose was not defined, 2) many patients were treated at unnecessary dose levels because the modified Fibonacci dose escalation schedule was not applied, and 3) the percentage of ineligible patients was high. Some of these issues were improved in 1997 or thereafter, but the maximum tolerated dose is still not defined in as many as 40% of trials. Recently, oncology phase I trials came to be conducted among fewer hospitals than before, as more participants were recruited in each hospital. This facilitated communication among phase I investigators, which is important to complete phase I trials safely.

Phase II trials play the central role in anticancer agent approval in Japan, because the approval can be granted based on the response rate in these trials. The quality of protocols for phase II trials suggested by eligibility criteria, sample size calculation, response criteria, and extramural review has been improved significantly. The study period of phase II trials, however, was and is still too long, as long as 4 years in recent trials. To increase participant hospitals, however, is not necessarily a desirable solution,

because a certain number of patients per hospital are needed to maintain the quality of trials by training doctors in the application of a new drug. Thus, enhancing patient recruitment in each hospital participating in the trial is the most important consideration.

A high standard of oncology clinical practice as the basis for clinical trials

Since a high standard of clinical practice is the basis for all clinical trials, the infrastructure for oncological clinical practice should be promptly advanced. The shortage of human resources including medical oncologists and oncology nurse practitioners in Japan is serious and acute. In the United States, medical oncology was established as a separate discipline by the American Board of Internal Medicine in 1971, and approximately 8,000 certified internists as of 2003 have been further certified by the Board in the subspecialty of medical oncology (Holland et al. 2003). In contrast, medical oncology has not been established as an academic unit or a regular university course in many medical schools in Japan. The Japanese Society of Medical Oncology was launched as an association in 1993, and framed the system of cancer medical specialists in 2003. A total of 1,479 doctors were certified as a tentative medical oncology supervisor between 2003 and 2005, and 47 doctors as a medical oncology specialist in 2005 (Table 3) (Japanese Society of Medical Oncology 2005).

To deal with complex cancer care, oncology nurse practitioners in the United States have become an integral part of the multidisciplinary team in the care of patients. As of 2002, more than 19,000 oncology nurse practitioners have been certified by the Oncology Nursing Society in the United States (Rieger 2003). In contrast, the number of oncology nurse practitioners registered in the Japanese Nursing Association was only 44 as of 2005 (Table 3) (Japanese Nursing Association 2005). Introduction of oncology nurse practitioners in clinical practice should lessen the burden on oncologists significantly and help them to have the incentive to take part in registration-directed clinical trials.

The infrastructure and human resources to support clinical trials

The infrastructure to support in-house clinical trials remains insufficient and even lacking in almost all institutes in Japan, while it has been advanced systematically in the United States. In the 1960s, General Clinical Research Centers were founded with the support of National Institutes of Health in 80 universities and academic institutions to provide the primary resources and optimal environment necessary for investigators to conduct clinical research. They include experienced nursing, laboratory, computer system, and biostatistical staff (Robertson and Tung 2001; General Clinical Research Centers 2005). To carry out a multicenter trial, a central data center

TABLE 3. Medical oncology professionals in Japan and the USA.

Professionals	*n* of medical oncology professionals	
	Japan	USA
Medical oncologists	47 [1]	8,000 [2]
Oncology nurse practitioners	44 [3]	19,000 < [4]
Clinical research coordinators	335 [5]	10,723 [6]

[1] Certified by the Japanese Society of Medical Oncology in 2005.
[2] Certified by the American Board of Internal Medicine as of 2003.
[3] Certified by the Japanese Nursing Association as of 2005.
[4] Certified by the Oncology Nursing Society as of 2002.
[5] Certified by the Japanese Society of Clinical Pharmacology and Therapeutics as of 2005.
[6] Certified by the Association of Clinical Research Professionals as of 2005.

is needed to deal with the increased administrative difficulties and quality assurance problems associated with this type of trial (Pollock 1994). The quality control and quality assurance system of the Japan Clinical Oncology Group has been significantly developed during the last two decades (Japan Clinical Oncology Group 2005). Using Internet resources may facilitate developing national and regional networks for clinical trials by reducing the burden associated with the extensive research time and considerable cost of all these processes (Paul et al. 2005).

The new GCP demands more of the clinical researchers in time, resources and money to enhance the science, credibility, and ethics of clinical trials for approval (Sweatman 2003). The clinical research coordinator (CRC) plays a key role in the clinical trial process by supporting investigators. The CRCs are involved in every aspect of registration-directed clinical trials, including protocol development, checking eligibility criteria, informed consent, organizing study schedules, checking clinical tests, filling in case report forms, and providing support for monitoring and auditing the trials (Rico-Villademoros et al. 2004; Sakamoto 2004). Association of Clinical Research Professionals in the USA has offered the CRC certification since 1992, and there are 10,723 CRCs to date (Association of Clinical Research Professionals 2006). The Japanese Society of Clinical Pharmacology and Therapeutics launched the certified CRC system in 2003, and there were 335 certified CRCs as of 2005 (Table 3) (The Japanese Society of Clinical Pharmacology and Therapeutics 2005).

In conclusion, clinical trials of anticancer agents for approval have been developing significantly, but still remain at an unsatisfactory level. Development of the infrastructure and human resources for clinical trials is an urgent task to complete good quality clinical trials for approval without delay.

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Phase II trial of carboplatin and paclitaxel in non-small cell lung cancer patients previously treated with chemotherapy

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Summary The purpose of this phase II trial was to evaluate the efficacy and toxicity of carboplatin plus paclitaxel in the treatment of advanced non-small cell lung cancer (NSCLC) previously treated with chemotherapy. Patients with a performance status (PS) of 0 or 1 who had received one or two previous chemotherapy regimens for advanced NSCLC were eligible. Paclitaxel 200 mg/m^2 was infused over 3 h and followed by carboplatin (area under the curve 6) infusion over 1 h, once every 3 weeks. Thirty patients were enrolled. A complete response was observed in 1 patient and a partial response in 10 patients, for an overall response rate of 36.7%. The median time to progression was 5.3 months. The median survival time was 9.9 months, and the 1-year survival rate was 47%. Hematological toxicity in the form of grade 3/4 neutropenia occurred in 54%, but grade 3 febrile neutropenia developed in only 3%. Non-hematological grade 3 toxicities were less frequent. There were no treatment-related deaths. The combination of carboplatin plus paclitaxel is an active and well-tolerated regimen for the treatment of NSCLC patients who have previously been treated with chemotherapy and have a good PS.

1. Introduction

Lung cancer remains a major cause of death from cancer in many countries. More than half of all patients diagnosed with non-small cell lung cancer (NSCLC) have advanced stage IIIB or IV disease at presentation, and patients with advanced NSCLC are candidates for systemic chemotherapy. Platinum-based chemotherapy is considered the standard first-line treatment for patients with advanced NSCLC, and prolongs survival, palliates symptoms, and improves quality of life [1,2]. Many patients with good performance status (PS) when progression occurs after first-line chemotherapy are suitable candidates for second-line chemotherapy [3].

The taxanes are an important class of new agents for the treatment of advanced NSCLC. Paclitaxel, in combination with carboplatin, is the most common regimen

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used as first-line chemotherapy for advanced NSCLC, and this combination has a more favorable toxicity profile and is more convenient to administer than other platinum-based regimens [4,5]. Docetaxel has been investigated more extensively than any other agent for second-line treatment of advanced NSCLC, and the results of two randomized phase III trials of second-line chemotherapy in patients with advanced NSCLC demonstrated that docetaxel monotherapy significantly improved survival compared with best supportive care or other single agents (vinorelbine or ifosfamide) [6,7].

Belani et al. recently reported that results of a phase III trial comparing a carboplatin plus paclitaxel regimen with a cisplatin plus etoposide regimen for first-line treatment of advanced NSCLC [8]. Carboplatin plus paclitaxel yielded a higher response rate (23% versus 15%), time to progression (121 days versus 111 days), and overall quality of life benefit than cisplatin plus etoposide, but the median survival time was better in the cisplatin plus etoposide arm than in the carboplatin plus paclitaxel arm (274 days and 233 days, respectively [$P = 0.086$]). The authors reported that a substantially greater proportion of patients in the cisplatin plus etoposide arm received second-line chemotherapy with a taxane-containing regimen than in the carboplatin plus paclitaxel arm, and suggested that treatment with taxanes in a second-line setting may have had an impact on the survival in their study. Remarkably, more than half of the regimens that were used in the second-line setting of their study consisted of paclitaxel alone or carboplatin plus paclitaxel, not docetaxel. While the efficacy of paclitaxel-containing regimens as first-line chemotherapy for advanced NSCLC has been established in many randomized phase III trials [9], the data on the efficacy of paclitaxel-containing regimens in second-line settings are limited [10,11].

Based these considerations we conducted a phase II trial to evaluate the efficacy and toxicity of carboplatin plus paclitaxel in the treatment of advanced NSCLC previously treated with chemotherapy.

2. Patients and methods

2.1. Eligibility criteria

The inclusion criteria were: pathologically confirmed advanced NSCLC patients with measurable disease who had received one or two previous chemotherapy regimens for their disease. Patients were required to submit evidence of failure of prior chemotherapy. Patients who were previously treated with carboplatin or paclitaxel were excluded if the best response was progressive disease (PD). Patients who had received prior radiotherapy were eligible provided that at least 30 days had elapsed between the completion of radiotherapy and entry into the study. Patients were also required to be 20–75 years of age, have an Eastern Cooperative Oncology Group PS of 0 or 1, and have adequate organ function as indicated by the following parameters: absolute neutrophil count $\geq 1500\,mm^{-3}$, platelet count $\geq 100{,}000\,mm^{-3}$, hemoglobin $\geq 9.0\,g/dl$, AST and ALT $\leq 2.0 \times$ the institutional upper normal limits, total bilirubin $\leq 1.5\,mg/dl$, creatinine $\leq 1.5\,mg/dl$, $PaO_2 \geq 65$ Torr. Exclusion criteria were: uncontrolled pleural or pericardial effusion, active concomitant malignancy, prior irradiation to areas encompassing more than a third of the pelvis plus spine, active infection, myocardial insufficiency or myocardial infarction within the preceding 6 months, uncontrolled diabetes mellitus or hypertension, any other condition that could compromise protocol compliance, pregnancy and/or breast-feeding. All patients were required to provide written informed consent before entry into the study. The study was approved by the institutional review board of our institution.

2.2. Treatment plan

Treatment was started within a week of entry into the study. Patients received paclitaxel $200\,mg/m^2$ diluted in 500 ml of 0.9% saline as a 3-h intravenous infusion followed by carboplatin (area under the curve [AUC] 6; Calvert formula) diluted in 250 ml of 5% glucose as a 1-h intravenous infusion, every 3 weeks. All patients were premedicated with dexamethasone (24 mg i.v.), famotidine (20 mg i.v.), and diphenhydramine (50 mg orally) 30 min before the paclitaxel infusion to prevent a hypersensitivity reaction. A 5-HT3-receptor antagonist was intravenously administered as an antiemetic before carboplatin. Therapy was continued for at least two cycles unless the patient experienced unacceptable toxicity or had PD. The maximum number of cycles of chemotherapy was six. In the event of grade 4 leukopenia or thrombocytopenia or of grade 3 neutropenic fever, the dose of carboplatin and paclitaxel was reduced to AUC 5 and $175\,mg/m^2$, respectively, in the following cycle of chemotherapy. The next cycle of chemotherapy was started if the neutrophil count was $\geq 1500\,mm^{-3}$, the platelet count $\geq 100{,}000\,mm^{-3}$, AST and ALT ≤ 100 IU/l, total bilirubin $\leq 2.0\,mg/dl$, creatinine $\leq 1.5\,mg/dl$, PS 0 or 1, and the patient was afebrile.

Pretreatment evaluation included a medical history, a physical examination, vital signs, height and body weight, PS, complete blood count, biochemical studies, arterial blood gas analysis, electrocardiogram, chest radiograph and computed tomography scan (CT), abdominal ultrasound or CT, and brain magnetic resonance imaging or CT. A complete blood count, biochemical studies, and chest radiograph were performed weekly during the first cycle of chemotherapy, and 2 weekly starting with the second cycle.

2.3. Response and toxicity assessment

Objective tumor response was assessed as complete response (CR), partial response (PR), stable disease ≥ 8 weeks (SD), or PD according to the Response Evaluation Criteria in Solid Tumors. Measurable lesions were defined as lesions whose longest diameter was $\geq 2\,cm$. Imaging studies were repeated every 4 weeks until the objective tumor response was confirmed. All responses were reviewed by an independent radiologist. Toxicity was graded using National Cancer Institute-Common Toxicity Criteria version 2.0.

2.4. Statistical analysis

The primary endpoint of this study was the response rate, defined as the proportion of patients whose best response was CR or PR among all enrolled patients in the intent-to-treat analysis. The secondary end points were toxicity and overall and progression-free survival (PFS) from the date of enrollment in this study.

According to Simon's minimax two-stage phase II study design, the treatment program was designed for a minimal response rate of 5% and to provide a significance level of 0.05 with a statistical power of 80% in assessing the activity of the regimen according to a 20% response rate. The upper limit for first-stage drug rejection was no response in 13 evaluable patients. The upper limit for second-stage drug rejection was three responses in 27 evaluable patients. Overall survival time was defined as the interval between enrollment in this study and death or the most recent follow-up visit. PFS was defined as the interval between enrollment in this study and the first documented PD, death, or the most recent follow-up visit. Survival was estimated by the Kaplan–Meier analysis method. All comparisons between proportions were performed by Fisher's exact test.

3. Results

3.1. Patient characteristics

Between October 2002 and November 2003, 30 patients were enrolled in this study, and their characteristics are shown in Table 1. Twenty-six (87%) patients were men, and 21 (70%) patients had adenocarcinoma. Median age was 60 years. The majority of the patients (93%) had received prior platinum-based chemotherapy, and seven (23%) patients had received two prior chemotherapy regimens. The platinum-based chemotherapy regimens that had been used were: cisplatin plus vinorelbine ($n=26$), cisplatin plus gemcitabine ($n=1$), and carboplatin plus gemcitabine ($n=1$). There were 15 (50%) responders to any of the prior chemotherapy regimens and 12 of them had experienced a response (CR/PR) to cisplatin-based chemotherapy. Twenty-one (70%) patients had a treatment-free interval of 3 or more months since the final dose of the prior chemotherapy regimen.

A total of 94 cycles of chemotherapy were administered, and the median number of cycles per patient was three (range, 1–6). Four patients had received only one cycle of treatment either because of toxicity (two patients, grade 3 rash), the patient's refusal (one patient), or PD (one patient).

3.2. Response and survival

Two patients were not evaluable for response because the protocol treatment had been terminated because of toxicity (grade 3 rash) during the first cycle of chemotherapy, and they subsequently received further chemotherapy without PD. There was 1 CR and 10 PRs among the 30 patients, and the objective response rate in the intent-to-treat analysis was 36.7% (95% confidence interval [CI], 19.9–56.1%) (Table 2). Treatment outcomes of all patients are listed in Table 3. The response rate of patients who experienced a response (CR/PR) to prior cisplatin-based chemotherapy was 43% (6/14), as opposed to 23% (3/13) among the non-response patients ($P=0.41$). The response rate of the patients who had received one prior chemotherapy regimen was 39% (9/23), as opposed to 28% (2/7) among the patients who had received two regimens ($P>0.99$). According to the treatment-free interval since the final dose of the prior chemotherapy regimen, the response rate of patients whose interval was 3 months or more was 33% (7/21), com-

Table 1 Patient characteristics

Characteristic	No. of patients (%)
Patients enrolled	30
Sex	
Male	26
Female	4
Age, years	
Median	60
Range	39–75
ECOG performance status	
0	7
1	23
Stage	
IIIB	11
IV	19
Histology	
Adenocarcinoma	21
Squamous cell carcinoma	7
Large cell carcnioma	2
Prior treatment	
Platinum-based chemotherapy	28 (93)
Docetaxel	5 (16)
Chest radiotherapy	4 (13)
No. of prior chemotherapy regimens	
1	23
2	7

Table 2 Treatment efficacy ($n=30$)

	No. of patients	%
Response		
Overall response rate	11	36.7
Complete response	1	3.3
Partial response	10	33.3
Stable disease	12	40
Progressive disease	5	16.7
Not evaluable	2	6.7
Survival		
Median (months)	9.9	
1 year (%)	47	
Progression-free survival		
Median (months)	5.3	

Table 3 Treatment outcomes of all patients

Patient No.	Prior first-line therapy		Prior second-line therapy		Time from last therapy (months)	CBDCA + PTX, best response	PFS (months)	Survival (months)
	Regimen	Best response	Regimen	Best response				
1	CDDP + VNR	SD	DOC	PD	1.8	SD	1.4	25.2
2	CBDCA + GEM	NE	Gefitinib	PD	0.8	PR	3.8	8.8
3	CDDP + VNR	SD	—		6.8	SD	7.6	18.1
4	CDDP + GEM	PR	—		9.5	PR	7.5	33.8+
5	CDDP + VNR	SD	—		4.8	SD	2.8	7.0
6	CDDP + VNR + DOC + RT	PR	—		6.0	PR	8.0	21.6
7	GEM + VNR	SD	—		23.0	PD	1.2	7.8
8	CDDP + VNR + RT	PR	—		13.6	SD	6.7	25.0+
9	CDDP + VNR	SD	—		5.0	SD	2.1	3.7
10	CDDP + VNR	SD	—		5.0	PD	1.2	6.7
11	CDDP + VNR	PR	—		8.9	NE	1.1	3.3
12	CDDP + VNR	SD	Gefitinib	CR	1.9	SD	6.3	6.3
13	CDDP + VNR	PR	—		5.4	NE	1.0	13.4
14	CDDP + VNR	PR	—		1.7	SD	4.8	5.7
15	CDDP + VNR + RT	PR	—		9.3	SD	5.0	15.7
16	CDDP + VNR	SD	—		2.8	PR	3.7	15.8
17	CDDP + VNR	SD	DOC + GEM	SD	3.8	SD	5.3	21.6+
18	CDDP + VNR + DOC + RT	PR	—		3.9	SD	4.5	9.0
19	CDDP + VNR	PR	—		12.9	PR	9.4	16.0
20	CDDP + VNR	PR	—		11.5	CR	24.8+	24.8
21	CDDP + VNR	PD	—		1.1	PR	9.2	23.6+
22	CDDP + VNR	SD	DOC	SD	4.5	PD	2.3	5.5
23	Gefitinib	SD	—		0.9	PR	8.8	12.7
24	CDDP + VNR	PR	—		11.1	PR	5.3	10.2
25	CDDP + VNR	PR	Gefitinib	PR	4.4	PR	5.5	9.9
26	CDDP + VNR	NE	—		11.7	PR	7.0	12.2
27	CDDP + VNR	PR	—		5.4	SD	6.2	9.4
28	CDDP + VNR	SD	—		0.8	PD	1.4	2.5
29	CDDP + VNR	PR	—		4.4	PD	0.2	8.4
30	Gefitinib	PD	CDDP + VNR	PD	0.9	SD	3.1	3.3

CBDCA, carboplatin; PTX, paclitaxel; PFS, progression-free survival; CDDP, cisplatin; VNR, vinorelbine; GEM, gemcitabine; DOC, docetaxel; RT, chest radiotherapy; SD, stable disease; NE, not evaluable; PR, partial response; PD, progressive disease; CR, complete response.

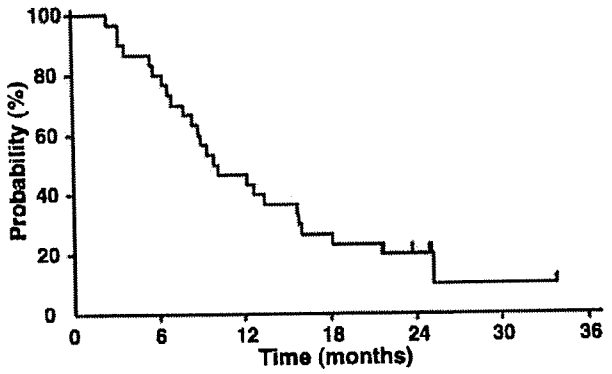

Fig. 1 Kaplan–Meier curve for overall survival.

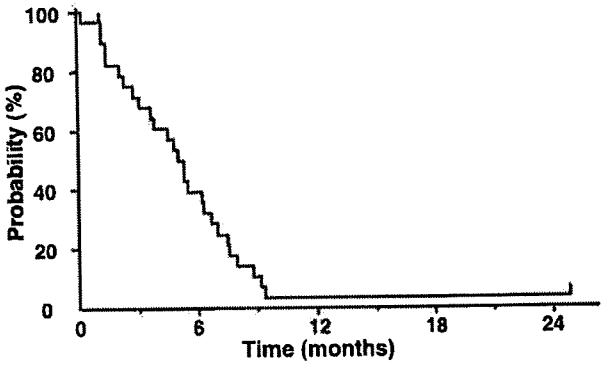

Fig. 2 Kaplan–Meier curve for progression-free survival.

pared with 44% (4/9) in patients in whom it was less than 3 months ($P=0.68$).

The median follow-up time was 24 months. The median survival time (MST) was 9.9 months (range, 2.5–33.8 months), and the 1-year survival rate was 47% (95% CI, 29–65%). The median PFS was 5.3 months. The Kaplan–Meier curve for overall survival and for PFS is shown in Figs. 1 and 2, respectively. Nineteen patients (63%) received at least one subsequent chemotherapy regimen, and their regimens are shown in Table 4. Fourteen of them were treated with gefitinib, and a PR was achieved in three of them.

3.3. Toxicity

The common toxicities associated with carboplatin plus paclitaxel are listed in Table 5. Grade 3/4 neutropenia occurred in 54% of the patients in our study, but grade 3 febrile neutropenia developed in only 3%. Grade 3/4 anemia and thrombocytopenia were observed in five patients (16%) and two patients (13%), respectively. Non-hematological grade 3 toxicities were less frequent. Grade 3 hyponatremia was observed in five (16%) patients, but they were all asymptomatic. Grade 2 neuropathy occurred in 33% of the patients. There were no treatment-related deaths.

Table 4 Post-study chemotherapy

Regimen	No. of patients	Responder (%)
Gefitinib	14	3 (21)
Docetaxel	9	0
Gemcitabine plus viborelbine	1	0

4. Discussion

Docetaxel, pemetrexed, and erlotinib have been approved for second-line treatment of advanced NSCLC on the basis of the results of phase III trials [6,7,12,13]. Hanna et al. reported a phase III study comparing 3-weekly pemetrexed 500 mg/m^2 with 3-weekly docetaxel 75 mg/m^2 as second-line treatment for advanced NSCLC. The overall response rate with pemetrexed and docetaxel was 9.1% and 8.8%, respectively, and MST was 8.3 months and 7.9 months, respectively. Although efficacy in terms of the outcome as measured by survival time and response rate was similar for both treatments, the pemetrexed group experienced less grades 3–4 hematological toxicity and alopecia of all grades [12]. In the trial reported by Shepherd et al. 731 NSCLC patients previously treated with chemotherapy were randomized to receive either erlotinib at a dose of 150 mg daily or placebo, and the response rate in the erlotinib group was 8.9%. MST was 6.7 months in the erlotinib group and 4.7 months in the placebo group ($P<0.001$). The results of their trial showed that erlotinib significantly prolonged the survival of patients with advanced NSCLC who had previously been treated with chemotherapy [13]. Despite the positive results of these phase III trials, the response rate of advanced NSCLC to second-line chemotherapy remains low, and the life expectancy of advanced NSCLC patients remains short. Alternative effective chemotherapy option is needed for second-line treatment of advanced NSCLC.

The combination of carboplatin plus paclitaxel has proved effective as one of the standard platinum-based doublet regimens for first-line treatment of advanced NSCLC [4,5,14]. However, since the efficacy of carboplatin plus paclitaxel used in a second-line setting had hardly been assessed, in the present study we evaluated the efficacy and toxicity of carboplatin plus paclitaxel in the second- or third-line treatment of advanced NSCLC. The results in the 30 patients with advanced NSCLC previously treated with chemotherapy indicated that the combination of carboplatin plus paclitaxel yielded an objective response rate of 36.7% and an MST of 9.9 months, with a 1-year survival rate of 47%. Our study had not included patients who were treated with the platinum/taxane combination chemotherapy. Most of the toxicity observed in our study was hematological. Grade 3/4 neutropenia, anemia, or thrombocytopenia occurred in 54, 16, or 13% of the patients in our study, respectively. Hematological toxicity of carboplatin plus paclitaxel used in first-line treatment for Japanese patients with advanced NSCLC has been reported that grade 3/4 neutropenia, anemia, or thrombocytopenia occurred in 88, 15, or 11% of the patients [15]. The toxicity observed in our study appeared similar to that of carboplatin plus paclitaxel, which was administered as the first-line treatment, although the number of patients in our study was not large. The combination of carboplatin plus paclitaxel seems to be effective and tolerable, not only as first-line therapy for advanced NSCLC but