that the expression level of H-FABP was significantly different between the responder (PR and CR) and nonresponder (PD) groups (P = 0.0031, Mann-Whitney U test) and also between the patients with MR or SD and the nonresponder group (P = 0.0047, Mann-Whitney U test). These results indicate that up-regulation of H-FABP in tumor tissues can be monitored by routine clinical methods.

Discussion

We identified 87 protein spots of which the intensity was statistically significantly different between samples from the responder (CR and PR) and nonresponder (PD) groups in the training set. Application of a data-mining procedure allowed identification of a set of nine protein spots that accurately distinguished between responders and nonresponders. The different expression levels of these nine protein spots allowed classification of 13 of 14 of our test PR and PD cases in accordance with their clinical response to gefitinib. These protein spots classified cases showing a MR to gefitinib (MR) into the responder group. The intermediate cases, SD, were categorized into both responder and nonresponder groups. The usefulness of our findings will be validated in a larger clinical data set.

Table 3. List of proteins for the response to gefitinib

Spots no.*	Rank	Accession no.†	Identified protein†	MW (DA)‡	pI‡	Ion charge state (+)	MZ (obs)§	Mass‖	δ¶	Miss**	Mascot ions score††	Peptide sequence
384	5	Q96RP9	Ig mu chain C region	49,557	6.35	2	810.3	1,617.7	0.91	0	74	QVGSGVTTDQVQAEAK
						2	640.1	1,277.5	0.63	0	47	YAATSQVLLPSK
671	1	P01876	Ig α-1 chain C region	37,655	6.08	2	919.2	1,836.0	0.32	0	68	QEPSQGTTTFAVTSILR
						2	771.8	1,540.7	0.91	0	54	DASGVTFTWTPSSGK
1090	7	Q9UNH7	SNX 6	46,649	5.81	2	636.5	1,270.5	0.55	0	73	NLVELAELELK
						2	577.0	1,152.2	−0.33	0	39	SLVDYENANK
1182	8	P50453	Cytoplasmic antiproteinase 3	42,404	5.61	2	816.4	1,629.8	0.95	0	82	IEELLPGSSIDAETR
						2	626.6	1,249.4	1.66	0	75	AFQSLLTEVNK
						2	591.0	1,179.5	0.47	0	63	LVLVNAIYFK
						2	757.5	1,513.6	−0.56	0	47	LQEDYDMESVLR +Oxidation (M)
1292	6	P40121	Macrophage capping protein	38,518	5.88	2	633.8	1,264.4	1.18	0	85	VSDATGQMNLTK
						2	676.8	1,351.4	0.05	0	79	YQEGGVESAFHK
						2	932.1	1,861.1	1.11	0	50	MQYAPNTQVEILPQGR +Oxidation (M)
						2	659.8	1,317.3	0.23	0	41	EGNPEEDLTADK
1711	3	Q8NBJ7	Sulfatase modifying factor 2	33,857	7.78	2	792.5	1,581.7	1.32	0	112	MGNTPDSASDNLGFR
						2	779.9	1,557.6	0.15	0	95	GASWIDTADGSANHR
						2	740.0	1,477.6	0.36	0	83	LPTEEEWEFAAR
						2	613.2	1,224.4	−0.02	0	66	FLMGTNSPDSR
						2	629.9	1,256.5	1.27	0	55	SVLWWLPVEK
						2	818.0	1,633.8	0.12	1	55	RLPTEEEWEFAAR
						2	837.7	1,672.9	0.48	0	47	LEHPVLHVSWNDAR
2091	9	P09211	Glutathione S-transferase P	23,225	5.44	2	647.5	1,292.5	0.44	0	36	MLLADQGQSWK +Oxidation (M)
2182	4	P02794	Ferritin heavy chain	21,094	5.30	2	823.4	1,643.8	1.04	0	91	MGAPESGLAEYLFDK +Oxidation (M)
						2	648.3	1,294.5	0.03	0	53	NVNQSLLELHK
2478	2	P05413	Fatty acid-binding protein, heart	14,727	6.34	2	735.2	1,467.5	0.81	0	103	LGVEFDETTADDR
						2	798.7	1,595.7	−0.32	1	73	LGVEFDETTADDRK
						2	603.3	1,204.3	0.26	0	70	WDGQETTLVR
						2	455.0	907.0	1.04	0	67	SLGVGFATR
						2	774.7	1,546.8	0.56	0	61	QVASMTKPTTIIEK
						2	438.0	873.0	0.88	0	54	NGDILTLK
						1	889.6	889.0	−0.41	0	45	SIVTLDGGK

Abbreviation: pI, isoelectric point.
*Spot numbers refer to those in Fig. 1B (Supplementary Fig. S3).
†Accession nos. of proteins were derived from Swiss-Prot and National Center for Biotechnology Information nonredundant databases.
‡Theoretical molecular weight and isoelectric point were obtained from Swiss-Prot and the ExPASy database (http://au.expasy.org).
§Experimental m/z value.
‖Relative molecular mass calculated from the peptide sequence.
¶Difference (error) between the experimental and calculated masses.
**Number of missed cleavage sites.
††Mascot ions score (http://www.matrixscience.com/search_form_select.html).

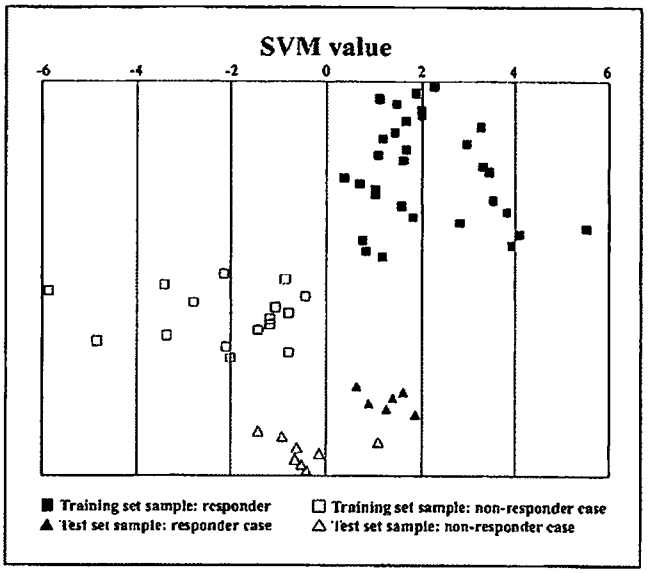

Fig. 2. Predictive performance of the nine spots was validated by examining the SVM value of each sample in the group.

We identified the proteins whose expression was correlated with response to gefitinib and found associations with the EGFR signal pathway and with the biology of lung cancer. Sorting nexin (SNX) 6 is a member of a SNX family that functions in the intracellular trafficking of plasma membrane receptors (33). SNXs form complexes with other SNXs and with plasma membrane receptors. In complexes with SNX1, SNX2, and SNX4, SNX6 interacts with the intercellular portion of the EGFR as well as with transforming growth factor-β receptor, insulin receptor, leptin receptor, and platelet-derived growth factor receptor (34). By binding to the kinase domain of the transforming growth factor-β receptor, SNX6 perturbs transforming growth factor-β signal transduction (34). The other SNX family, SNX1, decreases the expression of EGFR by activating the endosome-to-lysosome pathway with enterophilin-1 (35), although the functions of the complex of SNX6 and EGFR have not yet been reported. The functional association of SNX6 with oncogene product Pim-1, which has been implicated in the development of hematopoietic (36), gastric (37), and prostatic (38) malignancies, suggests the involvement of SNX6 in cancer biology. Kakiuchi et al. (21) reported that another SNX family member, SNX13, was correlated with the response to gefitinib in patients with NSCLC. These reports suggest that SNX6 might play an important role in signal transduction pathways that affect the phenotypes of lung cancer.

We tried to identify the proteins whose expression was associated with EGFR mutation. Because gefitinib is a specific inhibitor of EGFR and mutation of EGFR is considered to be a predictive marker for gefitinib sensitivity, we had expected some similarity between the set of proteins predicting sensitivity to gefitinib and the set of proteins reflecting EGFR mutation status. However, only sulfate modifying factor 2 was common to the two sets. Search of the PubMed database revealed no association of sulfate modifying factor 2 with the EGFR pathway and no evidence for its involvement in resistance to chemotherapy. Similarly, the other proteins correlated with EGFR mutation status had no obvious involvement in the EGFR pathway. Functional studies on these proteins will contribute to further understanding of EGF signaling in cells and to discovery of novel therapeutic targets in lung cancer.

2D-DIGE is a high-performance proteomic technology and a powerful tool to develop candidate biomarkers. However, 2D-DIGE requires expensive fluorescent dyes and well-trained operators to run the gels. Thus, routine clinical studies with multiple large-format two-dimensional gels and a 2D-DIGE protocol are unlikely to be practical. Application of our results requires a simple and cost-effective method that can be used routinely in the clinic. In addition, as we need to examine the expression of multiple proteins, a practical tool for simultaneously measuring the amount of the other proteins is required. With that in mind, we validated measurement of the differential expression of H-FABP by the use of a commercially available ELISA kit (MARLIF-M H-FABP) that is routinely used in hospitals for the early diagnosis of acute myocardial infarction using serum samples. The expression level of H-FABP in tumor tissues as monitored by the ELISA assay was highly correlated with that by 2D-DIGE, and a significant difference in H-FABP expression was observed between responders (CR + PR), minor responders (MR + SD), and nonresponders (PD). Thus, our results can provide a simple and direct method to predict the response to gefitinib.

H-FABP functions in intracellular lipid transport, storage, and metabolism. As H-FABP is highly expressed in heart and released into plasma after myocardial injury, it has been used as a plasma marker for early diagnosis of acute myocardial infarction and stroke. However, many lines of evidence also suggest an association of H-FABP with cancer biology. Higher expression of H-FABP was observed in a more tumorigenic small-cell lung cancer cell line (39) compared with its counterpart. Increased expression of H-FABP is associated with tumor aggressiveness, metastasis, and poor prognosis of gastric cancer (40). In contrast, H-FABP is known to have growth-inhibitory activity in breast cancer cells (41), and breast cancer does not express H-FABP because of gene silencing by hypermethylation (42). These observations suggest complexity in the way that H-FABP is involved in the progression of cancer. Recently, Loeffler-Ragg et al. (43) reported that another FABP family member, E-FABP, is up-regulated in gefitinib-resistant colon cancer cell lines compared with gefitinib-sensitive cell

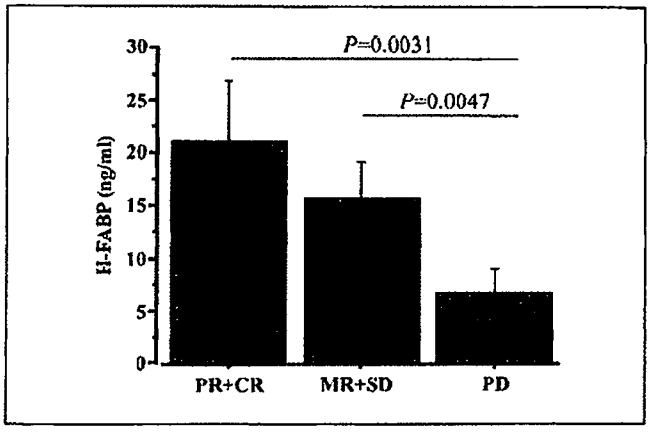

Fig. 3. ELISA assay for H-FABP. The differential expression level of H-FABP was validated by ELISA assay.

lines. Further study on the contribution of the FABP family to cancer phenotypes, including resistance to chemotherapy, will provide novel insights into cancer biology.

In conclusion, our proteomic study has identified proteins whose expression can predict the response to gefitinib in patients with recurrence of lung adenocarcinoma. Large-scale validation of the present results and functional analysis to elucidate the contribution and synergies of the identified proteins in the response to gefitinib will assist in developing novel therapeutic strategies for lung cancer.

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Proteomics-based identification of α-enolase as a tumor antigen in non-small lung cancer

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(Received December 17, 2006/Revised April 3, 2007/Accepted April 6, 2007/Online publication May 16, 2007)

Autoantibodies against tumor antigens represent one type of biomarker that may be assayed in serum for detection of cancer and monitoring of disease progression. In the present study, we used a proteomics-based approach to identify novel tumor antigens in non-small cell lung cancer (NSCLC). By combining two-dimensional electrophoresis, western blotting, mass spectrometry and enzyme-linked immunosorbent assay technology, we detected autoantibodies against α-enolase in a subset of NSCLC patients' sera. When 'Mean $OD_{\text{healthy control sera}}$ + 3 $SD_{\text{healthy control sera}}$' was used as the cut-off point, the prevalence of this autoantibody was 27.7% in patients with NSCLC (26 of 94), 1.7% in healthy control subjects (1 of 60), and not detectable in sera from 15 patients with small cell lung cancer, 18 patients with gastrointestinal cancer and nine patients with *Mycobacterium avium* complex infection of lung. Immunohistochemical staining showed that expression of α-enolase was increased in cancer tissues of NSCLC patients, and flow cytometric analysis confirmed the expression of α-enolase at the surface of cancer cells. The combined detection of autoantibodies against α-enolase, carcinoembryonic antigen and cytokeratin 19 fragment (CYFRA21-1) enhanced sensitivity for the diagnosis of NSCLC. Therefore, autoantibodies against α-enolase may constitute a promising biomarker for NSCLC. (*Cancer Sci* 2007; 98: 1234–1240)

Lung cancer is the leading cause of cancer death,[1] and NSCLC accounts for nearly 80% of lung cancer cases. There is an urgent need for a better understanding of the biological mechanisms of NSCLC as well as the identification of reliable biomarkers for its diagnosis and prognosis. To date, a number of NSCLC markers have been evaluated, including CEA, CYFRA 21-1, SCC antigen, CA125 and NSE.[2–8] Autoantibodies against several tumor antigens such as L-myc and c-myc, p53 and antineural/antinuclear antigens have also been investigated.[9–12] Recently, autoantibodies against PGP9.5, peroxiredoxin-I, annexin-I and annexin-II were identified in the sera of lung cancer patients using a proteomic approach.[13–15] However, the sensitivity and specificity of these biomarkers are not yet satisfactory and there are currently no data to support any particular method for screening for lung cancer.[16]

Autoantibodies against tumor antigens represent one type of biomarker that may be assayed in serum for detection of cancer and monitoring of disease progression. In spite of the fact that the quantity of any tumor antigen in cancer cells or in the circulation is usually very small, especially in the early stages of cancer, the body's immune response to such antigens represents a remarkable phenomenon of biological amplification of these weak signals from tumor antigens.[17] The identification of panels of tumor antigens that elicit an immune response may thus be useful for detecting potential specific biomarkers as well as for the initiation of immunotherapy against NSCLC. The aim of the present study was to identify novel candidate tumor antigens in NSCLC by means of a proteomics-based approach. One of these antigens was identified as α-enolase, and its immunogenicity was confirmed by western blotting using recombinant protein. The results obtained with enzyme-linked immunosorbent assay (ELISA) demonstrated that when 'Mean $OD_{\text{healthy control sera}}$ + 3 $SD_{\text{healthy control sera}}$' was used as the cut-off point, a humoral immune response directed against α-enolase occurred in 27.7% of NSCLC patients, but in only 1.7% of healthy control subjects. Immunohistochemical staining showed that α-enolase was overexpressed in cancer tissues of NSCLC patients. The combined detection of autoantibodies against α-enolase, CEA and CYFRA 21-1 enhanced sensitivity for NSCLC diagnosis. Therefore, autoantibodies against α-enolase may constitute a promising biomarker for NSCLC.

Materials and Methods

Subjects. Sera and tumor tissue were obtained at the time of diagnosis after informed consent had been given by the subjects. The experimental protocol was approved by the ethics committee of Osaka University. Sera from 94 patients with NSCLC, 15 patients with SCLC, 18 patients with gastrointestinal cancer (10 patients with gastric cancer, 8 patients with colon cancer) and nine patients with MAC were analyzed. In terms of TNM stages, the NSCLC patients comprised 17 cases of stage I, 14 cases of stage II, 34 cases of stage III and 29 cases of stage IV. The histological distribution of NSCLC was 73 adenocarcinoma cases and 21 SCC cases. Clinical data for the serum tumor marker CEA and CYFRA 21-1 were also collected for investigation. Sera from 60 asymptomatic healthy subjects, whose average age and sex were comparable to those of the NSCLC patient group, were used as controls.

1-DE and 2-DE. Proteins were extracted from NSCLC tumor tissues using the Complete Mammalian Proteome Extraction Kit (Calbiochem, Darmstadt, Germany). For 1-DE, extracted proteins

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Abbreviations: 1-DE, one-dimensional electrophoresis; 2-DE, two-dimensional electrophoresis; a.a., amino acids; CA, cancer antigen; CBB, Coomassie brilliant blue; CEA, carcinoembryonic antigen; CYFRA21-1, cytokeratin 19 fragment; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; IEF, isoelectric focusing; IHC, immunohistochemical; LC, liquid chromotography; MAC, Mycobacterium avium complex; MALDI-TOF, matrix-assisted laser desorption–ionization time-of-flight; MS, mass spectrometry; NSCLC, non-small cell lung cancer; NSE, neuronspecific enolase; OD, optical density; PCR, polymerase chain reaction; PMF, peptide mass fingerprinting; PVDF, polyvinylidene difluoride; SCC, squamous cell carcinoma; SCLC, small cell lung cancer; TNM, tumour–node–metastasis.

doi: 10.1111/j.1349-7006.2007.00509.x

were resolved by using 10% Bis-Tris Criterion XT Precast gels (Bio-Rad Laboratories, Hercules, CA, USA), transferred to PVDF membranes or stained with CBB. For 2-DE, IEF was carried out using the PROTEAN IEF cell (Bio-Rad Laboratories) according to the manufacturer's instructions. Extracted proteins were reconstituted in a rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2 mM tributyl phosphine (TBP), 0.0002% bromophenol blue (BPB), 0.2% bio-lyte ampholyte 3-10) and applied to ReadyStrip IPG strips (11 cm, pH 3–10). IEF was run for 45 000 Vh, and 2-DE was carried out using 10% Bis-Tris Criterion XT Precast gels. The gels were then stained with the Silver Stain MS Kit (Wako Pure Chemical Industries, Osaka, Japan) or used for protein transfer to PVDF membranes.

Western blotting. After blocking with 5% skim milk, the PVDF membranes were incubated with serum at a 1:100 dilution or rabbit anti-enolase antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:1500 dilution. The membranes were then incubated with sheep anti-human IgG or donkey anti-rabbit IgG (Amersham Biosciences UK, Buckinghamshire, UK). Membranes incubated with sheep anti-human IgG only were used as negative controls. Finally, the signals were visualized with an enhanced chemiluminescence reaction system (Perkin Elmer Life Sciences, Boston, MA, USA).

Identification of protein bands or spots. Protein bands on gels stained with CBB or protein spots on gels stained with silver, which corresponded to positive bands or spots on western blot membranes, were excised from the gel and digested with trypsin (Promega, Madison, WI, USA) according to published procedures.[18] For protein bands, the LC-MS/MS analysis was carried out using an LCQ ion trap mass spectrometer (ThermoElectron, San Jose, CA, USA) coupled on-line with Magic 2002 capillary high-performance liquid chromatography (Michrom BioResources, Auburn, CA, USA). For protein spots, all PMF spectra were obtained by using an ultraflex TOF/TOF MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). MS/MS or PMF data were then searched with Mascot software (Matrix Science, London, UK) against the NCBInr or swiss-prot databases. Protein database searching was carried out with following parameters for PMF: *Homo sapiens*, maximum of one missed, cleavage by trypsin, monoisotopic mass value, charge state of 1+, allowing a mass tolerance of 100 p.p.m., and carbamidomethyl modification of cysteine.

Preparation of recombinant protein. To prepare recombinant proteins, the human full-length α-enolase complementary DNA (1–434 a.a.) was amplified by PCR from the Hep3B cell line cDNA library using the primers: sense 5′-GTGGCTAGAAGTTCACCATG-3′, antisense 5′-TTACTTGGCCAAGGGGTTTC-3′. To map the autoepitope on α-enolase, three cDNA fragments that encode C-terminal deletion mutant proteins (α-Eno1, α-Eno2, α-Eno3) were similarly amplified. The nucleotide sequences of the primers for PCR were: α-Eno1 (1–334 a.a.) sense 5′-TGTCTATTCTCAAGATCCATGCC-3′, antisense 5′-TTACTCGTTCACGGCCTTGGC-3′; α-Eno2 (1–234 a.a.), sense 5′-TGTCTATTCTCAAGATCCATGCC-3′, antisense 5′-TTAAGCTTTCCCAATAGCAGTC-3′; and α-Eno3 (1–134 a.a.) sense 5′-TGTCTATTCTCAAGATCCATGCC-3′, antisense 5′-TTAGATGTGGCGGTACAGGGG-3′. These cDNA fragments were then subcloned into the pET-28a(+) vector (Novagen, Madison, WI, USA), resulting in expression of α-enolase or its fragments with a 6 × His tag. Recombinant proteins were produced in *Escherischia coli* BL21-CodonPlus (DE3)-RIL cells (Stratagene, La Jolla, CA, USA) and purified by affinity chromatography using Ni-NTA resin (QIAGEN, Tokyo, Japan). Recombinant human full-length or C-terminal deletion mutant α-enolase, rabbit β-enolase (Sigma, St Louis, MO, USA) and human γ-enolase (Calbiochem) were subjected to sodium dodecylsulfate–polyacrylamide gel electrophoresis, using a 4–20% precast gel, then stained with CBB or transferred to PVDF membrane and probed with anti-enolase antibody, anti-6 × His monoclonal antibody or sera as described above.

Flow cytometry. Human lung adenocarcinoma cell line A549 was maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells (10^6) were incubated with rabbit anti-enolase antibody at a 1:100 dilution and labeled with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin (BD Biosciences, San Jose, CA, USA). Normal rabbit IgG was used as a control. Stained cells were analyzed using a FACS Canto cytometer (Becton-Dickinson, Mountain View, CA, USA) and the results were analyzed using FlowJo software (Tree Star, Stanford, CA, USA).

ELISA. To assess the potential of these autoantibodies as a diagnostic marker, their frequencies in the sera were determined by means of ELISA using recombinant human full-length α-enolase protein. The ELISA was carried out as published elsewhere, with modifications.[19] Briefly, each well of a Microtiter plate (MaxiSorp; Nunc A/S, Roskilde, Denmark) was coated with 1 μg of recombinant human full-length α-enolase. After blocking with 1% bovine serum albumin, all wells were incubated with human serum at a 1:500 dilution at room temperature for 1 h. To reduce the background level originating from the non-specific reactivity of sera with bacterial proteins, the sera were diluted and incubated with 100 μg/mL *E. coli* BL21-CodonPlus (DE3)-RIL cell lysate for 2 h at room temperature before incubation with coated recombinant human α-enolase. The antigen–antibody complexes were detected with 1:5000-diluted HRP-conjugate sheep anti-human IgG with TMB (Dako, Carpinteria, CA, USA) as the substrate. OD was read at 450 nm. The antibody titer was expressed by using arbitrary binding units calculated according to the formula:

$$\text{binding units of sample} = (OD_{\text{sample}}/[\text{Mean } OD_{\text{healthy control sera}} + 3\ SD_{\text{healthy control sera}}]) \times 100.$$

Based on this formula, 100 binding units was used as the cut-off point.

IHC staining. After deparaffinization, tissue sections were treated with 100% cold methanol containing freshly prepared 0.3% hydrogen peroxide for 30 min, blocked in 10% normal goat serum for 20 min and incubated with rabbit anti-enolase antibody (Santa Cruz Biotechnology) at a 1:250 dilution overnight. Incubation of parallel sections omitting the first antibody was done to generate negative controls. Staining of sections was completed with a biotin-conjugated secondary antibody, HRP-conjugated streptavidin and diaminobenzidine.

Statistical analysis. Significant differences between groups were assessed with the χ^2-test and Fisher's exact test. $P < 0.05$ was considered significant.

Results

Detection of autoantigens associated with NSCLC by 1-DE western blotting and LC-MS/MS. In order to screen for autoantibodies against cancer cells in patients with NSCLC, proteins extracted from a given patient's tumor tissue were subjected to 1-DE, transferred to membranes, and incubated with sera from the same patient or from healthy control subjects. Membranes incubated with only the secondary antibody were used as negative controls. An approximately 47-kDa band was recognized only by a subset of NSCLC patient sera, whereas no such reaction was observed with healthy control sera or negative controls (Fig. 1a). To identify this 47-kDa protein, the corresponding band on the gel stained with CBB was digested and analyzed using LC-MS/MS. The eight proteins, including α-enolase and elongation factor 1-α 1, which were identified by database searching through Mascot software, are listed in Table 1. Many of these proteins

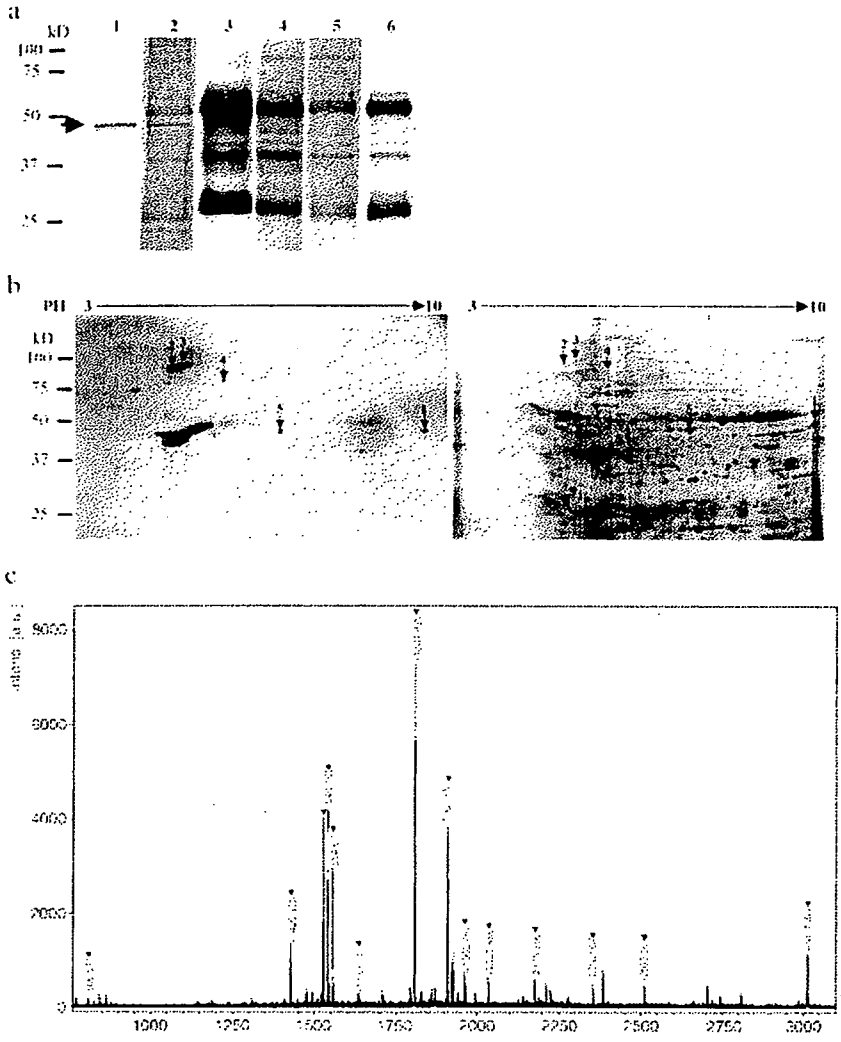

Fig. 1. (a) Screening by means of one-dimensional electrophoresis (1-DE) western blotting analysis for autoantigen associated with non-small cell lung cancer (NSCLC). Lane 1: ~47-kDa positive band (arrow), which was recognized by anti-enolase antibodies. Lanes 2, 3: 47-kDa positive band, which was recognized by NSCLC sera. Lane 4: no 47-kDa positive band was observed in a negative NSCLC case. Lane 5: no positive reaction was observed with healthy control sera. Lane 6: no positive reaction was observed in negative controls. (b) Detection by means of two-dimensional electrophoresis (2-DE) western blotting analysis of autoantigen associated with NSCLC. Left panel: Representative 2-DE western blotting analysis. Right panel: corresponding 2-DE silver-stained image. Protein spots recognized only by NSCLC sera are marked with arrows and numbers. (c) Peptide mass fingerprinting spectra of positive spot 5. For spot 5, 14 peptide masses were matched with human α-enolase by executing an NCBInr database search, yielding 52% protein sequence coverage. The matched mass peaks are marked with arrow heads.

Table 1. Mascot search results of the liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) data

Protein name	Swiss Prot Accession number	Molecular weight (Da)	pI	Score*	Peptide matched	Protein coverage (%)
Elongation factor 1-α 1	P68104	50 451	9.10	123	4	7
Cytokeratin 17	Q04695	48 230	4.97	271	13	25
α-Enolase	P06733	47 037	6.99	96	3	5
Elongation factor Tu	P49411	49 852	7.26	56	1	3
α-1-acid glycoprotein 1 precursor	P02763	23 725	4.93	160	4	18
Vimentin	P08670	53 545	5.06	115	5	9
Albumin precursor	P02768	71 317	5.92	106	4	6
Actin-like protein 3	P61158	47 797	5.61	46	2	7

*Scores > 39 indicate identity or extensive homology ($P < 0.05$). To identify the 47-kDa protein recognized only by non-small cell lung cancer patient sera, the corresponding band stained with Coomassie brilliant blue was digested and analyzed by LC-MS/MS. The eight proteins identified are listed.

are of similar molecular weight and one of them may be the autoantigen associated with NSCLC. We used western blotting with rabbit anti-enolase antibodies to confirm that the expression of α-enolase occurred at the same position as that of the 47-kDa positive band (Fig. 1a).

Autoantibodies against α-enolase present in NSCLC patient sera. To characterize autoantibodies in NSCLC sera, proteins extracted from a given patient's tumor tissue were separated by 2-DE, transferred to membranes, and incubated with sera from the same patient or from healthy control subjects. Compared with the sera of healthy control subjects, 2-DE western blotting with NSCLC patient sera showed five positive protein spots (Fig. 1b, left panel), including one spot (spot 5) that also had a molecular weight of approximately 47 kDa and a pI value of

doi: 10.1111/j.1349-7006.2007.00509.x

Table 2. Mascot search results of matrix-assisted laser desorption–ionization time-of-flight (MALDI-TOF) data

Spot no.	Protein name	Sequence coverage (%)	Molecular weight (Da)	pI
1	Chain D, myeloperoxidase	22	53 806	9.43
2	Tumor rejection antigen-1 (gp96)	17	92 696	4.76
3	Not identified			
4	α glucosidase II α subunit	15	86 236	5.71
5	α-enolase	52	47 037	6.99

To identify the immunoreactive spots in two-dimensional electrophoresis western blotting analysis recognized by non-small cell lung cancer patient sera, corresponding silver-stained spots were digested and analyzed by MALDI-TOF/mass spectrometry. 'Spot no.' corresponds to spots marked in Fig. 1b.

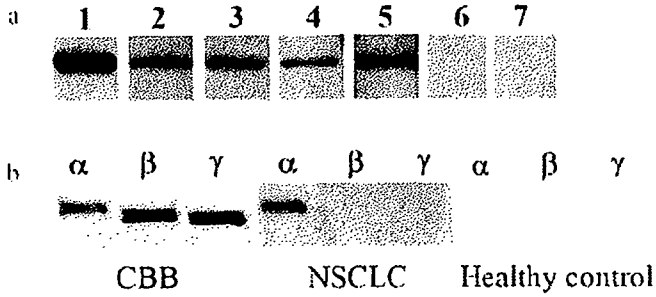

Fig. 2. (a) Western blotting analysis of recombinant human full-length α-enolase protein. Recombinant human full-length α-enolase protein was probed with rabbit anti-enolase antibodies (lane 1), with sera from non-small cell lung cancer (NSCLC) patients (lane 2–5), and with sera from healthy control subjects (lane 6–7). (b) Western blotting analysis of α, β and γ-enolase. α, β and γ-enolase protein were stained with Coomassie brilliant blue (left panel) or probed with sera from NSCLC patients (middle panel) and from healthy control subjects (right panel).

approximately 7.0. The corresponding spots on the silver-stained gel (Fig. 1b, right panel) were identified by MALDI-TOF/MS and database search. The identified proteins are summarized in Table 2. Spot 5 was recognized as α-enolase, as in the previous LC-MS/MS analysis. Its PMF spectrum is shown in Fig. 1c and the database search produced 14 peptide masses that coincided with human α-enolase, thus yielding 52% protein sequence coverage.

Next, western blotting with full-length recombinant human α-enolase protein was used to confirm and analyze the immunogenicity of α-enolase. Correct expression of the recombinant protein was verified by western blotting using rabbit anti-enolase antibodies (Fig. 2a). The recombinant proteins were then probed with sera from NSCLC patients or healthy control subjects, and positive bands were detected only in sera from the former, not from the latter (Fig. 2a). In addition, western blotting was used to determine the reactivity of NSCLC patient sera to enolase isoforms, which contain α, β and γ-enolase. The sera that were positive for autoantibodies against α-enolase reacted with neither β-enolase nor γ-enolase (Fig. 2b), whereas healthy control sera did not react with any of the enolase isoforms. This indicates the specificity of autoantibodies against α-enolase in NSCLC patient sera, and the overall results suggest that a subset of NSCLC patient sera contains autoantibodies against α-enolase.

Frequencies of autoantibodies against α-enolase in the sera were determined by means of ELISA using recombinant human full-length α-enolase protein. We tested 94 sera from patients with NSCLC, 15 from patients with SCLC, 18 from patients with gastrointestinal cancer (10 patients with gastric cancer, 8 patients with colon cancer), nine from patients with MAC infection of lung and 60 from healthy control subjects. When

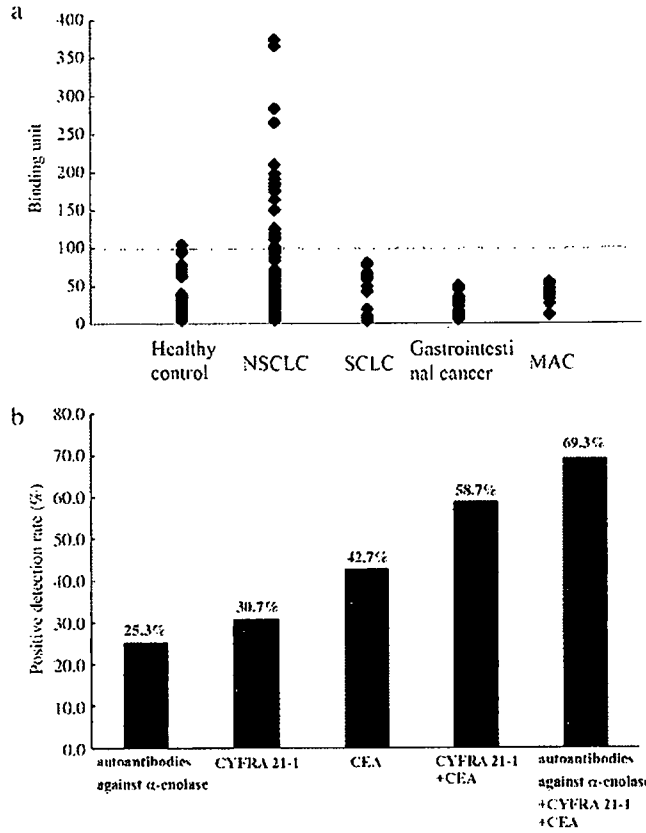

Fig. 3. (a) Prevalence of autoantibodies against α-enolase determined by enzyme-linked immunosorbent assay (ELISA). The *y*-axis denotes binding units. The solid horizontal line represents the positive cut-off limit. The prevalence of autoantibodies against α-enolase was 27.7% in patients with non-small cell lung cancer (NSCLC) (26 of 94), 1.7% in healthy control subjects (1 of 60), and not detectable in small cell lung cancer gastrointestinal cancer (10 patients with gastric cancer, 8 patients with colon cancer) and *Mycobacterium avium* complex infection of lung. (b) Positive detection rate of autoantibodies against α-enolase, cytokeratin 19 fragment (CYFRA21-1) and carcinoembryonic antigen (CEA) in NSCLC patients. Detection of CYFRA 21-1 and CEA in combination increased the positive detection rate to 58.7%. Furthermore, combined detection of autoantibodies against α-enolase, CEA and CYFRA 21-1 achieved a positive detection rate of up to 69.3%.

'Mean $OD_{\text{healthy control sera}} + 3\ SD_{\text{healthy control sera}}$' was used as the cut-off point, the prevalence of autoantibodies against α-enolase was 27.7% in patients with NSCLC (26 of 94), 1.7% in healthy control subjects (1 of 60), and not detectable in SCLC, gastrointestinal cancer or MAC infection of lung (Fig. 3a). These results

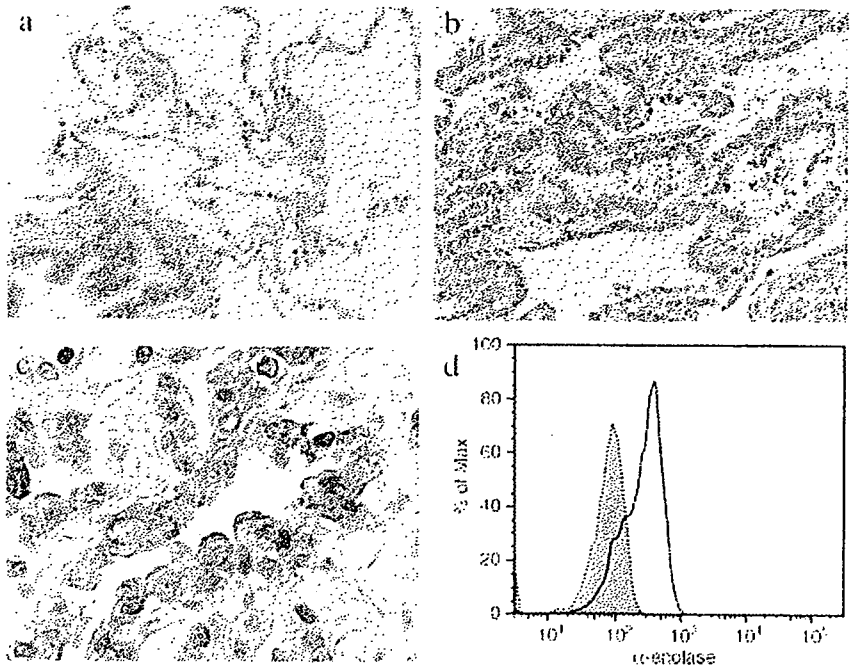

Fig. 4. Immunohistochemical and flow cytometric analysis of α-enolase. (a) Normal lung tissue (×100). (b) Lung adenocarcinoma (×100). (c) α-Enolase staining showing a mixture of cytoplasmic, and membranous immunoreactivity (×400). (d) A549 cells were stained with anti-enolase antibody, labeled with fluorescein-isothiocyanate-conjugated goat antirabbit immunoglobulin, and analyzed on a FACS Canto (open histogram). Shaded histogram indicates staining with control IgG.

showed that titers of autoantibodies against α-enolase are increased in a subset of NSCLC patients. Next, we examined the correlation between the prevalence of autoantibodies against α-enolase and clinicopathological features in NSCLC patients. Positive reactivity was detected in 21 of the 73 sera from adenocarcinoma patients (28.8%) and in five of the 21 sera from SCC patients (23.8%). There was no significant correlation between the occurrence of autoantibodies against α-enolase and pathological types ($P = 0.654$). In addition, there was a tendency for autoantibodies against α-enolase to be more prevalent in patients with advanced NSCLC cases (stage III/IV, 33.3%, 21 of 63) than in stage I/II cases (16.1%, 5 of 31), although the results of the statistical analysis suggest that the prevalence has no significant correlation with disease stage ($P = 0.08$). We also investigated the relationship between autoantibodies against α-enolase and other tumor markers (CEA, CYFRA 21-1) that have been applied to clinical practice in NSCLC patients. In a total of 94 NSCLC patients, clinical data of both CEA and CYFRA 21-1 were available for 75 patients. In these patients, 25.3% (19/75) were positive for autoantibodies against α-enolase, 42.7% (32/75) were positive for CEA, and 30.7% (23/75) were positive for CYFRA 21-1. The occurrence of autoantibodies against α-enolase didn't show a significant correlation with CEA ($P = 0.63$) or with CYFRA 21-1($P = 0.92$). Detection of CYFRA 21-1 and CEA in combination increased the positive detection rate to 58.7% (44/75). Furthermore, positive detection rate was enhanced up to 69.3% (52/75) when combined detection of autoantibodies against α-enolase, CEA and CYFRA 21-1 was used (Fig. 3b).

IHC and flow cytometric analysis of α-enolase. We used IHC staining to compare α-enolase expression in non-malignant and malignant lung tissues from 20 NSCLC patients, including 10 patients with autoantibodies against α-enolase and 10 patients without autoantibodies against α-enolase. The staining results showed that expression of α-enolase was increased in malignant lung tissue of NSCLC patients (Fig. 4a–c). Additionally, IHC staining showed not only cytoplasmic but also membranous immunoreactivity in cancer cells (Fig. 4c). Flow cytometric analysis of human lung adenocarcinoma cell line A549 also confirmed the expression of α-enolase at the surface of lung cancer cells (Fig. 4d).

Epitopes located at the N-terminal region (1–134 a.a.) of α-enolase that are recognized by autoantibodies. To locate the serological epitopes of α-enolase, full-length and C-terminal deletion mutant proteins (α-Eno1, α-Eno2, α-Eno3) were prepared. The full-length α-enolase (1–434 a.a.), α-Eno1 (1–334 a.a.), α-Eno2 (1–234 a.a.) and α-Eno3 (1–134 a.a.) recombinant proteins were clearly shown by an anti-6 × His antibody or stained with CBB, which verified their expression (Fig. 5). A commercially available rabbit anti-enolase antibody reacted only with the full-length α-enolase, α-Eno1 and α-Eno2 recombinant proteins (Fig. 5). However, sera from NSCLC patients who showed the presence of autoantibodies against α-enolase reacted with the α-Eno1, α-Eno2, and α-Eno3 recombinant proteins (Fig. 5), indicating that in NSCLC patients at least the N-terminal region of α-enolase contains epitopes.

Discussion

In the present study we used a proteomics-based screen test to identify proteins such as α-enolase and gp96 that may elicit a humoral immune response in NSCLC patients. We then confirmed that some NSCLC patients' sera contained autoantibodies against α-enolase by means of western blotting using recombinant protein. Furthermore, the results obtained with ELISA demonstrated that when 'Mean $OD_{\text{healthy control sera}} + 3\,SD_{\text{healthy control sera}}$' was used as the cut-off point, the humoral immune response directed against α-enolase occurred in 27.7% of NSCLC patients, but in only 1.7% of healthy control subjects. α-enolase is an isoenzyme of enolase, a key protein that catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate, which is the second of the two high-energy intermediates that generate ATP in glycolysis.[20] Three isoforms of enolase have been identified and are known as α, β and γ-enolase. α-enolase is present in most tissues and is predominant in early embryonic tissue, β-enolase is expressed in muscle tissue, and γ-enolase, also known as NSE, is found only in neuronal tissues.

Autoantibody responses to tumors are generally thought to be elicited in three ways. These are overexpression of specific proteins, especially on the cell surface, gene mutation or post-translational modification of proteins, which shows new epitopes

doi: 10.1111/j.1349-7006.2007.00509.x

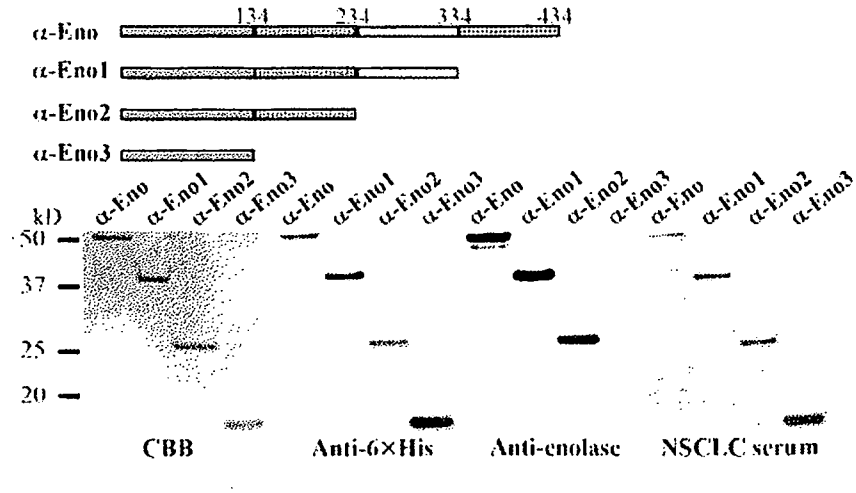

Fig. 5. Upper panel: preparation of recombinant human full-length α-enolase protein: α-Eno (1–434 a.a.), C-terminal deletion mutant α-enolase proteins: α-Eno1 (1–334 a.a.), α-Eno2 (1–234 a.a.) and α-Eno3 (1–134 a.a.). Lower panel: recombinant proteins were stained with Coomassie brilliant blue or probed with anti-6 × His antibody, anti-enolase antibody or non-small cell lung cancer sera.

as immunogens, and other types of protein processing in tumor tissue.[21] In the present study we used normal recombinant proteins to confirm the immunogenicity of α-enolase in NSCLC patients. In addition, although a past study reported that expression of α-enolase was downregulated in NSCLC tissues,[22] IHC staining used in our study showed that α-enolase expression is commonly increased in malignant lung tissue from NSCLC patients compared to the expression in non-malignant lung tissue, which is consistent with other previous reports.[23–27] Interestingly, IHC staining also showed not only cytoplasmic but also membranous immunoreactivity. Moreover, flow cytometric analysis demonstrated expression of α-enolase on the surface of lung cancer cells. We think that enhanced expression of α-enolase on cancer-cell surface might be one reason for autoantigenicity, and might be required for induction of autoantibody responses. However, α-enolase expression alone is insufficient for autoantibody production as increased expression was also found in these patients without autoantibodies against α-enolase. Further study is necessary to investigate the detailed mechanisms involved in this autoantibody response.

It is widely accepted that the propensity for glycolysis is enhanced in cancer cells because of increased cell proliferation. In fact, α-enolase, a key enzyme in the glycolysis pathway, was found to be overexpressed in 18 cancers.[25] Furthermore, although the mechanism of its surface expression and orientation on the membrane are not yet clearly understood, it is known that the C-terminal a.a. of α-enolase, lysine, is exposed at the cell surface and is involved in binding to plasminogen, which is then activated and converted to plasmin.[28] Once plasmin is stabilized at the cell surface, it in turn induces fibrinolysis.[29] In response to the upregulation of α-enolase expression, progression of the fibrinolytic system is markedly accelerated, and the resultant increase in local fibrinolysis may contribute to cancer cell invasion and metastasis. This is consistent with our finding that there was a tendency for autoantibodies against α-enolase to be more prevalent in patients with advanced NSCLC (stage III/IV) than in stage I/II cases.

Tumor markers for NSCLC are potentially useful for both diagnostic and therapeutic practice. To date, a variety of NSCLC tumor markers have been identified and the most extensively investigated circulating protein markers include CEA, CYFRA 21-1, SCC antigen, NSE and CA125.[5] The percentages of NSCLC patients who have elevated serum protein levels of the above markers are 26%–42% for CEA, 51%–74% for CYFRA 21-1, 20%–32% for SCC, 28–32% for NSE, and 46–55% for CA125, with variations depending on histology and stage.[5–8] However, because elevated serum protein levels of these markers have been observed in tumors other than NSCLC, their sensitivity and specificity are not satisfactory and their clinical applicability is limited. Our study demonstrated that when 'Mean $OD_{\text{healthy control sera}}$ + 3 $SD_{\text{healthy control sera}}$' was used as the cut-off point, autoantibodies against α-enolase occurred in 27.7% of NSCLC patients but not in those with SCLC, gastrointestinal cancer or MAC infection of the lungs. Moreover, combined detection of autoantibodies against α-enolase, CEA and CYFRA 21-1 enhanced sensitivity for the diagnosis of NSCLC. These results suggest that using autoantibodies against α-enolase has potential as a clinical biomarker for serological screening of NSCLC. Further large-scale validation studies will be needed to determine the sensitivity, specificity and positive prognostic value of this marker in real-world screening scenarios.

Autoantibodies against α-enolase have been detected in some autoimmune diseases,[20] and Fujii *et al.* report that in Hashimoto's encephalopathy one of these autoantibodies recognizes the N-terminal region of α-enolase.[29] Because our results show this same recognition in NSCLC, further experiments are warranted to compare epitopes in α-enolase as detected in NSCLC and in autoimmune diseases to determine its utility as a biomarker for NSCLC.

Acknowledgments

We would like to thank Dr S. Nomura for extremely helpful technical instructions for immunohistochemical staining. We also thank all members of our laboratory, especially Dr T. Hirano and Dr A. Ogata for their helpful discussions and support, and Ms T. Arimoto for her secretarial assistance. This study was supported by a Grant-in-Aid from the Ministry of Education, Science and Culture, Japan and the Osaka Foundation for Promotion of Clinical Immunology, Japan.

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doi: 10.1111/j.1349-7006.2007.00509.x

original article

Annals of Oncology 18: 317–323, 2007
doi:10.1093/annonc/mdl377
Published online 1 November 2006

Randomized phase III study of cisplatin plus irinotecan versus carboplatin plus paclitaxel, cisplatin plus gemcitabine, and cisplatin plus vinorelbine for advanced non-small-cell lung cancer: Four-Arm Cooperative Study in Japan

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Received 16 May 2006; revised 13 August 2006; accepted 30 August 2006

Background: To compare the efficacy and toxicity of three platinum-based combination regimens against cisplatin plus irinotecan (IP) in patients with untreated advanced non-small-cell lung cancer (NSCLC) by a non-inferiority design.
Patients and methods: A total of 602 patients were randomly assigned to one of four regimens: cisplatin 80 mg/m^2 on day 1 plus irinotecan 60 mg/m^2 on days 1, 8, 15 every 4 weeks (IP) carboplatin AUC 6.0 min × mg/mL (area under the concentration–time curve) on day 1 plus paclitaxel 200 mg/m^2 on day 1 every 3 weeks (TC); cisplatin 80 mg/m^2 on day 1 plus gemcitabine 1000 mg/m^2 on days 1, 8 every 3 weeks (GP); and cisplatin 80 mg/m^2 on day 1 plus vinorelbine 25 mg/m^2 on days 1, 8 every 3 weeks (NP).
Results: The response rate, median survival time, and 1-year survival rate were 31.0%, 13.9 months, 59.2%, respectively, in IP; 32.4%, 12.3 months, 51.0% in TC; 30.1%, 14.0 months, 59.6% in GP; and 33.1%, 11.4 months, 48.3% in NP. No statistically significant differences were found in response rate or overall survival, but the non-inferiority of none of the experimental regimens could be confirmed. All the four regimens were well tolerated.
Conclusion: The four regimens have similar efficacy and different toxicity profiles, and they can be used to treat advanced NSCLC patients.
Key words: carboplatin, cisplatin, gemcitabine, irinotecan, non-small-cell lung cancer, paclitaxel, randomized phase III study, vinorelbine

introduction

Nearly 60 000 patients in Japan died of lung cancer in 2004, and the mortality rate is still increasing [1]. Even old-generation cisplatin-based chemotherapy provides a survival benefit and symptom relief in patients with inoperable non-small-cell lung cancer (NSCLC) [2]. Several anticancer agents including irinotecan, paclitaxel, docetaxel, gemcitabine, and vinorelbine, were developed in the 1990s and most of them have mechanisms of action that differ from those of the old-generation agents [3–7]. The combinations of platinum and these new agents developed in the 1990s are more useful against advanced NSCLC than old-generation combination chemotherapy, and doublets of platinum and new-generation anticancer agents are considered standard chemotherapy regimens for advanced NSCLC, although no consistent standard regimens have yet been established [8–17].

Two phase III studies comparing cisplatin plus irinotecan (IP) with cisplatin plus vindesine for advanced NSCLC have been conducted in Japan [18, 19]. Fukuoka et al. [20] reported the results of a combined analysis of the 358 eligible stage IV patients in these studies. They carried out a multivariate analysis using the Cox regression model with adjustment for well-known prognostic factors, and the Cox regression analysis demonstrated that treatment with IP was one of significant independent favorable factor. Based on their data, we selected IP for the reference arm in our study.

The Ministry of Health, Labour and Welfare of Japan approved the prescription of paclitaxel, gemcitabine, and

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vinorelbine for NSCLC in 1999 and requested a phase III study to confirm the efficacy and safety of these agents. The Japanese investigators and the pharmaceutical companies decided to conduct a four-arm randomized phase III study for NSCLC, the so-called FACS, Four-Arm Cooperative Study. The purpose of the study was to compare the efficacy and toxicity of three platinum-based combination regimens, carboplatin plus paclitaxel (TC), cisplatin plus gemcitabine (GP), cisplatin plus vinorelbine (NP), with IP as the reference arm.

patients and methods

patient selection

Patients with histologically and/or cytologically documented NSCLC were eligible for participation in the study. Each patient had to meet the following criteria: clinical stage IV or IIIB (including only patients with no indications for curative radiotherapy, such as malignant pleural effusion, pleural dissemination, malignant pericardiac effusion, or metastatic lesion in the same lobe), at least one target lesion >2 cm, no prior chemotherapy, no prior surgery and/or radiotherapy for the primary site, age 20–74 years, Eastern Cooperative Oncology Group performance status (PS) of 0 or 1, adequate hematological, hepatic and renal functions, partial pressure of arterial oxygen (paO_2) ≥60 torr, expected survival >3 months, able to undergo first course treatment in an inpatient setting, and written informed consent. The study was approved by the Institutional Review Board at each hospital. Written informed consent was obtained from every patient.

treatment schedule

All patients were randomly assigned to one of the four treatment groups by the central registration office by means of the minimization method. Stage, PS, gender, lactate dehydrogenase (LDH) and albumin values, and institution were used as adjustment variables. The first group received the reference treatment, 80 mg/m^2 of cisplatin on day 1 and 60 mg/m^2 of irinotecan on days 1, 8, and 15, and the cycle was repeated every 4 weeks. The second group received 200 mg/m^2 of paclitaxel (Bristol-Myers K.K., Tokyo, Japan) over a 3-h period followed by carboplatin at a dose calculated to produce an area under the concentration–time curve of 6.0 min × mg/mL on day 1 and the cycle was repeated every 3 weeks. The third group received 80 mg/m^2 of cisplatin on day 1 and 1000 mg/m^2 of gemcitabine (Eli Lilly Japan K.K., Kobe, Japan) on days 1, 8 and the cycle was repeated every 3 weeks. The fourth group received 80 mg/m^2 of cisplatin on day 1 and 25 mg/m^2 of vinorelbine (Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan) on days 1, 8 and the cycle was repeated every 3 weeks. Each treatment was repeated for three or more cycles unless the patient met the criteria for progressive disease or experienced unacceptable toxicity.

response and toxicity evaluation

Response was evaluated according to the Response Evaluation Criteria in Solid Tumors, and tumor markers were excluded from the criteria [21]. Objective tumor response in all responding patients was evaluated by an external review committee with no information on the treatment group. Toxicity grading criteria in National Cancer Institute Common Toxicity Criteria Ver 2.0 were used to evaluate toxicity.

quality of life assessment

Quality of life (QoL) was evaluated by means of the Functional Assessment of Cancer Therapy—Lung (FACT-L) Japanese version and the QoL Questionnaire for Cancer Patients Treated with Anticancer Drugs (QoL-ACD), before treatment, immediately before the second cycles of chemotherapy, and 3 and 6 months after the start of treatment [22–24].

statistical analysis and monitoring

The primary end point of this study was overall survival (OS), and the secondary end points were response rate, response duration, time to progressive disease (TTP), time to treatment failure (TTTF), adverse event, and QoL. The 1-year survival rate of the control group in this study was estimated to be 43% based on the data in published papers, and the 1-year survival rate in the other treatment group was expected to be 50%. The lower equivalence limit for 1-year survival rate was set as '−10%'. The criterion for the non-inferiority of each treatment was a lower limit of the two-sided 95% confidence interval (CI) of the 1-year survival rate of treatment minus that of control larger than the lower equivalence limit. Because the non-inferiority of each treatment versus the control was to be evaluated independently, a separate null hypothesis was stated for each treatment, and for that reason no multiple comparison adjustment was included in the study. Based on the above conditions and binomial distribution, 135 patients were needed per arm for a one-sided Type I error of 2.5% and 80.0% power. In view of the possibility of variance inflation due to censoring, the sample size was set at 600 (150 per arm).

Central registration with randomization, monitoring, data collection, and the statistical analyses were independently carried out by a contract research organization (EPS Co., Ltd, Tokyo, Japan).

results

patient characteristics

From October 2000 to June 2002, a total of 602 patients were registered by 44 hospitals in Japan. All patients had been followed up for >2 years, and 447 patients had died as of June 2004. Of the 602 patients registered, 151 were allocated to the reference treatment, IP, and 150, 151, and 150 patients were allocated to TC, GP, and NP, respectively. Since 10 patients did not receive chemotherapy and 11 patients were subsequently found to be ineligible, 592 patients were assessable for toxicity and 581 patients were assessable for efficacy. Four patients did not receive chemotherapy due to electrolytic disorder, fever, symptomatic brain metastases, and rapid tumor progression in IP, two patients due to refusal and pneumonia in TC, four patients due to lower WBC counts (two patients), rapid tumor progression, and nephritic syndrome in NP. Two patents were ineligible due to wrong stage in IP, two patients were wrong stage and one patient had double cancer in TC, two patients were wrong diagnosis, one patient had massive pleural effusion, one patient received prior chemotherapy in GP, one patient had no target lesions in NP. Age, gender, PS, stage, and LDH and albumin values were well balanced in each arm (Table 1). Fewer patients with adenocarcinoma and more patients with squamous cell carcinona were, however, entered in three experimental arms than in IP.

objective tumor response and response duration

Objective tumor response is shown in Table 2. Forty-five partial responses occurred in the 145 assessable patients in the reference arm, IP, for an objective response rate of 31.0% with a median response duration of 4.8 months. The response rate and median response duration were 32.4% and 4.0 months in TC, 30.1% and 3.5 months in GP, and 33.1% and 3.4 months in NP. The response rates in TC, GP, and NP were not statistically different from the rate in IP according to the results of the χ^2 test.

Table 1. Patient characteristics and treatment delivery

	Cisplatin + irinotecan	Carboplatin + paclitaxel	Cisplatin + gemcitabine	Cisplatin + vinorelbine
Assessable patients	145	145	146	145
Gender (male/female)	97/48	99/46	101/45	101/44
Age, median (range)	62 (30–74)	63 (33–74)	61 (34–74)	61 (28–74)
PS (0/1)	44/101	44/101	45/101	45/100
Histology				
Adenocarcinoma	121	104	108	109
Squamous cell carcinoma	16	31	29	29
Others	8	10	9	7
Stage (IIIB/IV)	31/114	28/117	30/116	26/119
No. of cycles				
Mean ± SD	3.0 ± 1.3	3.5 ± 1.5	3.2 ± 1.2	3.1 ± 1.3
Median	3	3	3	3
Range	1–7	1–10	1–7	1–8

PS, performance status; SD, standard deviation.

Table 2. Survival, TTP, TTTF, response rate, and response duration

	N	Median survival, months	1-year survival (%)	Difference in 1-year survival from IP	2-year survival (%)	TTP (median), months	TTTF (median), months	Response rate (%)	Response duration (median), months
Cisplatin + irinotecan	145	13.9	59.2	–	26.5	4.7	3.3	31.0	4.8 ($n = 45$)
Carboplatin + paclitaxel	145	12.3	51.0	−8.2% (95% CI −19.6% to 3.3%)	25.5	4.5 ($P = 0.355$)[a]	3.2 ($P = 0.282$)[a]	32.4 ($P = 0.801$)[b]	4.0 ($n = 47$)
Cisplatin + gemcitabine	146	14.0	59.6	0.4% (95% CI −10.9% to11.7%)	31.5	4.0 ($P = 0.170$)[a]	3.2 ($P = 0.567$)[a]	30.1 ($P = 0.868$)[b]	3.5 ($n = 44$)
Cisplatin + vinorelbine	145	11.4	48.3	−10.9% (95% CI −22.3% to 0.5%)	21.4	4.1 ($P = 0.133$)[a]	3.0 ($P = 0.091$)[a]	33.1 ($P = 0.706$)[b]	3.4 ($n = 48$)

[a]Compared with IP by the generalized Wilcoxon test.

[b]Compared with IP by the χ^2 test.

CI, confidence interval; IP, cisplatin plus irinotecan; TTP, time to progressive disease; TTTF, time to treatment failure.

OS, TTP disease, and TTTF

OS and TTP are shown in Figure 1. Median survival time (MST), the 1-year, and 2-year survival rate in IP were 13.9 months, 59.2%, and 26.5%, respectively. The MSTs, 1-year, and 2-year survival rates were, respectively, 12.3 months, 51.0%, and 25.5% in TC; 14.0 months, 59.6%, and 31.5% in GP; and 11.4 months, 48.3%, and 21.4% in NP. The lower limits of the 95% CI of the difference in 1-year survival rate between IP and TC (−19.6%), GP (−10.9%), and NP (−22.3%) were below −10%, which was considered the lower equivalence limit (Table 2). Thus, the results did not show non-inferiority in three experimental regimens compared with reference treatment. Median TTP and median TTTF were 4.7 and 3.3 months, respectively in IP. Median TTP and TTTF were, respectively, 4.5 and 3.2 months in TC, 4.0 and 3.2 months in GP, and 4.1 and 3.0 months in NP. There were no statistical differences in either TTP or TTTF in TC, GP, or NP, compared with IP according to the results of the generalized Wilcoxon test (Table 2).

hematologic and non-hematologic toxicity

In IP, 47.6% and 83.7% of patients developed grade 3 or worse leukopenia and neutropenia, respectively (Table 3). The incidences of grade 3 or worse leukopenia (33.1%, $P = 0.010$) and neutropenia (62.9%, $P < 0.001$) were significantly lower in GP than in IP. The incidence of grade 3 or worse leukopenia (67.1%, $P < 0.001$) was significantly higher in NP than in IP. Grade 3 or worse thrombocytopenia developed in 5.4% of the patients in IP, and the incidence was significantly higher in GP (35.1%, $P < 0.001$). The incidence of febril neutropenia in IP was 14.3%, and was significantly lower in GP (2.0%, $P < 0.001$).

Grade 2 or worse nausea, vomiting, anorexia, and fatigue occurred in 60.5%, 51.0%, 65.3%, and 38.8%, respectively, of the patients in IP. The incidences of grade 2 or worse nausea (TC: 25.0%, $P < 0.001$, NP: 47.3%, $P = 0.022$), vomiting (TC: 22.3%, $P < 0.001$, NP: 36.3%, $P = 0.011$), and anorexia (TC: 32.4%, $P < 0.001$, NP: 49.3%, $P = 0.005$) were significantly lower in TC and NP than in IP. Grade 2 or worse diarrhea was

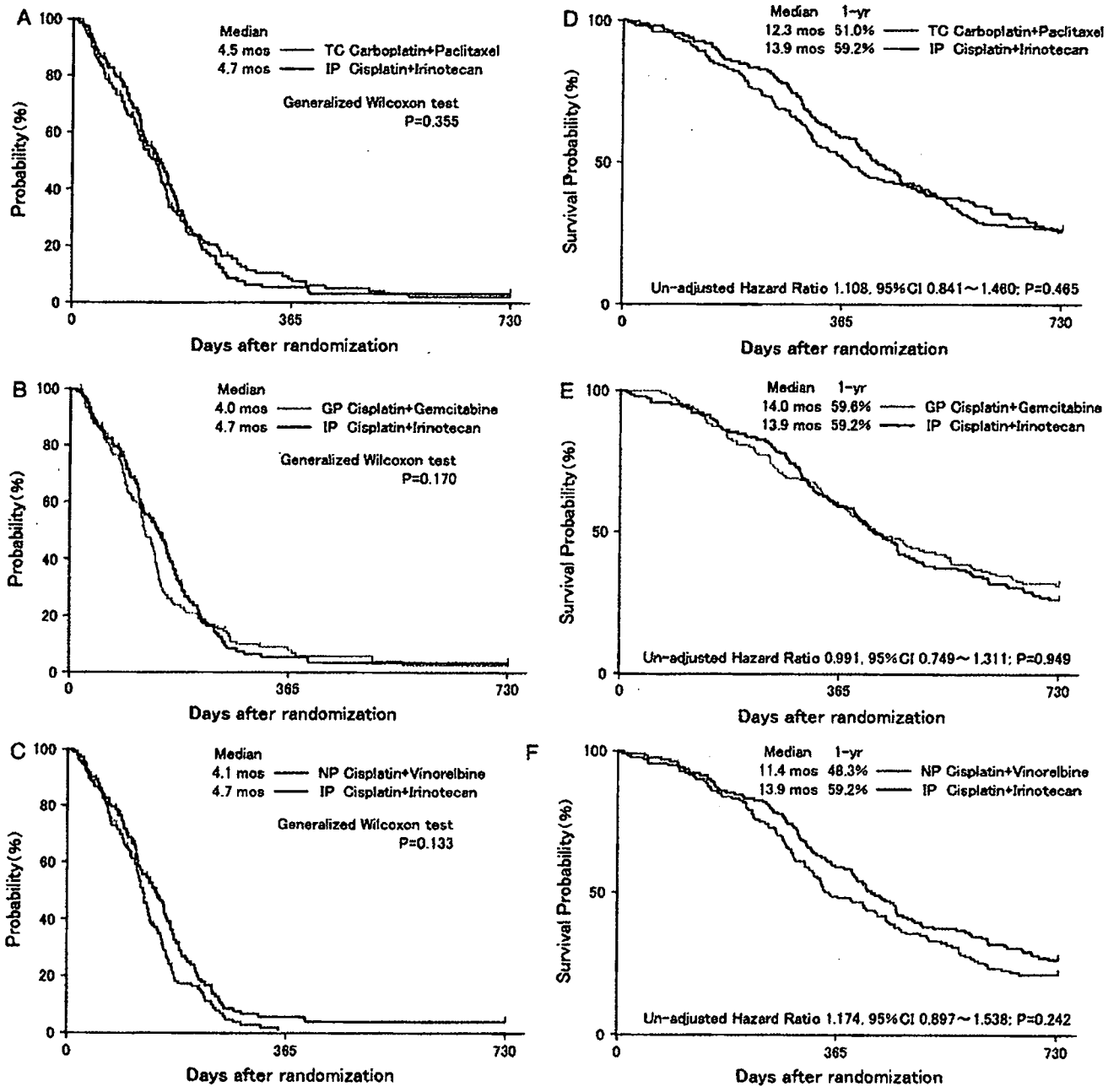

Figure 1. Overall survival (OS) and time to progressive (TTP) disease. TTP and OS in the carboplatin plus paclitaxel (TC) (A, D), cisplatin plus gemcitabine (GP) (B, E), and cisplatin plus vinorelbine (NP) (C, F) were not statistically significantly different from the values in the cisplatin plus irinotecan.

significantly less frequent in TC (6.8%), GP (8.6%), and NP (11.6%) than in IP (48.3%, $P < 0.001$). The incidences of grade 2 or worse sensory neuropathy (16.9%, $P < 0.001$), arthralgia (21.6%, $P < 0.001$), and myalgia (17.6%, $P < 0.001$) were significantly higher in TC than in IP. Grade 2 alopecia occurred in 30.6% of the patients in IP, and its incidence was significantly higher in TC (44.6%, $P = 0.013$) and significantly lower in GP (15.2%, $P = 0.001$) and NP (8.9%, $P < 0.001$). Grade 2 injection site reactions were more frequent in NP (26.7%) than in IP (4.8%, $P < 0.001$).

A total of five patients died of treatment-related toxicity: three in IP (cerebral hemorrhage, interstitial pneumonia, acute circulatory failure/disseminated intravascular coagulation: 2.0%), one in TC (acute renal failure: 0.7%), and one in NP (pulmonary embolism: 0.7%).

second-line treatment

Data on second-line treatment, but not third-line or later treatment, was available in this study, and they showed that

Table 3. Toxicity

	IP ($n = 147$)			TC ($n = 148$)			GP ($n = 151$)			NP ($n = 146$)		
	Grade (%)			Grade (%)			Grade (%)			Grade (%)		
	2	3	4	2	3	4	2	3	4	2	3	4
Leukocytes	42	43	5	39	42	3	40	31[a]	2[a]	25	51[b]	16[b]
Neutrophils	11	39	45	5	19	69	21	40	23[a]	5	16	72
Hemoglobin	42	24	7	42	13[a]	2[a]	44	22	5	43	25	5
Platelets	6	5	1	9	11	0	22	35[b]	0[b]	3	1[a]	0[a]
Febrile neutropenia	–	14	0	–	18	0	–	2[a]	0[a]	–	18	0
Nausea	32	29	–	14[c]	11[c]	–	35	23	–	33[c]	14[c]	–
Vomiting	38	13	0	17[c]	5[c]	0[c]	34	14	0	29[c]	7[c]	0[c]
Anorexia	30	33	2	15[c]	17[c]	1[c]	31	26	1	29[c]	20c	1[c]
Fatigue	27	12	1	26	2	1	17[c]	3[c]	0[c]	23[c]	3[c]	0[c]
Diarrhea	33	15	1	4[c]	3[c]	0[c]	7[c]	2[c]	0[c]	8[c]	4[c]	0[c]
Constipation	27	7	0	30	8	0	33	9	0	40[d]	14[d]	0[d]
Neuropathy, motor	1	0	0	1	1	1	0	0	0	0	0	0
Neuropathy, sensory	1	0	0	14[d]	3[d]	0[d]	0	0	0	0	0	0
Alopecia	31	–	–	45[d]	–	–	15[c]	–	–	9[c]	–	–
Arthralgia	2	0	0	20[d]	2[d]	0[d]	0	0	0	1	0	0
Myalgia	1	0	0	16[d]	2[d]	0[d]	0	0	0	1	1	0
Injection site reaction	5	0	–	5	0	–	5	0	–	27[d]	0[d]	–
Pneumonitis	0	1	1	0	1	0	0	0	0	0	1	0
Creatinine	8	1	0	2[c]	0[c]	0[c]	7	0	0	8	1	0
AST	7	1	1	5	1	0	6	3	0	1	3	0
Fever	2	0	0	5	1	0	1	0	0	1	0	0
Treatment-related death	3 (2.0%)			1 (0.7%)			0			1 (0.7%)		

[a]Incidence of grade 3 or 4 toxicity significantly ($P < 0.05$) lower than that with IP.
[b]Incidence of grade 3 or 4 toxicity significantly ($P < 0.05$) higher than that with IP.
[c]Incidence of grade 2 or worse toxicity is significantly ($P < 0.05$) lower than that with IP.
[d]Incidence of grade 2 or worse toxicity significantly ($P < 0.05$) higher than that with IP.
GP, cisplatin plus gemcitabine; IP, cisplatin plus irinotecan; NP, cisplatin plus vinorelbine; TC, carboplatin plus paclitaxel.
AST, aspartate aminotransferase; –, no category in the criteria.

60%–74% of the patients received chemotherapy and 6%–9% received thoracic irradiation as second-line treatment (Table 4). The percentages of patients in each treatment group who received second-line chemotherapy were not significantly different ($P = 0.081$).

quality of life

The details of the QoL analysis will be reported elsewhere. No statistically significant difference in global QoL was observed among the four treatment groups based on either the FACT-L Japanese version or the QoL-ACD. Only the physical domain evaluated by QoL-ACD was significantly better in TC, GP, and NP than in IP.

discussion

Many randomized phase III studies have compared platinum-plus-new-agent doublets in NSCLC, but, this is the first to evaluate the efficacy of an irinotecan-containing regimen in comparison with other platinum-plus-new-agent doublets in NSCLC [14–17]. Although non-platinum-containing chemotherapy regimens are used as alternatives, doublets of platinum and a new-generation anticancer agent, such as TC, GP, and NP, are considered standard chemotherapy regimens for advanced NSCLC worldwide [13–17, 25]. Although the non-inferiority of none of the three experimental regimens could be confirmed in this study, no statistically significant differences in response rate, OS, TTP, or TTTF were observed between the reference regimen and the experimental regimens. All four platinum-based doublets have similar efficacy against advanced NSCLC but different toxicity profiles. Nevertheless, IP was still regarded as the reference regimen in this study because the non-inferiority of none of the three experimental regimens could be confirmed.

OS in this study was relatively longer than previously reported. The estimated 1-year survival rate in the reference arm was 43%, but the actual 1-year survival rate was 59.2%, much higher than expected. The MSTs reported for patients treated with TC, GP, and NP in recent phase III studies have ranged from 8 to 10 months, and in the present study they were 12.3, 14.0, and 11.4 months, respectively [14–17]. One reason for the good OS in this study was the difference in patient selection criteria, for example exclusion of PS2 patients. Ethnic differences in pharmacogenomics have also been indicated as a possible reason for the good OS in this study [26]. The OS in IP in this study, however, was better than in previous Japanese studies [18, 19]. TTP in this study ranged from 4.0 to 4.7 months, and was similar to the TTP of 3.1–5.5 months reported in the literature [15, 16]. OS not TTP was longer in this study

Table 4. Second-line treatment

	Cisplatin + irinotecan	Carboplatin + paclitaxel	Cisplatin + gemcitabine	Cisplatin + vinorelbine	
Number of patients	145	145	146	145	
Chemotherapy	107 (74%)	87 (60%)	101 (69%)	95 (66%)	$P = 0.081$
Docetaxel	39	25	50	51	
Gefitinib	11	9	18	12	
Paclitaxel	15	14	7	11	
Gemcitabine	24	28	17	28	
Vinorelbine	9	12	2	9	
Irinotecan	15	4	3	3	
Thoracic irradiation	8	10	13	10	

than previously reported, and higher 2-year survival rates, 21.4%–31.5%, were observed in the minimum 2-year follow-up in this study. Second-line or later treatments may affect survival, because docetaxel has been established as standard second-line chemotherapy for advanced NSCLC [27, 28]. Gefitinib is also effective as second-line or later chemotherapy for advanced NSCLC, especially in Asian patients, never smokers and patients with adenocarcinoma [29–32].

The toxicity profile of each treatment differed and the toxicity of all four regimens was well tolerated. Overall QoL was similar in the four platinum-based doublets. Only physical domain QoL evaluated by the QoL-ACD was statistically better in TC, GP, and NP than in IP. This finding is presumably attributable to the fact that diarrhea is a statistically less frequent adverse effect of TC, GP, and NP than of IP.

In conclusion, all four platinum-based doublets had similar efficacy for advanced NSCLC but different toxicity profiles. All the four regimens can be used to treat advanced NSCLC patients in clinical practice.

appendix

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acknowledgements

This study was supported by Bristol-Myers K.K., Tokyo; Eli Lilly Japan K.K., Kobe; and Kyowa Hakko Kogyo Co. Ltd, Tokyo, Japan.

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British Journal of Cancer (2007) 97, 162–169

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Clinical Studies

Randomised phase III trial of carboplatin plus etoposide *vs* split doses of cisplatin plus etoposide in elderly or poor-risk patients with extensive disease small-cell lung cancer: JCOG 9702

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We compared the efficacy and the safety of a carboplatin plus etoposide regimen (CE) *vs* split doses of cisplatin plus etoposide (SPE) in elderly or poor-risk patients with extensive disease small-cell lung cancer (ED-SCLC). Eligibility criteria included: untreated ED-SCLC; age $\geqslant 70$ and performance status 0–2, or age <70 and PS 3. The CE arm received carboplatin area under the curve of five intravenously (IV) on day 1 and etoposide 80 mg m^{-2} IV on days 1–3. The SPE arm received cisplatin 25 mg m^{-2} IV on days 1–3 and etoposide 80 mg m^{-2} IV on days 1–3. Both regimens were given with granulocyte colony-stimulating factor support in a 21–28 day cycle for four courses. A total of 220 patients were randomised. Median age was 74 years and 74% had a PS of 0 or 1. Major grade 3–4 toxicities were (%CE/%SPE): leucopenia 54/51, neutropenia 95/90, thrombocytopenia 56/16, infection 7/6. There was no significant difference (CE/SPE) in the response rate (73/73%) and overall survival (median 10.6/9.9 mo; $P = 0.54$). Palliation scores were very similar between the arms. Although the SPE regimen is still considered to be the standard treatment in elderly or poor-risk patients with ED-SCLC, the CE regimen can be an alternative for this population considering the risk–benefit balance.
British Journal of Cancer (2007) **97**, 162–169. doi:10.1038/sj.bjc.6603810 www.bjcancer.com
Published online 19 June 2007

Keywords: small-cell lung cancer; carboplatin; cisplatin; etoposide; elderly; poor-risk

Approximately half of patients with small-cell lung cancer (SCLC) are older than 70 years, and the proportion of elderly SCLC patients is continuously increasing in Japan (Morita, 2002). However, since many investigators have arbitrarily excluded elderly patients from clinical trials, no standard chemotherapeutic regimen has been established for elderly patients with SCLC. The Japan Clinical Oncology Group (JCOG) has reported that carboplatin plus etoposide (CE) is an active and less toxic regimen in elderly patients with SCLC (Okamoto *et al*, 1999). However, other clinical trials have indicated that the combination chemotherapy of reduced (Souhami *et al*, 1997) or split doses of cisplatin plus etoposide (SPE) (Murray *et al*, 1998; Westeel *et al*, 1998) can be safely and effectively administered in elderly or poor-risk patients with SCLC. Therefore, we conducted a phase III trial comparing CE with SPE in elderly or poor-risk patients with SCLC. Although elderly is not the same as poor-risk, many clinical trials for the elderly have included both types of patients. Therefore, we decided to include both elderly and poor-risk patients with SCLC at the time of proposal for this phase III trial.

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Presented in part at the Forty-First Annual Meeting of the American Society of Clinical Oncology, Orlando, FL, May 13-17, 2005.
Received 18 October 2006; revised 25 April 2007; accepted 26 April 2007; published online 19 June 2007

PATIENTS AND METHODS

Patient selection

Eligibility criteria included patients with histologically or cytologically confirmed SCLC who were $\geqslant 70$ years of age and had an Eastern Cooperative Oncology Group performance status (PS) of 0–2, or who were <70 years in age and had a PS of 3. Additional criteria consisted of extensive disease (ED), chemotherapy-naive, evaluable or measurable disease, expected survival $\geqslant 2$ months, adequate organ functions (leucocyte count $\geqslant 4000\,\mathrm{mm}^{-3}$, platelet count $\geqslant 100\,000\,\mathrm{mm}^{-3}$, haemoglobin level $\geqslant 9.0\,\mathrm{g\,dl}^{-1}$, AST/ALT $\leqslant 2\times$ upper limit of normal range, total bilirubin $\leqslant 1.5\,\mathrm{mg\,dl}^{-1}$, creatinine $\leqslant 1.5\,\mathrm{mg\,dl}^{-1}$, 24-h creatinine clearance (Ccr) $\geqslant 50\,\mathrm{ml\,min}^{-1}$, and PaO2 $\geqslant 60$ mmHg), no symptomatic pericardial or pleural effusion requiring drainage, no active concomitant malignancy, no senile dementia, and written informed consent. Exclusion criteria included brain metastases requiring radiotherapy, superior vena cava (SVC) syndrome requiring radiotherapy, serious medical or psychiatric illness, or pregnancy or lactation. Staging procedures included chest X-ray, computed tomography (CT) scan of the chest, CT scan or magnetic resonance

imaging (MRI) of the brain, CT scan or ultrasound of the abdomen, isotope bone scanning, and bone marrow aspiration or biopsy.

Treatment protocol

Patients were randomised to either the CE arm or the SPE arm. The CE regimen consisted of carboplatin area under the curve (AUC) of five intravenously (IV) on day 1 and etoposide 80 mg m^{-2} IV on days 1, 2, and 3. The SPE regimen consisted of cisplatin 25 mg m^{-2} IV on days 1, 2, and 3 and etoposide 80 mg m^{-2} IV on days 1, 2, and 3. Cycles were repeated every 3–4 weeks for up to four courses. In our previous phase II study using the CE regimen for elderly patients with SCLC, carboplatin AUC of 5 on day 1 and etoposide 100 mg m^{-2} on days 1, 2, and 3 were administered every 4 weeks (Okamoto *et al*, 1999). However, because grade 3 or 4 neutropenia occurred in 91% of the patients, in the current phase III trial we decided to reduce the etoposide dosage to 80 mg m^{-2} on days 1, 2, and 3, and repeat the cycle every 3–4 weeks instead of every 4 weeks. Twenty-four-hour Ccr was substituted for glomerular filtration rate (GFR) in Calvert's formula. Antiemetic prophylaxis with 5-HT3 antagonists plus dexamethasone was used at the treating physician's discretion. According to the Japanese approved guideline, prophylactic use of recombinant human granulocyte colony-stimulating factor (G-CSF) was recommended for daily administration after day 4 until the leucocyte (neutrophil) count exceeded 10 000 (5000) mm^{-3}. If the leucocyte (neutrophil) count decreased to less than 3000 (1500) mm^{-3}, then G-CSF was restarted. However, the actual use of G-CSF was left at the discretion of the treating physician. Subsequent courses of chemotherapy were initiated when leucocyte count $\geqslant$3000 mm^{-3}; platelet count $\geqslant$75 000 mm^{-3}; Cr$\leqslant$1.5 mg dl^{-1}; AST/ALT$\leqslant$2.5 $\times$ upper limit of normal range; and either PS$\leqslant$2 and age$\geqslant$70 years, or PS$\leqslant$3 and age$<$70 years were satisfied both after day 21 and two or more days after the discontinuation of G-CSF. If the above criteria were not satisfied by the first day of the next course, treatment was withheld until full recovery. If more than 6 weeks passed from day 1 of the last course, the patient was removed from protocol treatment. Dose modifications were made based only on grade 4 haematologic toxicities. If grade 4 leucopenia or neutropenia lasting 4 days or more was present, or grade 4 thrombocytopenia occurred, the doses for the next course were carboplatin AUC of 4 on day 1, cisplatin 20 mg m^{-2} for 3 days, and etoposide 60 mg m^{-2} for 3 days. If the same haematologic toxicity was observed after dose reduction, the patient was removed from protocol treatment. If grade 3 or 4 non-haematologic toxicities, except for nausea/vomiting and hyponatraemia, occurred, the patient was removed from protocol treatment even if the toxicities improved thereafter.

Responders after four courses were not allowed to receive further chemotherapy until progressive disease (PD) developed. Although post-protocol treatment was left at the discretion of the physician, crossover treatment was prohibited.

Evaluation

Tumour responses were evaluated according to World Health Organization criteria (World Health Organization, 1979). Toxicities were evaluated according to JCOG Toxicity Criteria (Tobinai *et al*, 1993), which are similar to the National Cancer Institute-Common Toxicity Criteria (NCI-CTC ver 1) for the grading of toxicities.

Palliation score

Study-specific eight-item palliation scores were completed by patients before treatment and 3 weeks after the third course of chemotherapy. The attending physicians were not allowed to complete the scores. The items consisted of cough, pain, anorexia, shortness of breath, well-being, nausea, diarrhoea or constipation, and sleep. The items were scored as not at all present (0), a little (1), moderate (2), and very much (3). The sum of the total score for all eight items was compared between the baseline and post-treatment assessments. If the post-treatment score was below the baseline score, the palliation score for that patient was judged as having shown improvement.

Study design and statistics

This trial was designed as a multicentre, prospective, randomised phase III trial. The study protocol was approved by the Clinical Trial Review Committee of JCOG and the institutional review board of each participating institution before the initiation of the study. The primary endpoint was overall survival (OS). In this study, the experimental arm was the CE arm and the control was the SPE arm. The MST of our previous phase II trial for elderly patients with extensive disease small-cell lung cancer (ED-SCLC) using the CE regimen was 10.1 months. The MST of the SPE regimen for a similar population was not available at the time of the study proposal. Although Westeel and co-workers in 1998 and Murray and co-workers in 1998 reported an excellent MST of SPE plus concurrent chest radiotherapy for elderly or frail patients with limited disease (LD)-SCLC, an MST of the SPE regimen for elderly or frail patients with ED-SCLC was not available at that time. The only data available on the CAV/PE regimen for elderly or poor-risk patients with SCLC using reduced cisplatin (60 mg m^{-2} IV on day 1) were reported by Souhami and co-workers in 1997 and the MST of that study was 5.9 months. Therefore, for statistical calculations in the current phase III trial, we used the MST value of the Souhami trial for the control arm instead of the MST of the SPE regimen. In addition, an individualised AUC-based dosing strategy of carboplatin was expected to have greater efficacy and less toxicity compared with the SPE regimen at that time. This trial was designed as a superiority trial and the planned sample size was 110 patients in each arm for 80% power to detect a 0.67 hazard ratio for CE to SPE in OS at an alpha of 0.025 (one sided) (Schoenfeld and Richter, 1982). Patients were randomised to receive either CE or SPE with a minimisation method for balancing centre, PS (0–1 *vs* 2–3) and age ($\geqslant$70 years *vs* $<$70 years).

Survival distributions were compared by unstratified log-rank test. Proportion of improvement in palliation score was evaluated by Fisher's exact test. The change in each symptom score by treatment arm was evaluated by the Wilcoxon rank-sum test. The relationship between the interval of each chemotherapy course and the two regimens was evaluated by the Wilcoxon rank-sum test. Multivariate analysis was performed using Cox's proportional hazards model to evaluate the importance of seven clinically selected variables (treatment arm, PS, age, sex, lactate dehydrogenase level, alkaline phosphatase level, and leucocyte count) as prognostic factors. All *P*-values in this report are two sided, excluding *P*-values for OS and progression-free survival (PFS).

The interim analysis was performed after half of the planned number of patients had been enrolled in March 2002, with adjustment for multiplicity by the alpha-spending function (DeMets and Lan, 1994) with an O'Brien-Fleming type boundary. Because the interim analysis did not meet the prespecified stopping criteria, the study was continued and the planned accrual of 220 patients was randomised in this trial.

RESULTS

Patient characteristics

Between August 1998 and February 2004, a total of 220 patients were registered from 24 institutions. Baseline characteristics were well balanced between the arms. Median age was 74 years, 92% were 70 years or older, 88% were male, and 74% had a PS of 0 or 1 (Table 1). One patient in the CE arm was found to have LD after the completion of protocol chemotherapy due to protocol violation, and this patient was considered ineligible (Figure 1).

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Delivery of treatment

Reasons for termination of treatment are listed in Figure 1, and there were no major differences between the arms. Of the patients, 63% in the CE arm and 67% in the SPE arm completed four courses, and 11% in the CE arm and 8% in the SPE arm did not complete treatment because of toxicity or complications. Treatment-related death (TRD) occurred in four patients; three patients in the CE arm and one in the SPE arm. All TRDs of patients who were ⩾70 years old with a good pretreatment PS (all PS 1) were associated with neutropenic infection, which occurred after the first course of chemotherapy. Although the median interval of chemotherapy was slightly more prolonged in the CE arm than in the SPE arm, total delivered courses were similar between the arms (Table 2). One patient in the SPE arm never received chemotherapy due to the occurrence of delirium after registration. Dose reduction was more frequently observed in the CE arm than in the SPE arm: 29% *vs* 10%, $P<0.01$. Course delay, G-CSF delivery and total courses with G-CSF delivery were similar between the arms.

Toxicity and palliation score

Toxicities are listed in Table 3. Grade 3 or 4 leucopenia and neutropenia occurred in 54 and 95% of the CE arm *vs* 51 and 90% of the SPE arm, respectively. Grade 3 or 4 thrombocytopenia occurred more frequently in the CE arm than in the SPE arm: 56 *vs* 16%, $P<0.01$. Gastrointestinal toxicities including nausea or vomiting and diarrhoea were mild in both arms. There were few grade 3 or 4 toxicities and no remarkable differences between the arms. Other non-haematologic toxicities were similarly distributed between the arms. Grade 3–4 hyponatraemia, mainly caused by syndrome of inappropriate antidiuretic hormone (SIADH) secretion, occurred in 14–16% of the patients. More importantly, thrombocytopenia occurred more frequently in the CE arm, but none of the patients in either arm showed grade 3 or 4 bleeding. Only one patient in the CE arm showed grade 2 bleeding. Because no grading of febrile neutropenia was listed in JCOG toxicity criteria, the rate of the toxicity was not investigated in this study.

Baseline and post-treatment palliation scores were evaluated in 220/220 (100%) and 208/220 (95%) patients, respectively. We handled missing values by imputing the worst score. Improvement was achieved in 69 (63%) patients in the CE arm *vs* 61 (56%) patients in the SPE arm, although the difference was not statistically significant ($P=0.34$). Similarly, there were no statistical differences in the change of each symptom score between the arms (Table 4).

Objective tumour response, PFS and OS

The objective response rate of 73% was quite similar between the arms. Five CRs and 75 PRs were observed in each arm (Table 5). Progression-free survival curves and OS curves are shown in Figure 2A and B. Ninety-seven percent of the patients had progressed or died at the time of final analysis. Progression-free survival was quite similar between the arms ($P=0.20$, one sided).

Table 1 Patient characteristics

	CE ($n=110$)	SPE ($n=110$)	P-value
Age (years)			
Median (range)	74 (56–86)	73.5 (55–85)	0.34
⩾70 years old (%)	102 (93)	100 (91)	0.81
Sex (male/female)	95/15	98/12	0.68
ECOG PS, 0–1/2/3	81/21/8	81/19/10	0.80
⩾5% weight loss	26	38	0.18
LN metastasis			
Contralateral mediastinum	71	59	0.13
Supraclavicular	89	79	0.15
Distant metastasis			
Liver	30	30	1.0
Lung	31	30	1.0
Brain	18	18	1.0
Bone	25	17	0.23
Adrenal	13	7	0.24
Bone marrow	12	12	1.0

CE, carboplatin plus etoposide; ECOG, Eastern Cooperative Oncology Group; LN, lymph node; PS, performance status; SPE, split doses of cisplatin plus etoposide.

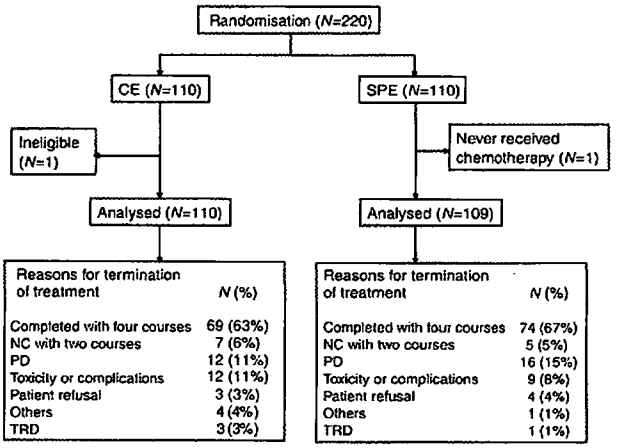

Figure 1 Flow diagram of randomised phase III trial of CE *vs* SPE in elderly or poor-risk patients with extensive disease SCLC.

Table 2 Compliance and drug delivery

	CE ($n=110$)	SPE ($n=109^a$)	P-value
Median interval of each chemotherapy (days) (range)			
1–2	27 (14–35)	23 (20–37)	0.02[b]
2–3	25 (21–56)	22 (20–35)	0.07[b]
3–4	27 (21–36)	24 (21–38)	0.05[b]
Total delivered courses/projected courses	353/440 (80%)	360/436 (83%)	
Dose reduction	32 (29%)	11 (10%)	<0.01[c]
Course delay	45 (41%)	40 (37%)	0.58[c]
G-CSF delivery	81 (74%)	84 (77%)	0.64[c]
No. of courses with G-CSF delivery/number of total courses	183/354 (52%)	203/362 (56%)	

CE, carboplatin plus etoposide; G-CSF, granulocyte colony-stimulating factor; SPE, split doses of cisplatin plus etoposide. [a]One patient never received chemotherapy due to delirium after registration. [b]Wilcoxon rank-sum test. [c]Fisher's exact test.