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Megakaryocyte potentiating factor as a tumor marker of malignant pleural mesothelioma: Evaluation in comparison with mesothelin

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KEYWORDS
Mesothelioma;
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Summary
Purpose: An early and reliable blood test is one deficiency in diagnosis of malignant pleural mesothelioma (MPM). Megakaryocyte potentiating factor (MPF) and mesothelin variants (MSLN), members of the mesothelin gene family, have been studied as candidate serum markers for MPM. We developed a novel enzyme-linked immunosorbent assay (ELISA) system to compare the diagnostic efficacy of MPF and MSLN in MPM and control groups.
Experimental design: MPF and MSLN were assayed with ELISA in 27 consecutive MPM patients and 129 controls including patients with lung cancer and asymptomatic asbestos-exposed subjects.

Abbreviations: MPM, malignant pleural mesothelioma; MPF, megakaryocyte potentiating factor; MSLN, mesothelin variants 1 and 3; ELISA, enzyme-linked immunosorbent assay.

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Results: Statistically significant elevation of serum MPF and MSLN levels was noted in MPM patients in comparison with every control group. The area under the receiver operating characteristic curve (AUC) was calculated for differentiation of MPM and lung cancer, healthy asbestos-exposed subjects, and healthy adults. While the AUC for serum MPF was 0.879, cut-off = 19.1 ng/ml (sensitivity = 74.1%, specificity = 90.4%), the AUC for serum MSLN was 0.713, cut-off = 93.5 ng/ml (sensitivity = 59.3%, specificity = 86.2%). Comparison between AUC for MPF and MSLN values shows that MPF is significantly superior to MSLN ($p = 0.025$). Finally, there was a significant correlation between MPF and MSLN values for MPM (Pearson's correlation coefficient = 0.77; $p < 0.001$).

Conclusions: These findings suggest that diagnostic value of MPF for MPM was better than that of MSLN although both markers showed almost equal specificity for MPM.

1. Introduction

Malignant pleural mesothelioma (MPM) is an aggressive tumor arising from mesothelial cells of serosal cavities. MPM may be asymptomatic at an early stage and is sometimes discovered by routine chest radiography. Common symptoms include chest pain and dyspnea, which are caused by tumor invasion of the chest wall or pleural effusion, and occur late during the disease progression. Although pemetrexed improves survival of unresectable MPM patients, overall median survival is only 12.1 months [1]. MPM is thought to be curable by early radical resection in combination with adjuvant chemoradiotherapy [2]. Even if surgical resection is not applicable, the early use of chemotherapy offers a prolonged period of symptom control and survival. MPM is often associated with past exposure to asbestos. There is a long latency period, often exceeding 20 years, between first exposure to asbestos and diagnosis of MPM [3]. The number of deaths from MPM is expected to increase in the next 20 years in Europe, Japan and Australia, where heavy use of asbestos has occurred [3–6]. There is thus a growing need for sensitive markers that can detect MPM in people at risk for this disease.

A 40-kDa membrane-bound mesothelin and a 31-kDa soluble megakaryocyte potentiating factor (MPF) originate from the same 69-kDa glycosyl-phosphatidylinositol-linked (GPI) glycoprotein precursor [7]. Mesothelin comprises three soluble forms of variants (MSLN). Variant 1 is a predominant mRNA expressed by both normal and tumor cells and its product is detectable in the ascites from ovarian cancer patients. It is thought to be proteolytically cleaved from the cell surface. Soluble variant 3 was detected as a small percentage of total mesothelin products from cell lines and tissues [8,9].

Scholler et al. prepared a monoclonal antibody, OV569, by immunizing mice with ovarian carcinoma cells. OV569 identified a 42- to 45-kDa protein with an N-terminal amino acid sequence identical to that of the membrane-bound portion of mesothelin and MPF, which was designated as soluble mesothelin related proteins (SMRP) [10]. A study utilizing OV569 showed an increase in SMRP concentrations in 37 of 44 patients (84%) with MPM [11]. The commercial assay kit for SMRP is designed to recognize the sequence within membrane-bound mesothelin and thus to measure mesothelin variants 1 and 3 [9]. SMRP is, therefore, identical to MSLN [11].

Later, Shiomi et al. showed that MPF is also secreted into the blood of mesothelioma patients and that the median levels of MPF in those patients were substantially elevated than in those in controls [12]. Onda et al. reported that serum MPF was elevated in 91% (51 of 56) of patients with mesothelioma and that measuring MPF may be useful for monitoring the response of mesothelioma to treatment [13]. Mesothelin variants and MPF thus appear to be promising targets for MPM diagnosis.

However, it has not yet been established whether MPF or MSLN is a more effective marker for differential diagnosis of MPM. To this end, we generated monoclonal antibodies (mAb) and prepared two enzyme-linked immunosorbent assay (ELISA) systems that each recognizes MPF or MSLN. In this paper we report the results of our studies comparing the discriminatory potency of MPF with that of MSLN for diagnosis of MPM.

2. Materials and methods

2.1. Patients and controls

Serum samples were collected from 27 consecutive patients with non-resectable MPM, whose diagnosis was confirmed by cytological or histopathological examination by pathologists skilled in diagnosis of this disease. The patient population included 13 with epithelial type MPM, three with sarcomatoid type, 5 with mixed type and 6 with unclassified type (diagnosed by cytological analysis). For control, we used 47 patients with lung cancer, 35 with other cancers (18 ovarian, 8 stomach and 9 colon cancers), 9 asbestos-exposed asymptomatic subjects and 38 healthy adults without a history of asbestos exposure. We obtained written and oral informed consent from all participants. This study was approved by our institutional review board.

2.2. Antigen preparation

Recombinant MPF protein was produced by amplifying the part coding for amino acids 1–288 from the cDNA encoding the transcript variant 1 for human mesothelin (Genbank accession no. NM_005823) with DNA polymerase

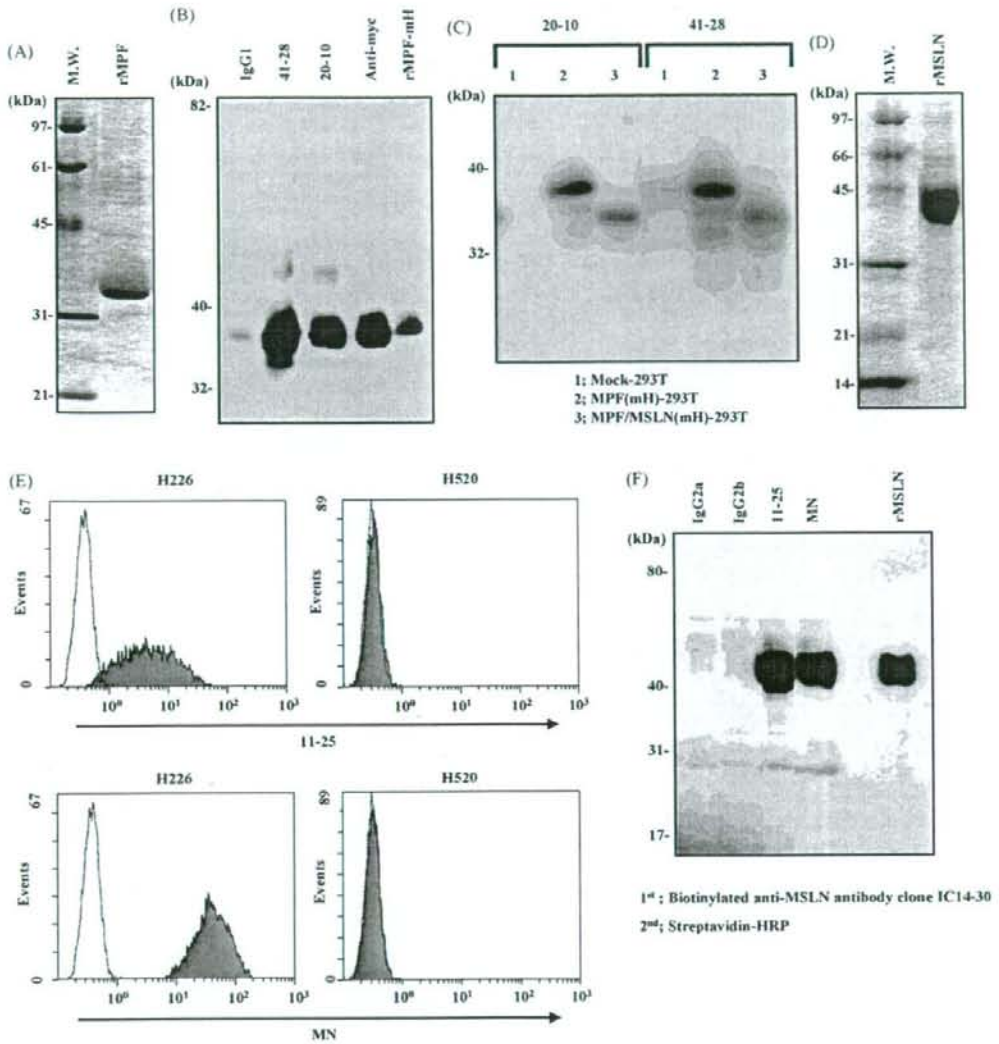


Fig. 1 Characterization of anti-Megakaryocyte potentiating factor (MPF) and anti-mesothelin variants (MSLN) antibodies. (A and D) Purity of the prepared recombinant MPF (A) and MSLN (D) proteins for immunization. Proteins were electrophoresed under reduced condition and stained with Coomassie Brilliant Blue. (B and F) Reactivity of anti-MPF antibodies to recombinant MPF protein (B) or anti-MSLN antibodies to MSLN in the culture supernatant of NCI-H226 cells (F). Anti-MPF antibodies (B, lanes 41–28 and 20–10) or anti-MSLN antibodies (F, lanes 11–25 and MN) were used for immunoprecipitation. Recovered proteins were separated by SDS-PAGE and electrotransfered to polyvinylidenefluoride (PVDF) membranes. Membranes were probed with anti-myc antibody for myc-His tagged MPF or anti-MSLN antibody clone IC14–30 for MSLN, respectively, and followed by development. Recombinant MPF protein (B, lane rMPF-mH) or recombinant MSLN protein (F, lane rMSLN) were applied for positive controls. (C), western blot analysis of anti-MPF antibodies. Supernatants of the HEK 293T transfectants with myc-His tagged MPF, full length of mesothelin variant 1 or mock vectors were separated by SDS-PAGE and electrotransfered to a PVDF membrane. The membrane was probed with anti-MPF antibodies (20–10 and 41–28), incubated with HRP-labeled anti-mouse IgG antibody and followed by chemiluminescence. Lane 1, Mock-transfected 293T; lane 2, myc-His tagged MPF-transfected 293T; lane 3, full length of mesothelin variant 1-transfected 293T. (E), expression of mesothelin on the surface of lung cancer cell lines. NCI-H226 and NCI-H520 were incubated with anti-MSLN antibodies (11–25 and MN) or an isotype-matched control antibody and followed by PE-conjugate anti mouse IgG. Antigen expression was detected by flow cytometry.

(recombinant Taq polymerase; Takara Bio Inc., Shiga, Japan) and using the primers 5′-CGGAATTCGCCGCCACC-ATGGCCTTGCCAACGGCTCGACCCCTGTTG-3′ and 5′-GCTCT-AGAGATGGTCCGTTCAGGCTGCCGCCAGGATGG-3′. The amplified DNA was inserted into the EcoRI/XbaI site of mammalian expression plasmid pcDNA3.1/myc-His (Invitrogen, Carlsbad, CA) and transfected into HEK 293T cells by lipofection (Lipofectamine2000; Invitrogen). The culture supernatant was applied to a TALON resin according to the manufacturer's instructions (Clontech, Mountain View, CA). The purified MPF protein thus obtained was dialyzed with 4.0 liter of PBS twice and kept frozen at −80 °C until use as an immunogen or as a standard polypeptide for sandwich ELISA. Purity of the recombinant MPF was confirmed by Coomassie Brilliant Blue staining after electrophoresis under reduced condition (Fig. 1A).

MSLN was produced by amplifying the part coding for amino acids 297–580 of the same cDNA and using the primers 5′-AAATTTCCCAAGCTTGTGGAGAAGACAGCCTGTCCTTCAG-GCAAG-3′ and 5′-AAGGAAAAAAGCGGCCGCGCCCTGTAGCC-CCAGCCCCAGCGTGTCCAG-3′. The amplified DNA was inserted into the HindIII/NotI site of expression vector pSecTag2B (Invitrogen). It should be noted that variants 1 and 3 of mesothelin share amino acids 297–580 of clone NM_005823 as a common sequence. The plasmid DNA was transfected into HEK 293T cells, and recombinant MSLN produced in the culture supernatant was purified and stored as above (Fig. 1D).

2.3. Antibody generation

To generate mAbs against MPF and against MSLN, 4- to 6-week old BALB/c mice were immunized with the respective purified protein i.p. on days 0, 7, 14, and 16 (10 μg/shot). Following the last immunization, lymphocytes of the spleen were collected and fused with P3U1 myeloma cells in a 50% polyethylene glycol 4000 solution (Wako, Osaka, Japan) on day 18. The fused cells were plated on 96-well plates with RPMI-1640 medium containing 15% fetal calf serum (FCS; Equitech-Bio Inc., Kerrville, TX), penicillin/streptomycin (Invitrogen) and HAT solution (Invitrogen). After 10 days incubation at 37 °C with 5% CO_2 in a humidified environment, culture supernatants were collected and screened for their ability to bind to the immunizing antigen by means of an indirect ELISA using recombinant MPF or soluble mesothelin, respectively. Selected positive hybridoma colonies were expanded and subcloned by limiting dilution. An isostrip kit (Hoffmann-La Roche, Basel, Switzerland) was used for antibody isotype determination according to the manufacturer's instructions. Antibody purification was carried out with protein A affinity chromatography (GE Healthcare, Buckinghamshire, UK). Following a competition assay for the immunogens among the clones thus obtained (data not shown), clone 20–10 (IgG1 κ) and clone 41–28 (IgG1 κ) were selected to construct an sandwich ELISA for the detection of MPF, while MN (IgG2a κ) and 11–25 (IgG2b κ) were chosen for construction of an MSLN ELISA as described elsewhere. Clone MN was previously obtained by Dr. Ira Pastan's laboratory as a specific monoclonal antibody against mesothelin [14] and used in this study under a licensing agreement with NIH. Clones 41–28 and 11–25 were biotinylated using ECL Protein Biotination Module (GE Healthcare).

2.4. Flow cytometry

Lung cancer cell lines NCI-H226 and NCI-H520 were cultured in RPMI medium (SIGMA) supplemented with 10% fetal bovine serum. The former cell line expresses both MPF and MSLN but the latter does neither [14]. After a treatment with PBS containing 5 mM EDTA for 3 min, detached cells were washed with PBS twice and incubated with 1 μg/ml of anti-MSLN Abs or isotype-matched controls for 30 min at 4 °C in PBS containing 0.5% BSA and 2mM EDTA. Following twice wash with the above buffer, PE-conjugate anti-mouse IgG was added and further incubated for 30 min at 4 °C. All flow cytometry was performed on Cytomics FC500 (Beckman Coulter).

2.5. Immunoprecipitation

The reactivity of anti-MPF Abs to recombinant MPF protein, or anti-MSLN Abs to recombinant MSLN protein in the culture supernatant of NCI-H226 was confirmed by immunoprecipitation. 15 μl of Protein G sepharose suspended in PBS containing 0.01% BSA (SIGMA) was incubated with 5 μg of anti-MPF Abs 20–10 and 41–28, or anti-MSLN Abs 11–25 and MN, for 2 h at 4 °C with gently rocking. During this step, 250 ng of the recombinant myc-His tagged MPF protein or 5 times concentrated culture supernatant of NCI-H226 and NCI-H520 were incubated with Protein G beads for 30 min at 4 °C with shaking to preclear the samples. The Protein G sepharose incubated with the antibodies were centrifuged at 1000 × *g* for 2 min and washed with PBS 3 times. Then the precleared samples were added to the tube containing the washed Protein G sepharose and rotated for over night at 4 °C. After the incubation, the beads were washed with PBS 3 times and boiled in 25 μl of 2× Laemmli's SDS sample buffer for 5 min. Proteins (20 μl of sample per lane) were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12.5% polyacrylamide gel and electrotransfered to a polyvinylidenefluoride (PVDF) membrane. The membrane blocked with 5% nonfat milk in PBS containing 0.05% Tween-20 (blocking buffer) was incubated with 1.0 μg/ml mouse anti-myc mAb (MBL) for detecting myc-His tagged MPF, or 1.0 μg/ml biotinylated anti-MSLN antibody clone IC14–30 (MBL) for detecting MSLN for 1 h at room temperature. After 4 times wash with PBS containing 0.05% Tween-20, the membrane was incubated with a horseradish peroxidase (HRP)-conjugated anti-mouse IgG (MBL) for MPF diluted 1:5000 with the blocking buffer or HRP-streptavidin (GE Amersham) for MSLN diluted 1:5000 with the blocking buffer. The chemiluminescence was developed by according to manufacture's procedure (ECL; GE Amersham). 50 ng of recombinant MPF protein or 250 ng of recombinant MSLN protein was applied as a positive control for Western blotting.

2.6. Western blot

HEK 293T cells were transfected by lipofection with expression vectors coding myc-His tagged MPF, full length of

mesothelin variant 1, or a mock vector as a negative control, and the culture media were collected after 72 h-incubation in a CO_2 incubator at 37 °C. Supernatants of the media were recovered by a centrifugation at 3000 × *g* for 10 min at room temperature, and then boiled with an equal volume of 2× SDS sample buffer for 5 min. 20 μl of samples per lane were loaded on a 12.5% SDS-polyacrylamide gel and separated by SDS-PAGE. After electrotransfer to a PVDF membrane, the membrane was treated with the blocking buffer containing 5% nonfat milk and incubated with 2.0 μg/ml anti-MPF Abs (20–10 and 41–28) for 1 h at room temperature. After 4 times wash with PBS containing 0.05% Tween-20, the membrane was incubated with HRP-labeled anti-mouse IgG (MBL) diluted 1:5000 with the blocking buffer. The chemiluminescence was done by according to manufacture's procedure (ECL; GE Amersham).

2.7. Sandwich ELISA

The serum concentrations of MPF and of the soluble form of mesothelin were measured by each specific sandwich ELISA constructed as follows: 96-well microtiter plates (Maxisorp; Nalgen Nunc International Corp., Rochester, NY) were coated with the capturing antibody, clone 20–10 for MPF or clone MN for MSLN, and adjusted to 5 μg/ml with 100 mM carbonate buffer (pH 9.6) at 4 °C overnight. The plates were blocked with 200 μl PBS (pH 7.4) containing 1.0% BSA, 5.0% sucrose, and 0.1% NaN_3 for 2 h and then incubated for 1 h with serum samples diluted to 1:40 with PBS (pH 7.4) containing 1.0% BSA, 0.1% Tween20, 50 μg/ml MAK33 (Roche), and 0.1% NaN_3. After washing with PBS (pH 6.7) containing 0.13% Tween-20, the wells were incubated for 1 h with 2.0 μg/ml biotinylated mAb 41–28 for detecting MPF, or incubated with 0.7 μg/ml biotinylated mAb 11–25 for detecting soluble mesothelin, and reacted with streptavidin-conjugated peroxidase (GE Healthcare) diluted to 1:60,000 for MPF or avidin-conjugated peroxidase (DAKO, Glostrup, Denmark) diluted to 1:10,000 for soluble mesothelin, respectively, with 20 mM HEPES (pH 6.5) containing 1.0% BSA, 0.135 M NaCl, 0.1% *p*-hydroxy phenyl acetic acid (Tokyo Chemical Industry, Tokyo, Japan), and 0.15% ProClin150 (Supelco, St. Louis, MO). Followed by four washes with PBS, 50 μl/well TMB (Moss Inc., Pasadena, MD) was added and the plates were incubated for 30 min. The color development was stopped by the addition of 0.36N H_2SO_4. Color intensity was determined with a microplate reader Model 680 (Bio-Rad Laboratories, Hercules, CA) at a wavelength of 450 nm with a reference wavelength of 620 nm. Analyte concentrations were calculated by referring to the standard curve using serial diluted recombinant MPF or MSLN, respectively (Fig. 2A for MPF and Fig. 2B for MSLN).

2.8. Statistical analyses

Nonparametric Mann Whitney's U test was used for comparison of the data. $p < 0.05$ was considered statistically significant. For drawing of receiver operating characteristic (ROC) curves and estimation of the area under the ROC curve (AUC) statistics software SPBS (Comworks, Saitama, Japan) was used to quantify the ability to differentiate

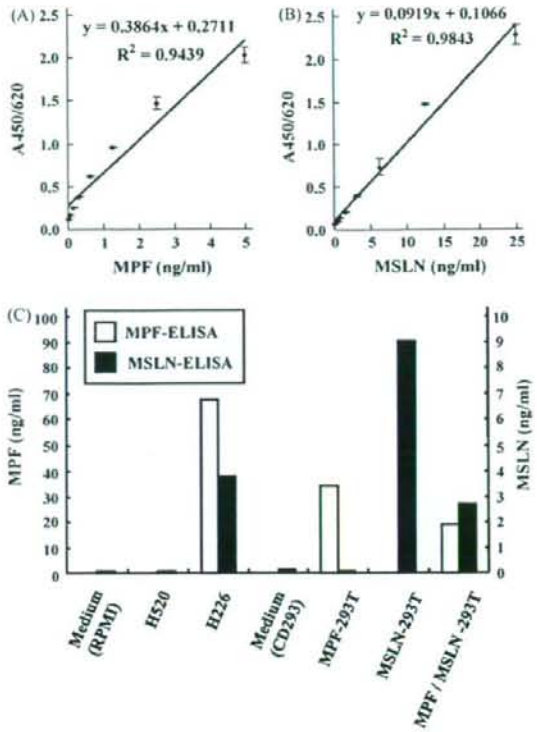


Fig. 2 Sandwich ELISA. Standard curves for MPF (A) and MSLN (B). ELISAs displaying the mean absorbance values from indicated concentrations of recombinant proteins. Points, mean; bars, S.D. (C), MPF and MSLN levels in the supernatants of cell lines and 293 transfectants measured by specific ELISA systems. The levels in the supernatants of 293 transfectants are indicated as those of 500 times diluted solution. Open bars, MPF; closed bars, MSLN.

between healthy volunteers and patients with indicated diseases. Pearson's correlation coefficient was calculated to determine the correlation between MPF and MSLN.

3. Results

3.1. Generation of sandwich ELISA specific for MPF and soluble mesothelin (MSLN)

To generate mAbs that specifically react with MPF, recombinant MPF protein corresponding to the amino acids 1–288 from the transcript variant 1 of human mesothelin was produced by 293T transfectants (Fig. 1A) and immunized to BALB/c mice. Supernatants of obtained hybridomas were tested for the binding activity to the microplate coated with immunizing antigen and further examined by a competition assay for the immunogen (data not shown). The specific reactivities of selected clone 20–10 and clone 41–28 to MPF were checked by immunoprecipitation using myc-His tagged recombinant MPF (Fig. 1B), and also performed by Western blot using the supernatants of 293T cells transfected with

expression vectors coding myc-His tagged MPF (Fig. 1C, lane 2) and the full length of mesothelin variant 1 (Fig. 1C, lane 3). A smaller band on lane 3 compared to the myc-His tagged MPF on lane 2 were detected by both anti-MPF Abs. MPF and mesothelin are produced together as a precursor form. Following cleavage by a protease furin, MPF is released as an N-terminal 31-kDa fragment from mesothelin [15]. The smaller band on the lane 3 seems to be the 31-kDa mature form of MPF without myc-His Tag. A sandwich ELISA for MPF was constructed using the anti-MPF antibodies 20–10 and 41–28. The standard curve using purified recombinant MPF was shown in Fig. 2A. Correlated with Fig. 1C, the MPF sandwich ELISA detected the antigen in the culture supernatant of the 293T transfectant expressing the full length of mesothelin variant 1 (Fig. 2C, MPF/MSLN-293T), as well as MPF-transfected cells (Fig. 2C, MPF-293T) but not the soluble mesothelin secreted in the culture supernatant of the MSLN-transfectants (Fig. 2C, MSLN-293T).

The monoclonal anti-MSLN antibody, clone 11–25, were generated by immunizing mice with recombinant MSLN (amino acids 297–580 of mesothelin variant 1). The specificity of 11–25 to MSLN was tested using a lung cancer cell line, NCI-H226, which has been clarified to express mesothelin [14]. 11–25 detected not only the antigen on the cell surface of NCI-H226 by flowcytometory (Fig. 1E, upper left panel), but also identified soluble antigen in the culture supernatant of NCI-H226 by immunoprecipitation (Fig. 1F, lane 11–25). Another anti-MSLN Ab, clone MN, which the Pastan's laboratory has previously developed, was used, in combination with 11–25, to establish a sandwich ELISA for MSLN [14]. The standard curve using recombinant MSLN was shown in Fig. 2B. The MSLN sandwich ELISA recognized the soluble antigen in the culture supernatant of NCI-H226 (Fig. 2C, H226) as well as the 293T transfectant expressing the full length of mesothelin variant1 (Fig. 2C, MPF/MSLN-293T). On the other hands, this MSLN ELISA did not detect recombinant MPF in the culture supernatant of the MPF-transfectants (Fig. 2C, MPF-293T).

3.2. MPF and MSLN in blood samples

We measured serum MPF and MSLN levels in all subjects. Serum MPF levels differed among the five groups (Fig. 3A and Table 1), with mean serum MPF values were higher for MPM patients (68.7 ± 101.1 ng/ml [mean ± standard deviation]) than for patients with lung cancer (16.6 ± 15.3 ng/ml), individuals with other cancers (15.1 ± 9.7 ng/ml), healthy asbestos-exposed subjects (9.7 ± 5.3 ng/ml) and healthy adults (9.0 ± 2.9 ng/ml). The difference in median values between MPM and every control group was statistically significant (Mann–Whitney's U test; $p < 0.001$). Mean serum MSLN levels in MPM, patients with lung cancer, individuals with other cancers, healthy asbestos-exposed subjects, or healthy adults were 130.0 ± 112.9 ng/ml, 83.4 ± 50.4 ng/ml, 74.4 ± 45.3 ng/ml, 59.5 ± 25.6 ng/ml and 61.4 ± 21.4 ng/ml, respectively (Fig. 3B and Table 1). The median serum MSLN level of MPM was significantly higher than in the control groups (Mann–Whitney's U test; MPM *vs.* lung cancer: $p = 0.028$, MPM *vs.* other cancers: $p = 0.005$, MPM *vs.* asbestos-exposed subjects: $p = 0.010$, MPM *vs.* healthy adults: $p < 0.001$). There was no significant difference in MPF or MSLN levels among MPM patients with different histologies (Table 1).

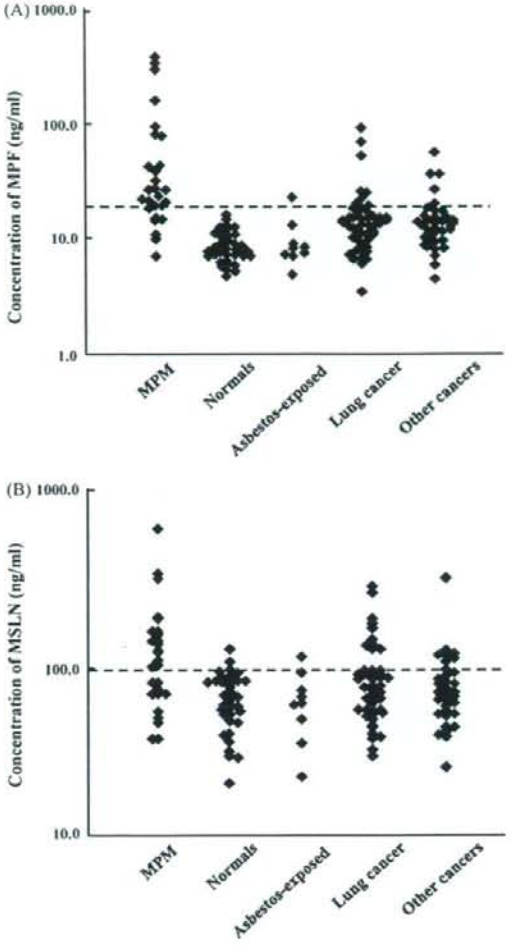


Fig. 3 Megakaryocyte potentiating factor (MPF) (A) and mesothelin variants (MSLN) (B) levels in the sera of malignant pleural mesothelioma (MPM) patients and control groups. Each dot represents one patient. The horizontal broken lines represent cut-off values: 19.1 ng/ml for MPF and 93.5 ng/ml for MSLN. MPM, malignant pleural mesothelioma; Normals, healthy volunteers; Asbestos-exposed, asbestos-exposed asymptomatic individuals; other cancers, ovarian, stomach and colon cancer patients.

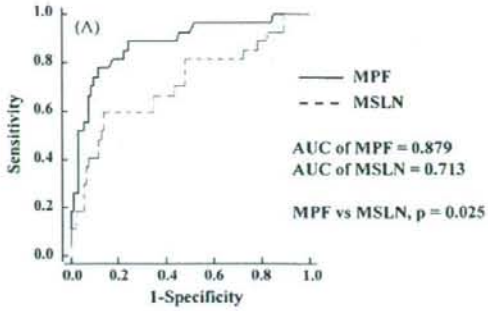
3.3. Cut-off value, sensitivities and specificities calculation of MPF and MSLN

To assess the clinical potential of MPF, we compared sensitivities and specificities of MPF with those of MSLN. The operating characteristics for the two tumor markers with their cut-off points for achieving the best individual accuracy are shown in Fig. 4A. The area under the receiver operating characteristic (ROC) curve (AUC) for serum MPF

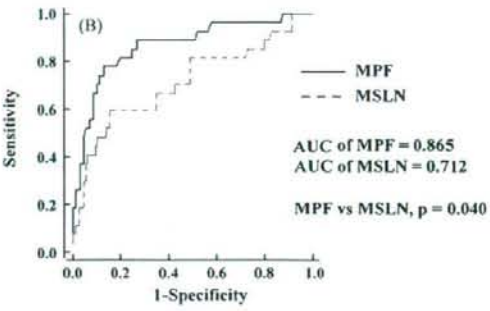
Table 1 Serum concentrations of megakaryocyte potentiating factor (MPF) and mesothelin variants (MSLN) in mesothelioma patients and controls

Diagnosis	Number of study participants	MPF (ng/ml)			MSLN (ng/ml)		
		Mean (S.D.)	Median	Range	Mean (S.D.)	Median	Range
MPM	27	68.7 (101.1)	26.3	6.9–370.1	130.0 (112.9)	101.5	35.9–576.1
Epithelial type	13	87.4 (112.6)	38.1	14.2–370.1	132.2 (67.7)	129.3	64.8–317.8
Sarcomatoid type	3	16.5 (5.8)	19.1	9.8–20.4	67.9 (38.6)	57.3	44.5–112.4
Mixed type	5	77.7 (143.7)	14.8	6.9–334.6	158.8 (233.6)	65.2	35.9–576.1
Unclassified type	6	47.0 (54.3)	24.7	15.3–156.3	132.3 (88.9)	109.7	35.9–296.6
Malignant condition							
Lung cancer	47	16.6 (15.3)	13.3*	3.4–89.4	83.4 (50.4)	70.7†	28.6–267.8
Other cancers	35	15.1 (9.7)	13.0*	4.4–54.5	74.4 (45.3)	66.4‡	24.6–298.2
Ovarian cancer	18	18.0 (12.5)	13.7	4.4–54.5	83.2 (56.9)	67.8	41.7–298.2
Stomach cancer	8	14.3 (3.7)	14.1	9.6–19.7	73.9 (29.0)	69.9	36.9–113.8
Colon cancer	9	9.7 (2.9)	8.7	5.8–14.0	57.0 (24.5)	51.6	24.6–106.6
Asbestos-exposed individuals	9	9.7 (5.3)	8.2*	4.9–22.7	59.5 (25.6)	56.7§	21.6–106.0
Healthy volunteers	38	9.0 (2.9)	8.0*	4.8–16.6	61.4 (21.4)	62.0*	19.6–117.4

* Significance of median values for the specified control groups compared with MPM. Mann–Whitney's *U* test; $p < 0.001$.
† Significance of median values for the specified control groups compared with MPM. Mann–Whitney's *U* test; $p = 0.028$.
‡ Significance of median values for the specified control groups compared with MPM. Mann–Whitney's *U* test; $p = 0.005$.
§ Significance of median values for the specified control groups compared with MPM. Mann–Whitney's *U* test; $p = 0.01$.



Marker	Cut-off (ng/ml) (n=94)	Sensitivity (%)	Specificity (%)
MPF	19.1	74.1	90.4
MSLN	93.5	59.3	86.2



Marker	Cut-off (ng/ml) (n=129)	Sensitivity (%)	Specificity (%)
MPF	19.1	74.1	89.1
MSLN	123.7	40.7	93.8

Fig. 4 Receiver operating characteristic (ROC) curves for megakaryocyte potentiating factor (MPF) and mesothelin variants (MSLN) for differentiation between malignant pleural mesothelioma ($n = 27$) and controls. Controls comprise lung cancer patients ($n = 47$), asbestos-exposed asymptomatic individuals ($n = 9$) and healthy volunteers ($n = 38$) for (A). For the analysis shown in (B), control groups include other cancers ($n = 35$) in addition to those specified in (A). The tables show the best statistical cut-off values for MPF and MSLN with pairs of sensitivity and specificity.

was 0.879 for differentiating MPM patients from controls comprising lung cancer patients, asbestos-exposed individuals and healthy adults, with a cut-off value of 19.1 ng/ml (sensitivity = 74.1%, specificity = 90.4%), whereas the AUC for serum MSLN was 0.713 with a cut-off value of 93.5 ng/ml (sensitivity = 59.3%, specificity = 86.2%) (Fig. 4A). Serum MPF levels were elevated in 20 (74.1%) MPM patients, eight (17.0%) lung cancer patients and five (14.3%) individuals with other cancers (Fig. 3A). Corresponding serum MSLN levels were elevated in 59.3%, 21.3% and 20.0% of the patients (Fig. 3B). Both MPF and MSLN showed elevated levels in

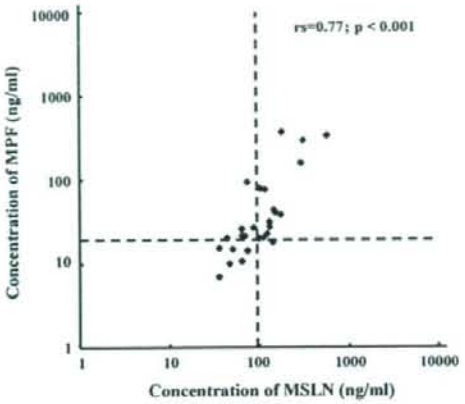


Fig. 5 Inter-marker correlation between megakaryocyte potentiating factor (MPF) and mesothelin variants (MSLN) for malignant pleural mesothelioma (MPM). Each dot indicates MPF and MSLN values for the same MPM patient ($n = 27$). r_s, Pearson's correlation coefficient. The horizontal dotted line represents the cut-off value for MPM (19.1 ng/ml) and the vertical dotted line for MSLN (93.5 ng/ml).

four (22.2%) ovarian cancer patients, while MPF values were elevated in one (11.1%) asbestos-exposed adult and none of the healthy donors, and MSLN levels were elevated in one (11.1%) asbestos-exposed adult and two (5.3%) healthy donors. Calculation of areas under the ROC curves showed a significant difference between the two markers ($p = 0.025$) (Fig. 4A). ROC curves for a comparison of MPM with all non-MPM patients and healthy adults, yielded an AUC of 0.865 for MPF and of 0.712 for MSLN (MPF *vs.* mesothelin, $p = 0.04$) (Fig. 4B). Sensitivity of MSLN decreased to 40.7% while that of MPF did not change (Fig. 4B). Comparison of the AUC values thus showed a better diagnostic performance by MPF than MSLN for discriminating MPM.

3.4. Inter-marker correlation

To examine inter-marker correlation between MPF and MSLN values for MPM, we plotted concentrations of MPF and MSLN in the same figure (Fig. 5) and found a significant correlation between MPF and MSLN values for MPM (Pearson's correlation coefficient, $r_s = 0.77$; $p < 0.001$). Concentrations for six patients were above the cut-off value for MPF (19.1 ng/ml) but below the cut-off values (93.5 ng/ml) for MSLN, whereas only one showed the opposite condition. Concentrations for 14 patients were above the cut-off values for both markers and for six patients they were below these values (Fig. 5).

4. Discussion

Recent studies identified increased levels of MSLN in the blood of MPM [11,16–18] and other histological cancer patients [9,10]. They suggested MSLN was useful for diagnosis of MPM and for monitoring disease progression. Recently, MPM patients were found to have raised serum concentrations of MPF [13,19], indicating that MPF can also be a candidate diagnostic marker for MPM. To determine which molecule, MPF or MSLN, is more sensitive for screening of MPM, we developed two novel sets of ELISA system. The one for MSLN recognizes mesothelin variants 1 and 3. In our study, higher MPF levels were found in 74.1% of MPM patients and elevated MSLN levels in 59.3%. Scherpereel et al. demonstrated that MPM epithelioid type had significant elevated values for MSLN than mixed type or sarcomatoid type [17]. Nevertheless, we and Creaney et al. did not find significant difference in levels of either MSLN or MPF in MPM patients with different histologies (Table 1) [20]. The sensitivity of 59.3% of MSLN established in our study was comparable to the sensitivity of 52% in Creaney' s study of 117 MPM patients [20] or 68% of Cristaudo' s study [21]. Regarding sensitivity of MPF to patients with MPM, we do not know any other reports.

When two or more tests are available for diagnostic purposes, comparison of the respective AUCs will often show which test is the most effective. ROC curves of MPF yielded an AUC of 0.879, and those of MSLN an AUC of 0.713 (MPF *vs.* MSLN, $p = 0.025$) (Fig. 4A). There was a significant correlation between MPF an MSLN values for MPM (Pearson's correlation coefficient (r_s) = 0.77). This indicates that MPF is probably more sensitive than MSLN for diagnosis of MPM. Though osteopontin and CA125 are also indicated as potential markers in the diagnosis of MPM, neither showed better sensitivity for MPM in comparison with MSLN [20,22].

Physiological cleavage from the mesothelin precursor protein at the furin cleavage site may be responsible for extracellular secretion of MPF [13]. Mesothelin and its variants, attached to cell membranes by GPI-anchors, are also readily released *in vivo*, but the mechanism of mesothelin release has not yet been identified. We transfected the full length of mesothelin variant 1 cDNA into 293 cells and part of mesothelin variant 1 is released from the cell surface and can be measured with the ELISA system (Fig. 2 C, MPF/MSLN-293T). A relevant example is carcinoembryonic antigen (CEA), a tumor-associated GPI-anchored glycoprotein that is commonly shed from the cell surface [23]. The as yet unknown process that mediates a release of the membrane-bound mesothelin variant 1 may be associated with the lower sensitivity of MSLN in comparison with that of MPF. However, additional studies are needed to identify and characterize the process of mesothelin secretion.

Just as MPF and mesothelin originate from the same mesothelin precursor, so adrenomedullin (ADM) and proadrenomedulin (PAMP) are potent vasodilatory peptides derived from a common precursor peptide. However, their short half-life and the presence of a binding protein have been obstacles for an accurate quantification. The ADM precursor is produced in quantities stoichiometric to ADM and PAMP, but is non-functional and stable, while the quantities of the ADM precursor thus produced directly reflect those of ADM and PAMP [24]. One of the reasons for the lower sensitivity of the mesothelin assay could be a lower stability of the protein, but the stability of both proteins needs to be examined and compared.

Pastan's group showed that most ovarian cancer cells express 40-kDa protein mesothelin at the cell surface [25]. MPF was initially isolated from supernatant of pancreatic

cancer cells [26]. Scholler et al. and Hassan et al. independently demonstrated elevated levels of SMRP in 23 of 30 (76.7%) and 40 of 56 (71%) sera from patients with ovarian carcinoma by using a monoclonal antibody, OV569, that was prepared by immunizing mice with ovarian carcinoma cells [10,18]. However, Beyer et al. reported SMRP values were increased in less than 10% of 111 samples collected from ovarian cancer patients [16]. In our study, serum concentrations of both MPF and MSLN were increased in only four of 18 (22.2%) individuals with ovarian cancer. Frequent positive immunostaining for mesothelin is reportedly associated with the non-mucinous type of ovarian cancers [27,28]. Although these reports did not include a description of histological subtype classification in the various blood samples studied, the different ratios of mucinous to non-mucinous type in the samples may partly account for the differences in positivity of serum mesothelin. For MPF, ours is the first reported study of serum levels in ovarian cancer patients.

5. Conclusion

We used ELISA systems developed by us to detect significant differences in the levels of serum MPF and MSLN between MPM patients and controls including lung cancer patients, asbestos-exposed individuals, and normal volunteers. In addition, it is suggested that MPF has superior specificity for MPM compared to MSLN. We are planning a further study to determine the relationship between serum MPF and disease stage, prognosis and histological subtypes.

Conflict of interest

None.

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Short Communication

Pleural MALT lymphoma diagnosed on thoracoscopic resection under local anesthesia using an insulation-tipped diathermic knife

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A 79-year-old man presented with back pain. Chest CT scan showed elevated nodular lesions in the right parietal pleurae with pleural effusion. There were no intrapulmonary or mediastinal abnormalities. Under local anesthesia, right thoracoscopy and subsequent thoracoscopic pleural resection were performed using an insulation-tipped diathermic knife (IT-knife). The resected pleura, 2.2 cm in diameter, had a rough granular surface. Lymphoid cells histologically infiltrated diffusely into the pleura. They were composed of centrocyte-like and monocytoid cells. On immunohistochemistry they were found to be positive for Bcl2, CD20, CD45RB and CD79a, but negative for CD3, CD5, CD10 and cyclin D1. EBV-encoded small RNA-1 (EBER-1) *in situ* hybridization was negative. A diagnosis of extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma) arising in the pleura was therefore made. To the authors' knowledge this is the first case in which IT-knife was used for diagnosis of a pleural lesion. This large, single-piece, only slightly crushed pleural specimen, enabled study of histopathological findings (listed here) that could not have been obtained on conventional biopsy: (i) lack of apparent evidence of plasmacytic differentiation; (ii) no recognition of lymphoid follicles; (iii) mesothelial cells not infiltrated by lymphoma cell clusters; (iv) thin layer of hyperplastic mesothelial cells continuously covering the surface; and (v) no proliferation of fibroblast-like submesothelial cells.

Key words: insulation-tipped diathermic knife, MALT lymphoma, pleura, thoracoscopy

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Extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma) arising in the pleura is extremely rare, with only four published cases.[1–3] Reported cases were diagnosed pathologically using surgically resected pleural specimen under thoracotomy[1,3] or under video-assisted thoracic surgery (VATS).[2] But in these cases the histopathological findings were not described in detail, probably because the obtained specimens were fragmented or highly crushed. To resolve these issues, large, single-piece and only slightly crushed resected specimens are necessary.

In Japan, endoscopic mucosal resection (EMR) of gastric mucosa by gastrointestinal endoscopists has been accepted as a treatment option for early gastric cancer (EGC).[4] For resection of larger EGC as a single-piece specimen, a new EMR method using an insulation-tipped diathermic knife (IT-knife; Olympus, Tokyo, Japan) was developed.[4–6]

Herein we present the first case of pleural MALT lymphoma that was diagnosed thoracoscopically using a large and single-piece specimen obtained using an IT-knife (thoracoscopic IT-knife) and describe the histopathological findings of pleural MALT lymphoma in detail.

CASE REPORT

Clinical history

A 79-year-old man who had smoked approximately 60 cigarettes per day for 40 years, consulted Osaka Prefectural Medical Center for Respiratory and Allergic Diseases complaining of back pain that had persisted for 2 months. Family

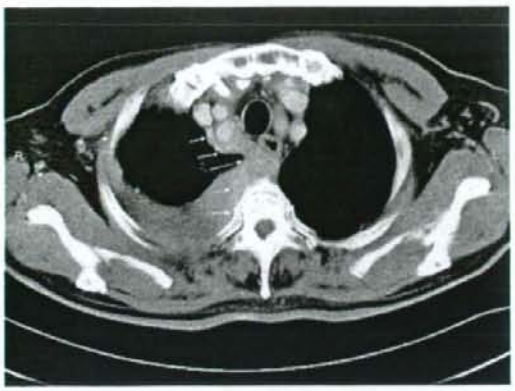

Figure 1 Chest CT scan showing diffuse prominent thickening of right mediastinal pleura (arrows) and pleural effusion.

history, past medical history and occupational history were unremarkable. Chest CT scan (Fig. 1) showed diffuse prominent thickening of the right mediastinal and costal pleurae. Right pleural effusion was present but there were no intrapulmonary or mediastinal abnormalities. Pleural effusion cytology did not show any evidence of neoplasia. Laboratory tests and CT scan of the abdomen and pelvis were unremarkable. Serum CEA, neuron-specific enolase, cytokeratin-19 fragment and soluble interleukin-2-receptor levels were 4.7 ng/mL (cut-off: 5 ng/mL) , 10.4 ng/mL (cut-off: 10 ng/mL), 1.3 ng/mL (cut-off: 3.5 ng/mL) and 857 U/mL (cut-off: 466 U/mL), respectively. A tentative diagnosis of pleural malignant mesothelioma was made. After removal of 1340 mL of pleural effusion, a thoracoscopic IT-knife was used to resect a nodular lesion in the costal parietal pleura under local anesthesia (Fig. 2a,b).

Macroscopic findings

The resected pleura measured 2.2 cm in diameter and had a rough granular surface (Fig. 2c). The specimen was serially examined at 4 mm intervals. The cut surface (Fig. 2d) was whitish and exhibited prominent thickening of the pleura.

Microscopic findings

Throughout the whole pleural specimen, from the superficial submesothelial layer to the deep margin, a diffuse infiltrate of small- to -medium lymphoid cells was recognized (Fig. 3a). These cells were composed of centrocyte-like and monocytoid cells (Fig. 3b). Plasmacytic differentiation was inconspicuous and lymphoid follicles were absent (Fig. 3b).

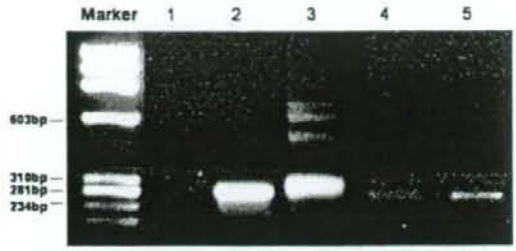

Figure 4 Clonal polymerase chain reaction (PCR). Clonal analysis of immunoglobulin heavy-chain (*IgH*) gene rearrangements was performed using semi-nested PCR. Lane 1, negative control; lane 2, positive control; lane 3, reactive lymphoid hyperplasia; lane 4, present case; lane 5, present case. Two samples (lanes 4, 5) taken from the right costal pleurae had a distinct single clonal band in the PCR assay for the *IgH* gene.

Mitoses were present in 3 figures/50 high-power fields. There was no necrosis or hemorrhage. Immunohistochemistry showed that these cells were positive for Bcl2, CD20, CD45RB and CD79a (Fig. 3c), but negative for CD3, CD5, CD10 and cyclin D1. EBV-encoded small RNA-1 (EBER-1) *in situ* hybridization was negative. Therefore, we made a final diagnosis of pleural MALT lymphoma. AE1/AE3 staining showed that the pleural lesions were covered with a thin continuous layer of hyperplastic mesothelial cells (Fig. 3d), but there was no infiltration of lymphoma cell clusters among the mesothelial cells, like the lymphoepithelial lesions typically seen in gastric lesions (Fig. 3e). There was no proliferation of submesothelial fibroblast-like cells (Fig. 3e).

Clonal analysis by polymerase chain reaction

Clonality analysis of immunoglobulin heavy-chain (*IgH*) gene rearrangements was performed using semi-nested polymerase chain reaction (PCR). The PCR conditions and the primers used (FR2A, LJH and VLJH) have been described previously.[7,8] This case had a distinct single clonal band in the PCR assay for the *IgH* gene (Fig. 4).

DISCUSSION

MALT lymphoma is an extranodal indolent lymphoma consisting of morphologically heterogenous small B-cells: marginal zone (centrocyte-like) cells, monocytoid cells, small lymphocytes, and scattered immunoblasts and centroblast-like cells.[9] The gastrointestinal tract is the most common site.[9] In the present case a diffuse infiltrate of small- to -medium lymphoid cells was recognized in the pleural specimens. Plasmacytic differentiation was inconspicuous and

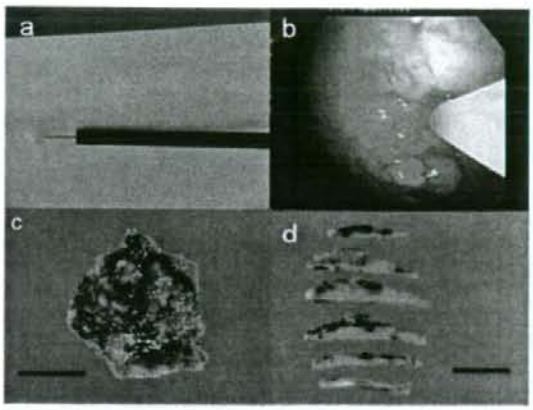

Figure 2 (**a**) Insulated-tipped diathermic knife (IT-knife): Needle knife with a ceramic ball at the top. (**b**) Thoracoscopic image showing a nodular lesion in the parietal pleura being incised. (**c**) Resected pleura with a rough granular surface. Bar, 1 cm (**d**) Cut surface showing prominent thickening of the pleura and a whitish appearance. Bar, 1 cm.

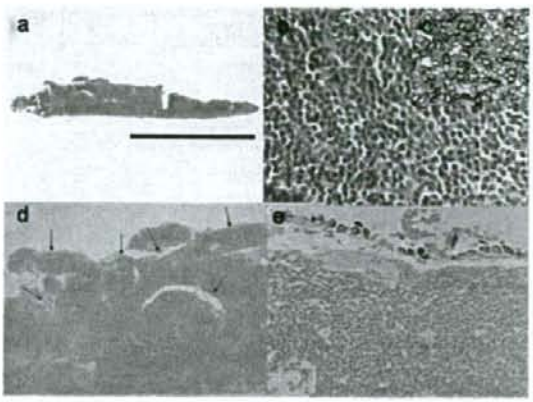

Figure 3 (**a**) Diffuse infiltrate of lymphoid cells through all layers of the pleura. Bar, 1 cm (**b**) Tumor cells composed of centrocyte-like and monocytoid lymphoid cells without apparent plasmacytic differentiation or follicular formation. (**c**) CD79a staining showing positivity for most tumor cells. (**d**) AE1/AE3 staining: thin layer of hyperplastic mesothelial cells (arrows). These cells covered the surfaces of pleural granular lesions. (**e**) AE1/AE3 staining did not show any proliferation of submesothelial fibroblast-like cells.

lymphoid follicles were completely absent histologically. These microscopic features also indicated another possibility of mature B-cell neoplasms including follicular lymphoma and mantle cell lymphoma. Most cases of follicular lymphoma are composed of small to medium-sized cells (centrocytes) and large transformed cells (centroblasts).[10] Large cell components were absent in the present case. Immunohistochemically, these tumor cells of follicular lymphoma are usually positive for CD10,[10] but this was negative in the present case. The neoplastic cells of mantle cell lymphoma are composed of small to medium-sized lymphoid cells most closely resembling centrocytes.[11] They are typically CD5 and cyclin D1 positive,[11] but in the present case these two markers were negative.

Until recently, MALT lymphoma primarily involving the pleura had not been documented, until Ahmad *et al.*[2] reported the first two cases of pleural MALT lymphoma; an additional two cases were reported thereafter.[1,3] Of four

cases, pathological diagnosis was made using a biopsy specimen obtained on VATS in two cases.[2] In the remaining two cases, decortication[1] or complete *en bloc* resection[3] was performed under thoracotomy.

In Japan, EMR for gastric mucosa has been accepted as a treatment option for EGC where the probability of lymph node metastasis is low.[4] The specimen obtained on EMR as a single specimen is available for accurate histopathological determination of the submucosal invasion, vessel involvement and marginal invasion. For EMR for a larger EGC, the IT-knife was developed.[4–6] The IT-knife can dissect the gastric submucosa safely, and completely remove a carcinoma as a large single-piece specimen without severe crushing. The use of the IT-knife during thoracoscopic examinations has not previously been reported anywhere. Using this procedure we first obtained a large, single-piece, full-thickness and only slightly crushed specimen of the pleura and were able to study its histopathological features in detail. Previously such thoracoscopic study has been impossible because thoracoscopic biopsy specimens were tiny or crushed by the biopsy forceps.

Beyond expectation, the thoracoscopic IT-knife allowed detailed histopathological findings of pleural MALT lymphoma to be obtained. New histopathological findings for pleural MALT lymphoma are as follows: (i) lack of apparent evidence of plasmacytic differentiation; (ii) no recognition of lymphoid follicles; (iii) mesothelial cells not infiltrated by lymphoma cell clusters; (iv) thin layer of hyperplastic mesothelial cells continuously covering the surfaces; and (v) no proliferation of fibroblast-like submesothelial cells. This new thoracoscopic IT-knife is very useful for the detailed pathological study of pleural lesions including MALT lymphoma.

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Vinorelbine plus gemcitabine followed by docetaxel versus carboplatin plus paclitaxel in patients with advanced non-small-cell lung cancer: a randomised, open-label, phase III study

Kaoru Kubota, Masaaki Kawahara, Mitsumasa Ogawara, Yutaka Nishiwaki, Kiyoshi Komuta, Koichi Minato, Yuka Fujita, Satoshi Teramukai, Masanori Fukushima, Kiyoyuki Furuse, on behalf of the Japan Multi-National Trial Organisation

Summary

Background Platinum-containing two-drug combinations improve survival and cancer-related symptoms in patients with advanced non-small-cell lung cancer (NSCLC). However, survival benefit is modest and platinum-containing regimens cause substantial toxic effects. We did a prospective randomised open-label phase III study to compare an experimental platinum-free, triplet, sequential regimen of vinorelbine plus gemcitabine followed by docetaxel with the standard platinum-containing, doublet regimen paclitaxel plus carboplatin in patients with advanced NSCLC.

Methods Between March, 2001, and April, 2005, patients with stage IIIB (positive pleural effusion) or IV NSCLC, performance status 0 to 1, and adequate organ function, were randomly assigned to experimental treatment or to standard treatment. Randomisation was done centrally by use of a dynamic balancing algorithm. Patients were stratified by weight loss, lactate dehydrogenase concentration, and disease stage. Patients in the experimental group were scheduled to receive intravenous vinorelbine (25 mg/m²) plus gemcitabine (1000 mg/m²) on days 1 and 8 every 21 days for three cycles, followed by intravenous docetaxel (60 mg/m²) on day 1 every 21 days for three cycles. Patients in the standard group were scheduled to receive intravenous paclitaxel (225 mg/m²) plus carboplatin (area under the curve=6) for 3 h on day 1, every 21 days for six cycles. The primary endpoint was overall survival, and secondary endpoints were progression-free survival, response, and toxic effects. Analyses were by intention to treat. This trial is registered with ClinicalTrials.gov, number NCT00079287.

Findings Of the 401 patients enrolled and randomised in the trial, five patients in the experimental group and three in the standard group were ineligible for analysis; thus 196 patients in the experimental group and 197 in the standard group were included in analyses. Patient characteristics were well-balanced between the two groups with regard to major prognostic factors. Median overall survival was 13·6 months (range 12·0–16·4) in the experimental group versus 14·1 months (11·9–17·5) in the standard group (p=0·97). 49 of 196 patients (25%) in the experimental group had a partial response compared with 73 of 197 patients (37%) in the standard group (p=0·012). There were no complete responses. Median progression-free survival was 5·5 months (95% CI 4·9–6·3) in the experimental group compared with 5·8 months (5·3–6·1) in the standard group (p=0·74). The incidence of grade 3 and 4 neutropenia, neuropathy, arthralgia, and myalgia was lower in the experimental group than in the standard group, although the incidence of pulmonary toxic effects was higher.

Interpretation Although platinum-containing regimens remain the standard treatment for advanced NSCLC, non-platinum regimens could provide equivalent efficacy with a different toxicity profile.

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See Reflection and Reaction page 1116

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Introduction

Lung cancer is the leading cause of cancer death worldwide and a growing concern in an ageing society.[1] Non-small-cell lung cancer (NSCLC) accounts for 85% of lung cancer histology. Several third-generation agents are available for the treatment of NSCLC, including docetaxel, paclitaxel, gemcitabine, and vinorelbine, and the combination of one of these agents with a platinum compound (ie, cisplatin or carboplatin) has been considered the standard treatment option for advanced NSCLC on the basis of several randomised studies.[2–4]

Combination chemotherapy containing cisplatin has substantial toxic effects, including vomiting and renal impairment, making treatment of elderly patients or outpatients with this agent difficult. Carboplatin has fewer toxic effects than cisplatin, although it still causes vomiting and myelosuppression. Non-platinum, two-drug combinations using third-generation agents have shown an equivalent outcome compared with platinum-containing regimens in patients with NSCLC.[5,6] In the newer non-platinum combinations, vinorelbine plus gemcitabine has shown activity and a good toxicity profile.[7,8] Vinorelbine plus gemcitabine has also shown

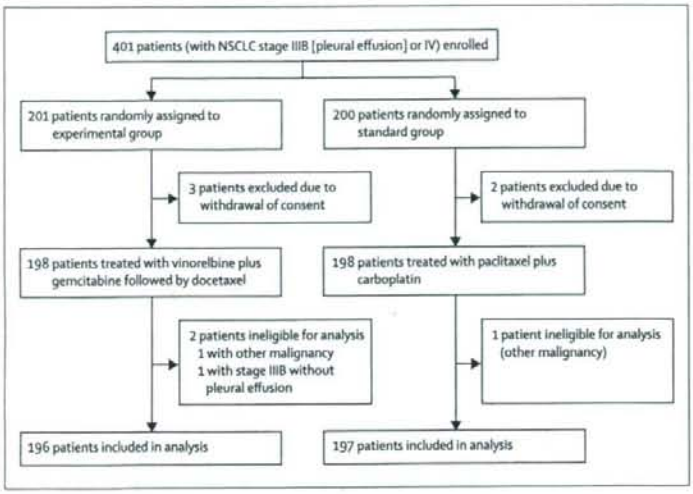

Figure 1: Trial profile

significantly better survival than vinorelbine plus carboplatin in a randomised trial.[9] Docetaxel is active against NSCLC and shows survival benefit in both chemotherapy naive patients, and patients previously treated with chemotherapy.[10,11] Docetaxel might be effective against subpopulations of lung-cancer cells (clones) resistant to first-line chemotherapy,[12] and some residual resistant clones might be eradicated by sequential administration of docetaxel before they grow and relapse.

We previously did a phase II trial of a sequential, non-platinum, triplet combination consisting of three cycles of vinorelbine (25 mg/m²) plus gemcitabine (1000 mg/m²) followed by three cycles of docetaxel (60 mg/m²). The resulting outcomes—21 of 44 patients [47·7%] had partial response, median overall survival was 15·7 months, and 1-year survival was 59%—were encouraging.[13] Therefore, we designed this phase III trial to identify whether vinorelbine plus gemcitabine followed by docetaxel offers better survival than the standard paclitaxel plus carboplatin regimen.

Methods

Patients

All patients enrolled in this study had histologically or cytologically confirmed NSCLC (categorised as squamous cell, large cell, adenocarcinoma, or NSCLC not otherwise specified), with stage IIIB (positive pleural effusion) or stage IV (no brain metastases) disease according to the International Staging System. Other eligibility criteria included: measurable or assessable disease; Eastern Cooperative Oncology Group performance status of 0 or 1; neutrophil count of at least $1·5\times10^9$ cells per L; platelet count above institutional lower limits of normal; haemoglobin concentration of a least 90 g/L; serum creatinine concentrations less than the institutional upper limit of normal (ULN) and a calculated or measured creatinine clearance of at least 50 mL/min; bilirubin, aspartate aminotransferase (AST) or alanine aminotransferase (ALT), and alkaline phosphatase concentrations of 2×ULN or less, or 4×ULN or less if the patient had liver metastases. Patients were excluded if they had grade 2 or higher peripheral neuropathy or previous chemotherapy or biological therapy. Stratification at the time of registration was by weight loss (<5% *vs* ≥5% from measurements taken 6 months before enrolment), disease stage (IIIB *vs* IV), and serum lactate dehydrogenase concentration (normal *vs* abnormal). All patients provided written informed consent. This protocol was approved by the institutional review boards of all participating institutions and of the data centre (Translational Research Informatics Centre, Kobe, Hyogo, Japan).

Treatment

Patients were randomly assigned to either the experimental regimen or the standard regimen (figure 1). Central randomisation to each group was applied by use of a dynamic balancing algorithm to obtain a good balance between groups in terms of the stratified factors. Randomisation was done centrally by members of the Japan Multi-National Trial Organisation (JMTO) data centre at the Translational Research Informatics Centre, Kobe, Hyogo, Japan. After obtaining written informed consent, patients were registered via fax, and, if eligibility was confirmed, patients were allocated to one of the treatment groups by computer. Neither patients nor physicians were blinded to allocated treatment. In the experimental group, patients were assigned intravenous vinorelbine (25 mg/m²) and gemcitabine (1000 mg/m²) on days 1 and 8 every 21 days for three cycles. Single-agent docetaxel (60 mg/m²) was subsequently given intravenously on day 1 every 21 days for a further three cycles. Premedications, such as antiemetic agents or corticosteroids, were given as needed. All patients were assigned 8 mg of dexamethasone orally before docetaxel administration. The standard regimen consisted of intravenous paclitaxel (225 mg/m²) plus carboplatin (area under the curve [AUC]=6) for 3 h on day 1. Treatment was repeated every 3 weeks for six cycles. Patients in the standard group were assigned premedication with dexamethasone, diphenhydramine, and ranitidine or cimetidine. Use of additional antiemetics was left to the physician's judgment. Erythropoietin-stimulating agents were not approved in Japan for chemotherapy-related anaemia, and were thus not used. G-CSF was permitted at any time during the study except for prophylactic use in both groups. In the absence of progressive disease or intolerable toxic effects, patients in both groups were treated for six cycles.

Complete blood-cell count was checked either on the treatment day or the day before planned treatment during

	Experimental group (N=196)	Standard group (N=197)
Median age (range), years	64 (39–81)	65 (33–81)
Sex, n (%)		
Men	143 (73)	136 (69)
Women	53 (27)	61 (31)
Smoking, n (%)		
Non-smokers	47 (24)	51 (26)
Former smokers	52 (27)	55 (28)
Smokers	88 (45)	82 (42)
Unknown	9 (5)	9 (5)
Histology, n (%)		
Squamous cell	46 (23)	30 (15)
Adenocarcinoma	130 (66)	149 (76)
Other	20 (10)	18 (9)
Stage, n (%)		
IIIB	33 (17)	35 (18)
IV	163 (83)	162 (83)
Performance status, n (%)		
0	79 (40)	78 (40)
1	117 (60)	119 (60)
Weight loss, n (%)		
<5%	160 (82)	161 (82)
≥5%	36 (18)	36 (18)
LDH concentration, n (%)		
Normal	141 (72)	142 (72)
Abnormal	55 (28)	55 (28)

Experimental treatment=vinorelbine and gemcitabine followed by docetaxel. Standard treatment=paclitaxel and carboplatin. LDH=lactate dehydrogenase.

Table 1: **Characteristics of assessable patients**

	Treatment		p value
	Experimental group (N=196)	Standard group (N=197)	
Tumour response, n (%)			
Complete	0 (0)	0 (0)	–
Partial	49 (25)	73 (37)	–
No change	90 (46)	76 (39)	–
Progressive disease	32 (16)	20 (10)	–
Non assessable	25 (13)	28 (14)	–
Overall response (95% CI), %	25 (19·1–31·7)	37·1 (30·3–44·2)	0·012
Progression-free survival (PFS)			
Median (95% CI), months	5·5 (4·9–6·3)	5·8 (5·3–6·1)	–
1-year PFS	15·4%	12·0%	–
2-year PFS	6·7%	5·8%	–
HR* (95% CI)	0·966 (0·79–1·19)	1†	0·742
Overall survival (OS)			
Median (95% CI), months	13·6 (12·0–16·4)	14·1 (11·9–17·5)	
1-year OS	57·1%	56·6%	
2-year OS	28·7%	30·1%	
HR* (95% CI)	0·966 (0·78–1·27)	1†	0·974

Experimental treatment=vinorelbine and gemcitabine followed by docetaxel. Standard treatment=paclitaxel and carboplatin. HR=hazard ratio. *Adjusted for disease stage, weight loss, and lactate dehydrogenase concentration. †Reference group.

Table 2: **Treatment outcomes**

each of the cycles. During the vinorelbine plus gemcitabine cycles, serum AST and ALT were assessed. If neutrophil count was less than $1{\cdot}5\times10^9$ cells per L, platelet count less than 100×10^9/L, or AST or ALT more than 100 IU/L on day 1 of each cycle, vinorelbine plus gemcitabine administration was delayed by a week. If neutrophil count was less than $1{\cdot}0\times10^9$ cells per L, platelet count less than 70×10^9/L, or AST or ALT more than 100 IU/L, vinorelbine plus gemcitabine was not given on day 8. Docetaxel administration was delayed by a week when the neutrophil count was less than $1{\cdot}5\times10^9$ cells per L or platelet count was less than 75×10^9/L on day 1 of each cycle. Treatment dose was decreased to 80% if grade 4 leucocytopenia or neutropenia were present, if platelet count was less than 20×10^9/L, or if other unacceptable toxic effects, including grade 3 neutropenic fever or grade 3 or higher non-haematological toxic effects, were present during the preceding treatment cycle. The first cycle of docetaxel was given at full dose even if toxic effects were noted in the previous vinorelbine plus gemcitabine cycles. The dose of docetaxel was decreased to 80% only when toxic effects were noted subsequent to docetaxel administration.

Dose modifications for paclitaxel and carboplatin were consistent with the Southwest Oncology Group Trial (SWOG) S0003.[14] In brief, if the neutrophil nadir was less than $0{\cdot}5\times10^9$ cells per L, the platelet nadir less than 50×10^9/L, or the patient had febrile neutropenia, the dose of carboplatin was decreased to an AUC of 5. If a patient developed grade 2 neurotoxicity at any time during a cycle, the dose of paclitaxel was decreased to 200 mg/m^2. Chest pain or arrhythmia during infusion resulted in immediate discontinuation and patient assessment. Patients with symptomatic arrhythmias, atrioventricular block (except first degree), or a documented ischaemic event discontinued the study.

Pretreatment and follow-up assessments

Baseline assessment and staging consisted of a physical examination; chest radiography; brain, chest, and abdominal CT or MRI; complete blood-cell count and serum chemistry; a bone scan if clinically indicated; and an electrocardiogram. A physical examination and complete blood work-up were done before each cycle. Scans or radiographs used to assess response were obtained every two cycles. Once treatment was finished, a follow-up assessment was done every 3 months.

Response and toxicity criteria

Patients were assessed every two cycles for an objective response, according to the Response Evaluation Criteria in Solid Tumors.[15] Confirmed responses required repeat measurements at a minimum of 4 weeks. Responses

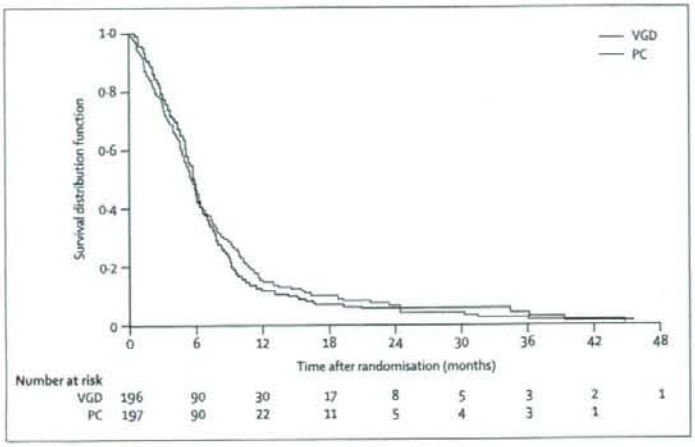

Figure 2: **Kaplan-Meier estimates of progression-free survival**
VGD=vinorelbine and gemcitabine followed by docetaxel. PC=paclitaxel and carboplatin.

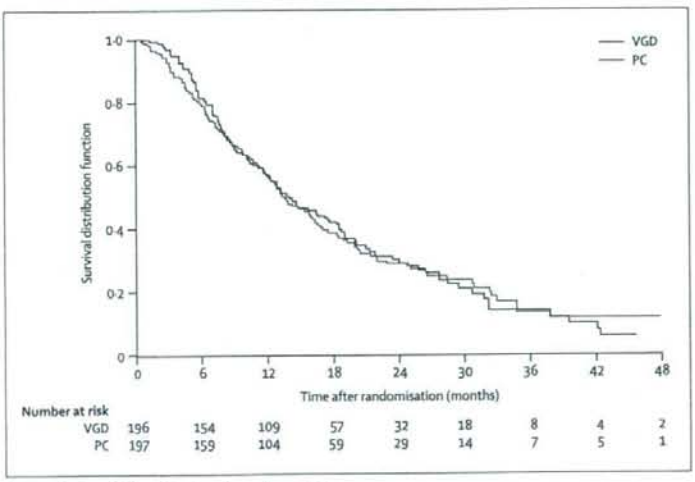

Figure 3: **Kaplan-Meier estimates of overall survival**
VGD=vinorelbine and gemcitabine followed by docetaxel. PC=paclitaxel and carboplatin.

were assessed by attending physicians, and a central review was not done for response evaluation. Grading of toxic effects was done in accordance with the US National Cancer Institute Common Toxicity Criteria, version 2.0.[16] Patients were removed from the study as a result of progression of disease, toxic effects, or at the patient's request. Appropriate procedures were undertaken for all unexpected or fatal toxic effects.

Statistical analyses

The primary objective of this study was to determine whether the experimental regimen (vinorelbine and gemcitabine followed by docetaxel) produced a survival advantage compared with the standard regimen (paclitaxel plus carboplatin) in patients with advanced NSCLC. The primary endpoint was overall survival. Secondary endpoints were progression-free survival, response, and toxic effects. Analyses were done by intention to treat.

We calculated our sample size based on an anticipated median overall survival of 8·0 months in the standard group[17] and an expected 40% increase in median overall survival (to 11·2 months) in the experimental group (chosen on the basis of the findings of our previous phase II study of the experimental regimen, which showed a median survival of 15·7 months[11]). This difference in median overall survival is equivalent to a 2-year survival of 12·5% in the standard group compared with 22·6% in the experimental group (HR 0·714). On the basis of these assumptions, we calculated that we would need 200 patients per group to detect such a difference, with a power of 0·85 using a two-sided Log-rank test at a significance level of 0·05. Survival curves were estimated by the product-limit method and compared by use of the Log-rank test, stratified by predetermined prognostic factors.[18,19] Cox regression analysis was used to estimate hazard ratios (HRs) for overall survival and progression-free survival.[20] Fisher's exact test was used to test the difference between treatment groups for response and toxic effects. Unless otherwise indicated, all reported p values are two sided. A planned interim analysis was done by the data monitoring committee when 300 patients had been enrolled, with early study termination to occur if the null hypothesis of no difference for the experimental group was rejected at the one-sided 0·0025 level.

Role of the funding source

This trial was sponsored by the JMTO, and members of JMTO were responsible for the design, set-up, and data collection of the trial. All authors had full access to the raw data in the study and the corresponding author had final responsibility for the decision to submit for publication.

Results

401 patients were enrolled in the trial between March 29, 2001, and April 13, 2005, from 45 institutions in Japan. Eight patients (2·0%) were ineligible for analysis: five withdrew informed consent, two had other malignancies, and one had stage IIIB disease without pleural effusion. Thus, 393 patients were eligible for analysis, 196 in the experimental group and 197 in the standard group. Patient characteristics are listed in table 1. Although the proportion of patients with adenocarcinoma histology was higher in the standard group than in the experimental group, there was no significant difference between the two groups.

Overall response (ie, best confirmed response during study treatment) was 25% (49 of 196 patients with PR) in the experimental group and 37% (73 of 197 patients with PR) in the standard group (p=0·012; table 2). No patients

had a complete response. 17 of 196 patients (8·7%) had a partial response after three cycles of treatment with vinorelbine plus gemcitabine, as reported by the attending physicians. The difference in response between the two groups was larger in patients with squamous-cell histology (15% [seven of 46 patients] in the experimental group *vs* 63% [19 of 30 patients] in the standard group; p<0·0001) than in patients with adenocarcinoma histology (26% [34 of 130 patients] in the experimental group *vs* 32% [47 of 149 patients] in the standard group; p=0·356), and the proportion of patients with squamous-cell histology who had progressive disease was higher in the experimental group (33% [15 of 46]) than in the standard group (13% [four of 30]). The comparison of response by the Mantel-Haenszel test (adjusted for the distribution imbalance of histology) was also significant (p=0·007). Median progression-free survival was 5·5 months (95% CI 4·9–6·3) in the experimental group and 5·8 months (5·3–6·1) in the standard group (p=0·742; figure 2 and table 2), and median overall survival was similar between groups, at 13·6 months (12·0–16·4) in the experimental group and 14·1 months (11·9–17·5) in the standard group (p=0·97; figure 3 and table 2). For overall survival, the HR was 1·06 (95% CI 0·80–1·41; p=0·688) in patients with adenocarcinoma histology and 0·94 (0·56–1·57; p=0·802) in patients with squamous-cell histology. For progression-free survival, the corresponding values were 0·98 (0·77–1·25; p=0·848) and 1·04 (0·65–1·68; p=0·861), respectively. Thus, there was no interaction between treatment and histology ($p_{interaction}$=0·794 and 0·773, respectively). In terms of other factors (ie, age, sex, smoking history, Eastern Cooperative Onology Group performance status, weight loss, disease stage, and lactate dehydrogenase concentration), there were no significant interactions between treatment and factor (data not shown).

196 patients in the experimental group and 197 patients in the standard group were assessable for toxic effects (table 3). The standard regimen resulted in a significantly increased incidence of grade 3 or 4 neutropenia, neuropathy, arthralgia, and myalgia compared with the experimental regimen. However, the incidence of pulmonary toxic effects was significantly higher in the experimental group than in the standard group. Only one patient assigned the standard regimen developed grade 1 to 4 drug-related pneumonitis compared with 17 patients assigned the experimental regimen (p<0·0001). Of these 17 patients, 14 developed pneumonitis during vinorelbine plus gemcitabine treatment, whereas the remaining three patients had pneumonitis during docetaxel treatment. Almost all patients improved with corticosteroids. There was no significant difference in neutropenic fever, anaemia, and thrombocytopenia between the two groups. Treatment-related death occurred in two patients. One patient had pneumonitis after the fourth cycle of the experimental regimen. Despite improvement of pneumonitis with corticosteroids, steroid-induced exacerbation of hepatitis C, followed by deterioration of pneumonitis, resulted in death due to respiratory failure. Another patient died of pneumonia after the fourth cycle of the experimental regimen.

	Treatment		p value
	Experimental group (N=196), n (%)	Standard group (N=197), n (%)	
Haematological toxic effects			
Leucopenia	79 (40·3)	89 (45·2)	0·359
Neutropenia	116 (59·2)	137 (69·5)	0·035
Neutropenic fever	23 (11·7)	24 (12·2)	1·000
Thrombocytopenia	6 (3·1)	14 (7·1)	0·106
Anaemia	9 (4·6)	16 (8·1)	0·214
Non-haematological toxic effects			
Allergic reaction	0 (0)	4 (2·0)	0·123
Fatigue	10 (5·1)	14 (7·1)	0·528
Constipation	3 (1·5)	7 (3·6)	0·337
Nausea	8 (4·1)	17 (8·6)	0·097
Vomiting	2 (1·0)	6 (3·0)	0·284
Anorexia	16 (8·2)	22 (11·2)	0·394
Neuropathy (motor)	1 (0·5)	8 (4·1)	0·037
Neuropathy (sensory)	1 (0·5)	19 (9·6)	<0·0001
Arthralgia	0 (0)	17 (8·6)	<0·0001
Myalgia	0 (0)	14 (7·1)	<0·0001
Dyspnoea	11 (5·6)	3 (1·5)	0·032
Drug-related pneumonitis	9 (4·6)	1 (0·5)	0·011
Pneumonia	14 (7·1)	1 (0·5)	0·0004
Liver dysfunction	6 (3·1)	5 (2·5)	0·771

Experimental treatment=vinorelbine and gemcitabine followed by docetaxel. Standard treatment=paclitaxel and carboplatin.

Table 3: **Grade 3 and 4 toxic effects occurring in ≥3% of patients in at least one group**

The median number of cycles delivered was six (range one to six) for the experimental regimen and four (one to six) for the standard regimen. There was no difference in the number of patients receiving four or more cycles between the groups. The proportion of patients receiving six cycles was significantly higher in the experimental group (97 of 196 [49%]), than in the standard group (57 of 197 [29%]; p<0·0001). The proportion of patients who needed a dose reduction was 29% (57 or 196) in the experimental group and 51% (100 of 197) in the standard group (p<0·0001).

128 of 196 patients (65%) in the experimental group and 133 of 197 patients (68%) in the standard group received post-protocol chemotherapy of any type. In the experimental group, 44 of 196 patients (22%) received paclitaxel plus carboplatin, 35 (18%) received gefitinib, 17 (9%) received additional docetaxel, nine (5%) received vinorelbine plus gemcitabine, and 23 (12%) received other regimens. In the standard group, 40 of 197 patients (20%) received gefitinib, 38 (19%) received docetaxel, 23 (12%) received vinorelbine plus gemcitabine, six (3%) received additional paclitaxel plus carboplatin, and 26 (13%)

received other regimens as second-line chemotherapy. In both groups, around 35% of patients received more than one additional line of chemotherapy. Gefitinib was used as any line treatment after the study protocol in 75 of 196 (38%) patients in the experimental group and in 78 of 197 (40%) patients in the standard group, respectively.

Discussion

This study assessed whether a non-platinum, sequential, triplet (vinorelbine and gemcitabine followed by docetaxel) regimen[13,21] produced a survival advantagecompared with a standard platinum-containing regimen in patients with advanced NSCLC. The experimental regimen did not result in better overall survival than the standard regimen of paclitaxel plus carboplatin.

Although some baseline imbalances existed in terms of histology between the two groups, histology (adenocarcinoma *vs* others) was not an independent prognostic factor for overall survival (adjusted HR 0·96 [95% CI 0·73–1·27]; p=0·80) and the effect of imbalance on the endpoints was small. The proportion of patients receiving six cycles of treatment was higher in the experimental group than in the standard group; however, a median number of four cycles (range one to six) with standard treatment is the usual standard of care, and therefore, it is unlikely that the difference in number of cycles affected the outcomes of the study.

Although there was no difference between the regimens in terms of efficacy, the experimental, non-platinum regimen did show some benefits compared with the platinum-containing regimen. The regimen was well tolerated and 97 of 196 (49%) patients completed the planned six cycles. Furthermore, the incidence of grade 4 neutropenia, grade 3 or 4 neuropathy, arthralgia, and myalgia was significantly higher in the standard group; however, the incidence of pulmonary toxicity was higher in the experimental group. Of the 17 (8·7%) cases of grade 1 to 4 drug-related pneumonitis in the expermental group, 14 (82%) occurred during treatment with vinorelbine plus gemcitabine. In another Japanese study using vinorelbine plus gemcitabine, grade 3 or higher drug-related pneumonitis occurred in two of 62 patients (3%), resulting in one death.[22] A few cases of drug-related pneumonitis have also been reported when vinorelbine or gemcitabine is combined with cisplatin.[5] Interstitial lung disease due to inhibitors of epidermal growth factor receptors (EGFR) is also problematic in Japan,[23,24] either because of ethnic differences in drug-related pneumonitis or greater vigilance in diagnosing pneumonitis in Japan. Further studies are thus crucial.

Because docetaxel is active as second-line chemotherapy, sequential administration of docetaxel after other chemotherapy regimens might be effective for clones resistant to previous chemotherapy.[12] Edelman and colleagues[25] did a randomised phase II trial of carboplatin plus gemcitabine followed by paclitaxel, or cisplatin plus vinorelbine followed by docetaxel. Both regimens resulted in survival data comparable to platinum-based two-drug combinations and few patients showed an improvement in response with sequential taxane therapy.[25] In the present study, of the patients who achieved partial response in the experimental group, about a third (17 of 49) had their best response during treatment with vinorelbine plus gemcitabine, whereas nearly two-thirds (32 of 49) achieved partial response with docetaxel monotherapy. Although we should be careful when interpreting these data, because central review for response assessment was not done and the protocol specified response assessment every two cycles, which might have been too early to detect the real response to treatment with vinorelbine plus gemcitabine, data from this study suggest that alternative sequential therapy could be effective for NSCLC if highly active regimens are selected and administered in the optimum sequence. Preliminary findings from a randomised phase III study comparing immediate with delayed second-line chemotherapy in patients with stage IIIB or IV NSCLC suggest that median overall survival might be improved by giving docetaxel immediately after completion of a full course of first-line treatment (median overall survival 11·9 months for immediate docetaxel and 9·1 months for delayed docetaxel; p=0·071).[26] Pemetrexed has comparable efficacy to docetaxel as second-line chemotherapy.[27] Pemetrexed plus cisplatin showed similar overall survival to gemcitabine plus cisplatin in chemotherapy-naive patients with advanced NSCLC, and overall survival was better with pemetrexed plus cisplatin than with gemcitabine plus cisplatin in adenocarcinoma and large-cell carcinoma histology.[28] By contrast, a significant improvement in overall survival was shown with gemcitabine plus cisplatin in patients with squamous-cell histology.[28] Maintenance chemotherapy with pemetrexed after four cycles of platinum-based chemotherapy improved overall survival compared with supportive care in patients with non-squamous NSCLC, with a median survival of 14·4 months for pemetrexed and 9·4 months for the placebo group.[29] Furthermore, a subgroup analysis of a randomised trial of maintenance gefitinib after chemotherapy versus chemotherapy alone showed a significantly better overall survival favouring gefitinib in patients with adenocarcinoma.[30] Although the present study did not show better survival with the experimental regimen than with the standard regimen of carboplatin plus paclitaxel, further study of sequential chemotherapy in selected patients with stage IIIB or IV NSCLC is warranted.

The present study was done as a JMTO-SWOG common-arm trial with identical eligibility, staging, response, and toxicity criteria to SWOG S0003.[14] Dose, schedule, and dose modifications for paclitaxel and carboplatin were consistent with SWOG S0003. Patient baseline characteristics were similar in the two studies. Overall survival in patients