

tively early event in the genesis of bronchial squamous cell carcinoma, and that increasing histological atypia is accompanied by further diminution in the expression of these molecules. Finally, reduced levels of E-cadherin and/or catenins might play a critical role in local invasion beyond the basement membrane and the development of the advanced stage of squamous cell lung carcinoma.

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Phase II trial of postoperative adjuvant cisplatin and etoposide in patients with completely resected stage I-IIIa small cell lung cancer: The Japan Clinical Oncology Lung Cancer Study Group Trial (JCOG9101)

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Objective: Indications for surgical intervention for very limited small cell lung cancer have not yet been determined. The objective of this study is to determine whether resection followed by cisplatin and etoposide is feasible.

Methods: From September 1991 through December 1996, 62 patients with completely resected small cell lung cancer who were less than 76 years of age from 17 centers were entered in the trial. Of 62 patients, 61 were eligible, with a median follow-up of 65 months. Chemotherapy consisted of 4 cycles of cisplatin (100 mg/m², day 1) and etoposide (100 mg/m², days 1-3). There were 49 (80%) male patients, 44 with clinical stage I disease, 10 with stage II disease, and 6 with stage IIIa disease.

Results: Forty-two (69%) patients received 4 cycles of cisplatin and etoposide. No treatment-associated mortality was noted. Median survival time was not reached in patients with pathologic stage I disease, was 449 days in patients with stage II disease, and was 712 days in patients with stage IIIa disease. Three-year survival was 61% overall, 68% in patients with clinical stage I disease, 56% in patients with stage II disease, and 13% in patients with stage IIIa disease ($P = .02$). Recurrence was noted in 26 (43%) patients overall. Local failure was noted in 6 (10%) patients. Locoregional recurrence tends to be found more frequently in patients with stage IIIa disease. Distant failure was found in 21 (34%) patients overall. Brain metastasis was found in 15% of the patients.

Conclusion: Major lung resection followed by postoperative cisplatin and etoposide is feasible, with a favorable survival profile. Because nodal metastasis appears to be a major prognostic factor, preoperative evaluation of nodal status remains a major concern.

The prognosis of lung cancer remains poor, and this disease is the leading cause of cancer mortality worldwide. Small cell lung cancer (SCLC) comprises approximately 20% of lung cancer cases. Without treatment, SCLC has the most aggressive clinical course of any other type of lung cancer, resulting in a very short median survival time of approximately 2 to 4 months. Although surgical resection is generally indicated for early stage non-small cell lung cancer, this is not always the case with SCLC. This can be explained by the fact that

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dissemination to regional lymph nodes or distant organs would be found in most patients with SCLC at the time of initial presentation.¹ Therefore, localized forms of treatment, such as surgical resection or radiation therapy, rarely produce long-term survival, and systemic treatment with current chemotherapy regimens is usually incorporated into the treatment program.

Indications for surgical resection for SCLC have not yet been determined, although several authors have reported that a small minority of patients with limited-stage disease and adequate lung function might benefit from surgical resection.¹⁻⁹ According to these reports, the prognosis of resected SCLC was not so poor, especially when no pathologic nodal involvement was observed. The 5-year survival ranged from 26% to 61% in these trials if the tumor was stage I. Because SCLC tends to be disseminated and the results of surgical intervention alone for this disease have been reported to be poor,^{1,10} postoperative chemotherapy has been used in most studies. However, the chemotherapy was not standardized, and various chemotherapy protocols were often used. Furthermore, most previous studies were retrospective and thus suffered from the inherent weakness of any retrospective assessment of a given treatment.

Because the combination of cisplatin and etoposide has been considered to be standard in the treatment of SCLC,¹¹ this combination was selected as a postoperative adjuvant regimen. We conducted a prospective study of surgical resection plus adjuvant chemotherapy for stage I through IIIA SCLC to investigate the efficacy of this treatment strategy.

Patients and Methods

Eligibility

Patients who were given postoperative diagnoses of SCLC histologically or cytologically were eligible for enrollment in the study. The patients had to have completely resected pathologic stage I, II, or IIIA disease according to the TNM classification of the International Union Against Cancer.¹² Histologic typing was determined according to the World Health Organization classification.¹³ Inclusion criteria included an Eastern Cooperative Oncology Group performance score of 0 or 1, age between 20 and 75 years, no prior treatment for lung cancer, no other concurrent or previous malignancies, a leukocyte count of greater than 3500/ μ L, a platelet count of greater than 100,000/ μ L, a hemoglobin level of greater than 9.5 g/dL, a serum creatinine level of less than 1.5 mg/dL, and aspartate aminotransferase–alanine aminotransferase values of less than twice the institutional upper limit of normal. Exclusion criteria included a history of myocardial infarction within the past 3 months, hepatic cirrhosis, and/or severe cardiopulmonary dysfunction that required oxygen therapy. The following preoperative investigations were performed before entry into the study: computed tomographic (CT) scanning of the chest, upper abdomen, and brain; bronchoscopy; chest plain film; radionuclide bone scanning; complete blood cell count and serum chemistry; and physical examination. Preoperative mediastinoscopy was performed in

some cases. All patients provided written informed consent before entering the study.

Treatment Schedule

Major lung resection, such as pulmonary lobectomy or pneumonectomy, was required as a surgical procedure for SCLC. Complete hilar and mediastinal lymph node dissections were recommended on the basis of the lymph node map defined by Naruke and colleagues.¹⁴ After confirming complete resection and histologic typing of SCLC histologically, eligible patients were registered in the study.

Chemotherapy consisted of cisplatin (100 mg/m² on day 1) and etoposide (100 mg/m² on days 1-3; PE regimen). This regimen was repeated every 4 weeks and was administered in 4 courses. The dose was modified according to the blood cell count and renal function on the day of chemotherapy. Chemotherapy was administered unless the leukocyte count was less than 3000/ μ L or the platelet count was less than 75,000/ μ L. Chemotherapy was withheld until the counts recovered. If grade 4 hematologic toxicity, according to World Health Organization (WHO) criteria,¹⁵ was seen, the dose of etoposide was reduced to 75%. Chemotherapy was permanently discontinued at any time when the serum creatinine level was 2.0 mg/dL or greater or the blood urea nitrogen level was 30 mg/dL or greater. To assess toxicity, we subjected all patients to complete blood cell counts and blood chemistry evaluations, such as for aspartate aminotransferase–alanine aminotransferase, blood urea nitrogen, and serum creatinine, as well as chest plain film and urinalyses at least once per week during treatment. Toxicity criteria were evaluated on the basis of the WHO criteria.¹⁵

Patients were followed up at the outpatient department every 3 months postoperatively and underwent CT scans of the chest, upper abdomen, and brain, as well as radionuclide bone scanning every 6 months, even when they were asymptomatic. No postoperative radiotherapy was applied until relapse was apparent.

Sites of relapse were determined by clinical, radiologic, or histologic criteria at initial recurrence. Local failure was defined as recurrence at the primary lung site or hilar–mediastinal lymph nodes. Distant failure was defined as recurrence in the contralateral lung, bone, brain, liver, or other extrathoracic regions.

Statistical Analysis

The trial was designed as a prospective phase II trial. The primary goal of the study was to estimate the survival. A sample size of 30 was considered to provide a power of 90% for detecting a significant improvement in the 3-year survival (from 20% to 50%) in a 1-sided test with an α value of .025 and a β value of .10. The median follow-up period for 35 surviving patients was 65 months. The length of survival was defined as the interval in months between the day of surgical resection of lung cancer and the date of death from any cause or the last follow-up. The survival curves were constructed by using the Kaplan-Meier method,¹⁶ and curves were compared with the log-rank test.

Results

Patient Characteristics

Between September 1991 and December 1996, 62 patients were entered in this phase II trial at the 16 institutions that

TABLE 1. Patient characteristics

Total	61
Sex	
Male	49
Female	12
Age (y)	
Range	22-74
Median	64
Histologic subtype defined by WHO*	
Oat cell type	9
Intermediate type	45
Combined type	7
Clinical stage	
I	44
II	9
IIIA	8
Side of primary tumor	
Right	32
Left	29
Operative procedure	
Lobectomy	57
Pneumonectomy	4
Extent of lymph node dissection†	
Complete hilar and mediastinum	59
Only hilar	2
Pathologic stage	
I	35
II	8
IIIA	18
Performance status	
0	32
1	29

*Histologic subtyping was determined on the basis of the World Health Organization (WHO) classification. †The extent of lymph node dissection was defined by Naruke and associates.¹⁴

participated in the study. One patient was excluded because his final histologic category was changed from SCLC to large cell carcinoma. Thus, 61 patients were eligible for assessment of survival data, and their characteristics are shown in Table 1. The median age was 64 years (range, 22-74 years). According to histologic typing defined by the WHO, oat cell, intermediate, and combined types were found in 9, 45, and 7 patients, respectively. Forty-four patients had clinical stage I disease, 9 had stage II disease, and 8 had stage IIIA disease. Pathologically, stage I, II, and IIIA disease was found in 35, 8, and 18 patients, respectively.

Treatment Administration

As a surgical procedure, pulmonary lobectomy was performed in 57 (93%) patients, and pneumonectomy was performed in the other 4 patients. Among 4 pneumonectomies, 3 were on the left side, and 1 was on the right side. Complete hilar and mediastinal lymph node dissection was performed in 59 (97%) patients.

TABLE 2. Treatment delivery

Total no. of patients	61
No. of chemotherapy courses	
0	1 (2%)
1	5 (8%)
2	8 (13%)
3	5 (8%)
4	42 (69%)

A total of 204 courses were administered (Table 2). Forty-two (69%) patients underwent a full course of chemotherapy. The other 19 patients did not complete postoperative chemotherapy because of progressive disease in 3 patients, adverse effects in 7 patients, refusal of chemotherapy in 8 patients, and death from pneumonia in 1 patient.

Treatment-Related Toxicity

No treatment-associated deaths were found. Postoperative bronchopulmonary fistula was found in 1 (2%) patient who underwent pulmonary lobectomy after completion of the first cycle of chemotherapy. Chemotherapy-related toxicity is shown in Table 3. Grade 4 toxicity was found in 9 (15%) patients: leukopenia in 4 patients, thrombocytopenia in 2 patients, nausea in 2 patients, and cardiac failure in 1 patient. One patient died of pneumonia 2 months after the first course of chemotherapy, but this was not considered to be chemotherapy related.

Survival

Survival data are shown in Table 4. Among the 61 eligible patients, 35 were still alive after a median follow-up of 65

TABLE 3. Chemotherapy-related toxicity in 60 eligible patients treated for resected stage I to IIIA SCLC

Toxicity	WHO grade				4 (%)
	1	2	3	4	
Anemia	9	29	16	0	0
Leukocytopenia	7	17	26	4	6.5
Thrombocytopenia	11	8	14	2	3.2
Infection	2	1	0	0	0
Nausea	24	13	13	2	3.3
Diarrhea	8	2	2	0	0
Azotemia	35	0	0	0	0
Renal failure	18	0	0	0	0
Stomatitis	14	1	1	0	0
Dyspnea	5	0	0	0	0
Fever	10	7	0	0	0
Skin	4	2	0	0	0
Alopecia	13	23	11	0	0
Cardiac dysfunction	5	2	1	1	1.7
CNS	1	1	1	0	0
Peripheral neuropathy	5	1	0	0	0

WHO, World Health Organization; CNS, central nervous system.

TABLE 4. Survival in patients with resected SCLC who underwent postoperative chemotherapy

	Median survival time (d)	Survival	
		3 y	5 y
Clinical stage			
IA	Not reached	70%	66%
IB	Not reached	65%	65%
II	Not reached	56%	56%
IIIA	530	13%	13%
Pathologic stage			
IA	Not reached	78%	73%
IB	Not reached	67%	67%
II	449	38%	38%
IIIA	712	39%	39%

months. The overall estimated 3- and 5-year survivals were 61% and 57%, respectively (Figure 1). The 5-year survival was 66%, 56%, and 13% in patients with clinical stage I, II, and IIIA disease, respectively (Figure 2). Among the 44 patients with clinical stage I disease, 27 were classified as having clinical stage IA disease, and the other 17 were classified as having clinical stage IB disease. There was no significant difference in prognosis between clinical stage IA and IB disease. Similar results were obtained regarding the pathologic stage. Pathologic stage I disease showed a significantly better prognosis (Figure 3). The 5-year survivals in the 23 patients with pathologic stage IA disease and the 12 patients with stage

IB disease were 73% and 67%, respectively. No significant differences in survival were observed between patients with pathologic stage IA and IB disease.

Patterns of failure. Recurrence was noted in 26 (43%) patients, and the sites of initial relapse at a median follow-up time of 65 months are shown according to the pathologic stage in Table 5. Recurrence was found in 30% of patients with stage IA disease, 25% of patients with stage IB disease, 50% of patients with stage II disease, and 67% of patients with stage IIIA disease.

Local failure was noted in 6 (10%) patients: 4 in the mediastinal lymph nodes and 2 in the bronchial stump. Locoregional recurrence tended to be found more frequently in patients with stage IIIA disease (22%) than in patients with stage I or II disease. Relapse at the bronchial stump was only seen in patients with stage IIIA disease.

Distant failure was found in 22 (36%) patients overall: 6 (26%) with stage IA disease, 2 (17%) with stage IB disease, 4 (50%) with stage II disease, and 9 (50%) with stage IIIA disease. Distant failure was most frequently noted in the brain, followed by the liver. The incidence of brain metastasis was 15% overall, 17% in patients with stage IA disease, and 11% in patients with stage IIIA disease. Bone metastasis was noted exclusively in patients with stage IIIA disease.

Discrepancy between clinical and pathologic stages. Table 6 shows the relationship between the clinical stage and the pathologic stage. Among 44 patients with clinical stage I disease, only 33 (75%) had pathologic stage I disease, and

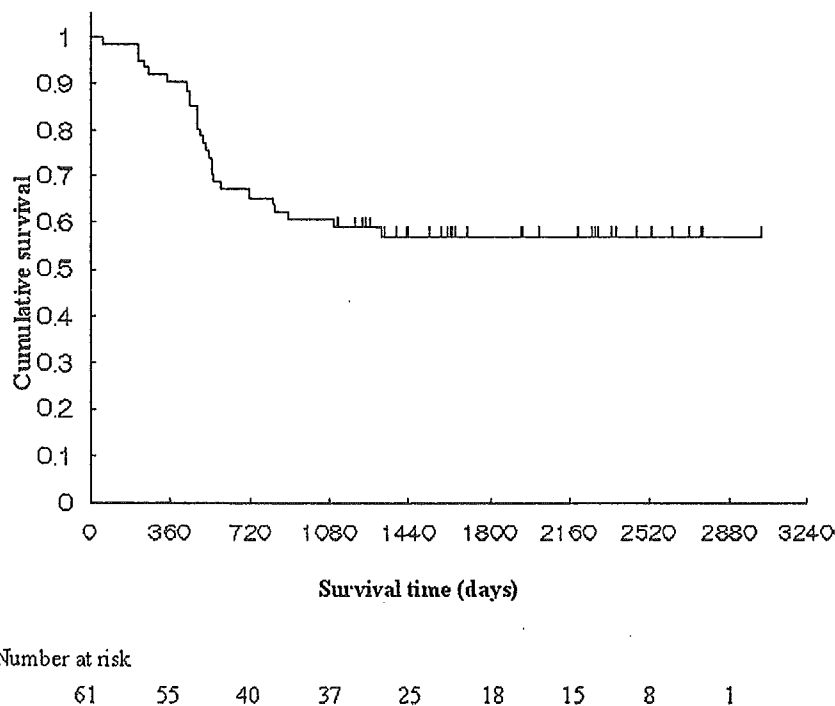


Figure 1. Survival curve for overall patients with resected SCLC.

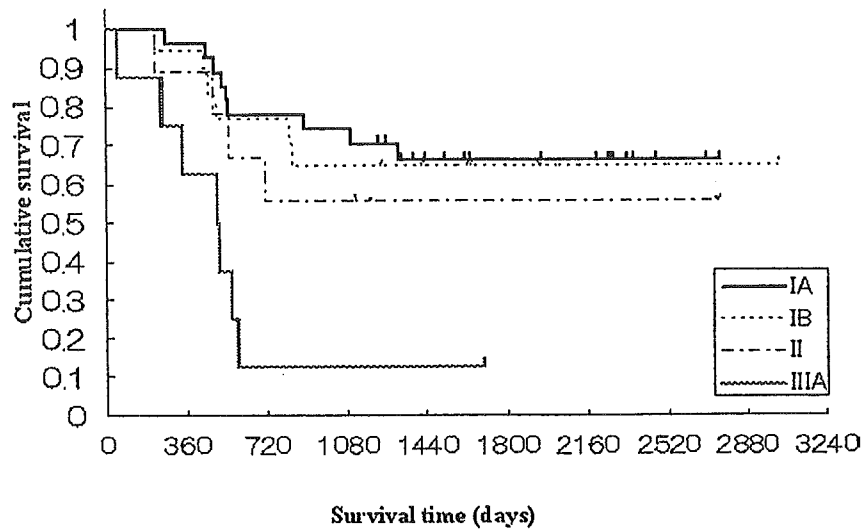


Figure 2. Survival curves for patients with resected SCLC by clinical stages.

6 had stage IIIA disease. Five patients with clinical stage IA disease had mediastinal lymph node metastasis. According to the Bowker test of symmetry, these differences were statistically significant.

Discussion

This phase II trial showed that postoperative PE for patients who underwent surgical resection of stage I to IIIA SCLC was feasible, and the outcome was acceptable. Survival was excellent in patients with stage I disease and did not appear

to be inferior to that with chemoradiotherapy in patients with stage II or IIIa disease.

On the basis of the results of the British Medical Research Council, radical radiotherapy has been preferable to surgical resection for SCLC,^{17,18} and the indications for surgical resection for SCLC are still controversial. An operation would be indicated for limited SCLC because the most common relapse site after radiotherapy was locoregional, and surgical intervention might improve local control.¹⁹ Several authors have reported that a small minority of

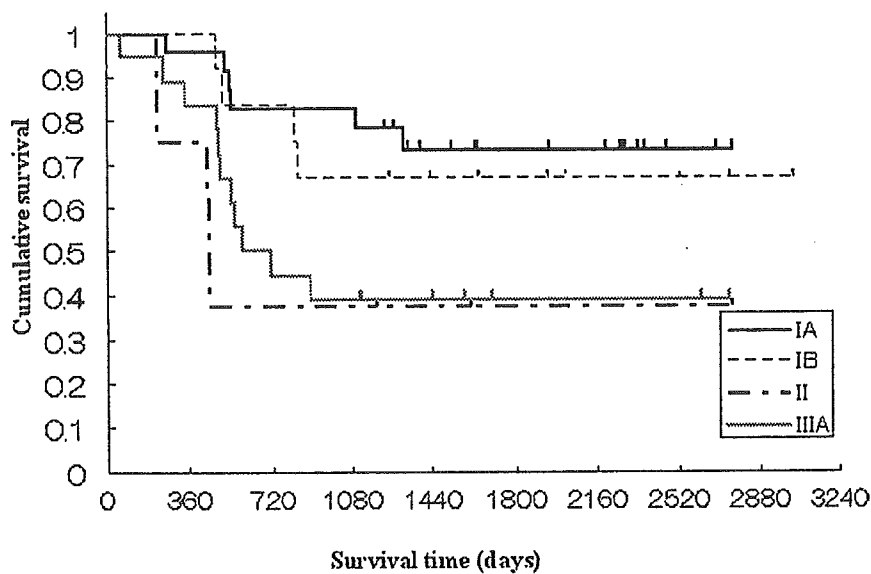


Figure 3. Survival curves for patients with resected SCLC by pathologic stage.

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TABLE 5. Site of the first relapse by pathologic stages*

Variables	Overall	Stage IA	Stage IB	Stage II	Stage IIIA
No. of patients	61	23	12	8	18
No. of recurrence	26 (43%)	7 (30%)	3 (25%)	4 (50%)	12 (67%)
Recurrence					
Local					
Overall	6 (10%)	1 (4%)	1 (8%)	0 (0%)	4 (22%)
Mediastinum	4	1	1	0	2
Bronchial stump	2	0	0	0	2
Distant					
Overall	22 (36%)	6 (26%)	2 (17%)	4 (50%)	9 (50%)
Brain	9 (15%)	4 (17%)	0 (0%)	3 (38%)	2 (11%)
Bone	3	0	0	0	3
Liver	7	1	1	1	4
Lung	2	0	1	0	1
Small intestine	2	1	0	0	1

limited-stage SCLCs could be managed with an operation and postoperative chemotherapy.¹⁻⁹ According to those reports, the 5-year survivals were 28% to 36% overall and 26% to 61% in patients with stage I disease. However, most of those reports were retrospective and used various combinations of chemotherapy. Therefore, a prospective trial of adjuvant chemotherapy for patients with resected SCLC using standardized chemotherapy has been needed. Our survival data suggest that postoperative PE after major lung resection and hilar and mediastinal lymph node dissection is a feasible and promising treatment, especially for patients with stage I SCLC. The 3- and 5-year survivals for patients with stage I disease were 78% and 73%, respectively, and the median survival time was not reached. As for patients with stage II or IIIA disease, the results were not definitive, and a further prospective study is needed. This study dealt with postoperatively proved SCLC. As to the indication for surgical intervention for preoperatively diagnosed SCLC, controversies still remain. Our recommendation is as follows. When a patient has SCLC of clinical N1 or N2 status, chemoradiotherapy should be considered because a survival after an operation alone would not be good enough. Surgical intervention should be considered, however, for patients with clinical stage I disease because an operation followed by chemotherapy offers a good prognosis, as shown in this

study, and because such SCLC sometimes turns out to be non-SCLC postoperatively. A phase III trial comparing chemoradiotherapy with surgical intervention followed by chemotherapy is interesting. However, the number of patients with SCLC with clinical stage I or II disease is very small, and we do not think it is possible to perform the phase III trial in this population.

Because clinical stage and pathologic stage were significant prognostic factors in our trial, preoperative staging, intraoperative staging, or both should be a major concern for the treatment of very limited SCLC. Actually, the following preoperative investigations were performed before entry into the study in this cohort: CT scans of the chest, upper abdomen, and brain; bronchoscopy; chest plain film; radio-nuclide bone scans; complete blood cell count and serum chemistry; and physical examination. If the diagnosis of SCLC was made preoperatively, we recommend the same preoperative workup as done by us in this study. Furthermore, if swollen lymph nodes are detected on thoracic CT scans, we absolutely recommend mediastinoscopy for such cases. As for positron emission tomography, we have no recommendation thus far because this modality has recently begun to be evaluated, although it could be useful for staging N1 disease. Intraoperatively, hilar and mediastinal lymph node sampling or dissection was performed in 59 (97%) patients. This intraoperative staging is also important for deciding on the treatment strategy.

The site of the first relapse was another fruit of our study. This clinical trial did not use postoperative mediastinal irradiation or prophylactic cranial irradiation (PCI). We should discuss the importance of these strategies for very limited SCLC. As to locoregional recurrence, approximately 10% of the patients showed relapse in the mediastinal lymph nodes, bronchial stump, or both. Five percent of patients with stage I or II disease eventually have locore-

TABLE 6. Relationship between clinical and pathologic stages

Clinical stage	Pathologic stage			P value*
	I	II	IIIA	
I	33	5	6	.011
II	1	3	5	
IIIA	1	0	7	

*P value in Bowker's test of symmetry.

gional recurrence, whereas this is seen in 22% of patients with stage IIIA disease. These results suggest that patients with stage IIIA disease, at least, could benefit from postoperative mediastinal irradiation, whereas those with stage I or II disease might not need to undergo radiotherapy. Thus, postoperative chemoradiotherapy might be used in a future trial for stage IIIA disease.

Auperin and associates²⁰ reported that PCI improved both overall survival and disease-free survival among patients with SCLC in complete remission. Surgically resected SCLC would be considered SCLC in complete remission, and PCI would be indicated. Overall, 15% of the patients in our study showed brain metastasis. Even among patients with stage IA disease, more than 10% of the patients had brain metastasis. Therefore, PCI might be necessary for all patients with completely resected SCLC, whereas some authors have insisted that patients with pathologic stage IA SCLC can be cured without any adjuvant treatment.¹⁹

Noda and coworkers²¹ reported that combination chemotherapy consisting of irinotecan (CPT-11) and cisplatin was superior to PE for extensive SCLC. Although concurrent radiotherapy with CPT-11 would be harmful, we would use the new regimen for very limited SCLC, especially for stage II or IIIA SCLC.

Major lung resection with complete hilar and mediastinal lymph node dissection followed by postoperative PE is a feasible treatment and results in a favorable survival profile. Survival was especially good for patients with stage I disease. Our strategy could be used as a standard treatment arm in a future trial for very limited SCLC.

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REGULAR ARTICLE

Clinical-scale high-throughput human plasma proteome analysis: Lung adenocarcinoma

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Clinical proteomics requires the stable and reproducible analysis of a large number of human samples. We report a high-throughput comprehensive protein profiling system comprising a fully automated, on-line, two-dimensional microflow liquid chromatography/tandem mass spectrometry (2-D μ LC-MS/MS) system for use in clinical proteomics. A linear ion-trap mass spectrometer (ITMS) also known as a 2-D ITMS instrument, which is characterized by high scan speed, was incorporated into the μ LC-MS/MS system in order to obtain highly improved sensitivity and resolution in MS/MS acquisition. This system was used to evaluate bovine serum albumin and human 26S proteasome. Application of these high-throughput μ LC conditions and the 2-D ITMS resulted in a 10-fold increase in sensitivity in protein identification. Additionally, peptide fragments from the 26S proteasome were identified three-fold more efficiently than by the conventional 3-D ITMS instrument. In this study, the 2-D μ LC-MS/MS system that uses linear 2-D ITMS has been applied for the plasma proteome analysis of a few samples from healthy individuals and lung adenocarcinoma patients. Using the 2-D and 1-D μ LC-MS/MS analyses, approximately 250 and 100 different proteins were detected, respectively, in each HSA- and IgG-depleted sample, which corresponds to only 0.4 μ L of blood plasma. Automatic operation enabled the completion of a single run of the entire 1-D and 2-D μ LC-MS/MS analyses within 11 h. Investigation of the data extracted from the protein identification datasets of both healthy and adenocarcinoma groups revealed that several of the group-specific proteins could be candidate protein disease markers expressed in the human blood plasma. Consequently, it was demonstrated that this high-throughput μ LC-MS/MS protein profiling system would be practically applicable to the discovery of protein disease markers, which is the primary objective in clinical plasma proteome projects.

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Abbreviations: ABC, ammonium bicarbonate; AID-HP, albumin- and IgG-depleted human plasma; IAM, iodoacetamide; Ig, immunoglobulin; ITMS, ion-trap mass spectrometry; μ LC, microflow liquid chromatography; NSI, nanoelectrospray ionization; SCX, strong cation exchange; TCEP, tris[2-carboxyethyl]phosphine; TPX, methylpentene polymer

1 Introduction

Human blood plasma is generally the most informative proteome from a medical viewpoint, because it is the primary clinical specimen and it also represents the largest and deepest version of human proteome present in any sample [1–3]. Almost all body cells communicate with the plasma either directly or through tissues/biological fluids, and many of these cells release at least a part of their contents into the plasma upon damage or death. A comprehensive, systematic characterization of the plasma proteome in the healthy and



diseased states will greatly facilitate the development of biomarkers for early disease detection, clinical diagnosis, and therapy of cancer and other diseases. However, broad characterization of the human plasma proteome may pose one of the greatest challenges. This is because it can contain low-level proteins, which are secreted by solid tissues, as well as other important proteins (tissue leakage proteins at pg/mL levels) in the presence of several relatively dominant, high-abundance proteins (particularly HSA at 35–50 mg/mL). The dynamic range of plasma protein concentrations minimally spans nine orders of magnitude. For clinical and diagnostic proteomics using human plasma, it is essential to develop a comprehensive system, which has a high resolution and a wide dynamic range, for large-scale proteome analysis.

Recently, multi-dimensional LC-MS/MS has been developed as a powerful tool, particularly for comprehensive identification of highly complex proteins. This method can achieve a resolving power that is equal to or higher than 2-DE [4–6]. Broad protein identification techniques can detect specific proteins present in low concentrations in a highly complex protein matrix. To characterize the human plasma proteome, Smith *et al.* have achieved a protein identification dynamic range of more than eight orders of magnitude using 2-D LC combined with conventional ion-trap MS/MS instrumentation [6]. This approach has resulted in the identification of >800 plasma proteins from 5 μ L plasma without the depletion of highly abundant HSA and/or immunoglobulins (Ig). The multi-dimensional LC-MS/MS techniques reported thus far indicate the potential usefulness of broad protein identification with high resolution and wide dynamic range for cataloging the plasma contents. However, these approaches require further improvement in terms of both ease of use and industrial applicability to routine clinical use, because their application to clinical research requires stable and reproducible analyses of a large number of human samples.

The establishment of a simple, robust, and high-throughput protein profiling system as a global platform is extremely important from the viewpoint of clinical proteomics. This is because a large number of human tissue/biological fluid samples could then be quantitatively analyzed, in a routine and reproducible manner, for expressed proteins. Such a system would help discover any protein that is significantly associated with a specific disease status. We have constructed a technically well integrated and high-throughput LC-MS/MS system with RP microflow LC (μ LC) and a conventional ion-trap MS/MS equipped with a nanoelectrospray ionization (NSI) interface to detect lung cancer biomarkers and to analyze apoptotic mechanisms [7, 8]. Additionally, the system has been combined with on- or off-line strong cation-exchange (SCX) chromatography to result in a multi-dimensional protein profiling system. This protein profiling system using off-line 2-D SCX/RP μ LC-MS/MS was successfully applied to broad protein identification of human plasma proteins [9]. We have also established protein depletion, in-solution digestion, and data-integrating/mining sys-

tems with an automated operation for large-scale human plasma proteome analysis.

The dynamic range and sequence coverage that results from protein identification by LC-MS/MS analysis depends on both the quality of the separation(s) applied and the MS platform [6]. When resolution performance is not considered, the quality of the 2-D LC is substantially related to the number of fractionation steps for the first dimension chromatography and the analytical running time for the second dimension chromatography. Since the dynamic range of the MS platform is based on the performance of the instrument used, the number of the MS/MS acquisition in one run strictly depends on both the scan speed and the analytical time required by the LC-MS/MS analysis. Recently, linear ion-trap MS/MS (2-D ITMS) instruments with a higher scan speed and sensitivity than conventional 3-D ITMS instruments have emerged as new generation instruments. Therefore, we applied the new 2-D ITMS instrument to the fully automated on-line 2-D μ LC-MS/MS system developed by us [10].

In this study, we evaluated the performance of the μ LC-MS/MS system using the 2-D ITMS instrument for extending the sensitivity, dynamic range, and coverage for comprehensive protein identification. BSA and human 26S proteasome were used as the authentic protein sample and the protein complex sample, respectively. The system, together with the on-line 2-D μ LC-MS/MS system, was then applied to proteome analysis of human plasma; HSA- and IgG-depleted samples were obtained from a few healthy individuals and lung adenocarcinoma cases.

2 Materials and methods

2.1 Materials

HPLC-grade ACN, formic acid, and TFA were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Milli-Q grade water (Millipore, Bedford, MA, USA) was used. BSA, ammonium formate, ammonium bicarbonate (ABC), and iodoacetamide (IAM) were purchased from Sigma (St. Louis, MO, USA). Human 26S proteasome (PW9310) was obtained from Affiniti Research Products (Devon, UK). Tris[2-carboxyethyl]phosphine (TCEP) was purchased from Pierce (Rockford, IL, USA). Sequencing grade-modified trypsin was purchased from Promega (Madison, WI, USA).

2.2 Preparation of the digested BSA and human 26S proteasome samples

BSA (1 nmol) was diluted with 225 μ L ABC (aq., 100 mM); then, 12.5 μ L TCEP (10 mM) was added for reduction and the solution mixture was kept at 37°C for 45 min. Further, 12.5 μ L IAM (50 mM) was added, and the solution mixture was alkylated in the dark at 24°C for 1 h. The resulting solution was digested with trypsin (trypsin:protein = 1:50, w/w),

and the resultant 250 μL solution was incubated in the dark at 37°C for 15 h. In-solution digestion of the 26S proteasome sample was carried out as follows: 26S proteasome sample (50 μg) was diluted with ABC (aq., 50 mM) containing 10% v/v ACN to a final volume of approximately 190 μL . For reduction, 2.5 μL TCEP (10 mM) was added, and the solution mixture was kept at 37°C for 45 min. Subsequently, 2.5 μL IAM (50 mM) was added, and the solution mixture was alkylated in the dark at 24°C for 1 h. For digestion, trypsin (2 μg) was added to 5 μL ABC (50 mM), and 200 μL of the resulting solution was incubated in the dark at 37°C for 18 h. All reactions were performed in methylpentene polymer (TPX) microtubes (Hitech Inc. Tokyo, Japan) using an Eppendorf thermomixer R (Brinkmann, Westbury, NY, USA) for 1.5 mL microtubes; the resulting solution was interval-mixed (10 s) at 850 rpm. The digested 26S proteasome sample (50 μL) was diluted with 125 μL of 2% v/v ACN (aq.) containing 0.005% v/v TFA (aq.) after adjusting the pH to approximately 3 with 50 μL of 1% v/v TFA (aq.); the samples (25 μL) were then injected into the $\mu\text{LC-MS/MS}$ system described in this paper.

2.3 Sample preparation of the digested human plasma protein mixture

The human blood plasma samples treated with heparin were obtained from Tokyo Medical University (Tokyo, Japan) and acquired from three, healthy, anonymous, male donors (samples: H-N, H-I, and H-S) and two male donors who were diagnosed with adenocarcinoma on the basis of clinical and laboratory criteria (stage: IIIA, samples: AC88 and AC94), after obtaining their informed consent. HSA and IgG in the human plasma samples (500 μL) was removed by affinity adsorption chromatography using Bio-Rad's Affi-Gel Blue Gel and protein A column (Bio-Rad Hercules, CA, USA), respectively (details not shown). The final concentration of the resulting HSA- and IgG-depleted human plasma (AID-HP) samples were 4.1 (H-N), 8.6 (H-I), 6.6 (H-S), 5.8 (AC88), and 7.2 mg/mL (AC94) in ABC (25 mM). Subsequently, 100 μL of the AID-HP sample was diluted with 400 μL ABC (25 mM) containing 32% v/v ACN. For reduction, 25 μL TCEP (50 mM) was added and the solution mixture was kept at 37°C for 45 min. Subsequently, 25 μL IAM (250 mM) was added and the solution mixture was alkylated in the dark at 24°C for 1 h. For digestion, trypsin (5 μg) was added and the resulting solution (555 μL) was incubated in the dark at 37°C for 16 h. All these reactions were carried out using the Eppendorf thermomixer R for 1.5 mL TPX microtubes, and the mixing was carried out at 850 rpm with periods and intervals of 10 s each. For the 1-D $\mu\text{LC-MS/MS}$ analysis, the digested AID-HP samples (20 μL) were diluted with 20 μL of 1% v/v TFA (aq.) and 160 μL with 2% v/v ACN (aq.) containing 0.1% v/v TFA, in a TPX auto sampler tube; 20 μL of the resultant samples was injected into the system. In order to prepare individual mixture samples of the healthy and adenocarcinoma groups for 1-D and 2-D $\mu\text{LC-MS/MS}$ analy-

ses, 33.3 μL of each of the three AID-HP samples from the healthy group and 50 μL of each of the two AID-HP samples from the adenocarcinoma group were mixed with 100 μL of 1% v/v TFA (aq.), respectively; subsequently, 4 μL of the resulting sample solutions was used in these analyses.

2.4 1-D RP and 2-D SCX/RP $\mu\text{LC-NSI-MS/MS}$ analyses

The 1-D and 2-D LC-MS/MS system with RP- μLC comprised a Paradigm MS4 dual solvent delivery system (Michrom BioResources, Auburn, CA, USA) for HPLC, an HTS PAL auto sampler with two 10-port injector valves (CTC Analytics, Zwingen, Switzerland), Finnigan LCQ Deca XP plus 3-D ion-trap, and Finnigan LTQ linear ITMS (Thermo Electron, San Jose, CA, USA) equipped with NSI sources (AMR Inc., Tokyo, Japan). Sample injection for the 1-D and 2-D $\mu\text{LC-MS/MS}$ analyses as well as SCX separation for 2-D analysis were automatically carried out using the HTS PAL auto sample injection system with no change in the configurations. The SCX separation was performed on an SCX microtrap cartridge (12 μm , 300 \AA , 8 \times 1.0 mm i.d., Michrom) by step-wise elution on the first injector valve. The solvent system containing 2% v/v ACN was composed of 0.005% v/v TFA (aq.) and 1 M ammonium formate (aq.) adjusted to pH 2.8 with TFA, and the elution solvents (25, 50, 100, 150, 200, and 500 mM) were prepared by mixing these. The effluent from all the SCX fractions was flowed serially into a peptide Cap-Trap cartridge (2.0 \times 0.5 mm i.d., Michrom), present on the second injector valve, for concentration and desalting. After desalting with 0.1% v/v TFA (aq.) containing 2% v/v ACN, the sample was loaded onto a capillary RP column, MAGIC C₁₈ (3 μm , 200 \AA , 50 \times 0.2 mm i.d., Michrom), for 2-D separation. Digested samples for the 1-D RP analysis were also injected directly into a peptide CapTrap cartridge for concentration and desalting and then applied to RP separation. Solutions of 2% and 90% v/v ACN (aq.) were used as the mobile phases A and B, respectively, and both contained 0.1% v/v formic acid. The gradient conditions in the chromatographic run were as follows: B 5% (0 min) \rightarrow 65% (20 min) for the digested samples of BSA and 26S proteasome, and B 5% (0 min) \rightarrow 40% (70 min) \rightarrow 95% (80 min) for plasma samples. Effluent solvent at 1.0–1.2 $\mu\text{L}/\text{min}$ from the HPLC was introduced into the mass spectrometer by the NSI interface *via* an injector valve with a CapTrap cartridge and the RP column. The NSI needle (FortisTip, Omniseparo-TJ, Hyogo, Japan), which was connected directly to the RP column outlet, was used as the NSI interface and the voltage was 1.8 kV, while the capillary was heated to 200°C [11]. No sheath or auxiliary gas was used. Further, the mass spectrometer was operated in a data-dependent acquisition mode in which MS acquisition with a mass range of m/z 450–2000 was automatically switched to MS/MS acquisition under the automated control of the Xcalibur software. The most intense ion of the full MS scan was selected as the parent ion and it was subjected to MS/MS scan with an isolation width of m/z 2.0; the activation amplitude parameter

was set at 30%. For the human plasma samples, the full MS scan was acquired followed by two successive MS/MS scans of the two most intense precursor ions detected in the full MS scan. The trapping time was 100 ms under the auto gain control mode. Data was acquired using the dynamic mass-exclusion windows that had an exclusion of 3.0-min duration and exclusion mass widths of -0.5 and $+1.5$ Da.

2.5 Database searches

All MS/MS data were investigated using the Mascot search engine (Matrix Science, London, UK) [12] against the Swiss-Prot database. The data acquired for BSA digests were investigated against other mammalian subsets of the sequences. The MS/MS data of the human 26S proteasome and plasma samples were investigated against the *Homo sapien* subsets of the sequences. The database searches allowed for fixed modification on the cysteine residue (carbamidomethylation, +57 Da), variable modification on the methionine residue (oxidation, +16 Da), peptide mass tolerance at ± 2.0 Da, and fragment mass tolerance at ± 0.8 Da.

3 Results and discussion

3.1 Evaluation of the μ LC-MS/MS analysis using the linear 2-D ITMS instrument

We developed the μ LC-MS/MS system with RP separation (1-D RP), which corresponds to the second dimension separation for the on-line and off-line 2-D μ LC-MS/MS system [9, 10]. This system comprises a microflow LC system with a variable splitter, a versatile auto-sampler equipped with an injector valve, and a LCQ 3-D (ITMS) with an NSI stage. A flow rate of 1.0–1.2 μ L/min *via* the injector valve and the RP column (0.2 mm i.d.) has been adopted as a convenient and efficient condition for routine proteome analysis with high sensitivity and reproducibility. The detection limit for identification of proteins in protein digests was approximately a few fmol. In order to evaluate the sensitivity of the new linear 2-D ITMS instrument (LTQ) for protein identification, we connected our RP μ LC system to the LTQ instead of the conventional 3-D ITMS (LCQ) instrument. BSA digests (5–500 fmol) were applied to μ LC-MS/MS analysis using the LTQ and LCQ instruments under identical conditions except those used for the mass spectrometer. The base-peak chromatogram for BSA digests (500 fmol) is shown in Fig. 1a. A comparison of the coverage, in terms of protein identification, between the LCQ and LTQ instruments revealed that 25% coverage of the BSA sequence was acquired from 5 fmol of the digests using the LTQ instrument, as shown in Fig. 2. Since the same coverage was obtained from 50 fmol of the digests using the LCQ instrument, the results indicated that the protein identification improved markedly as the sensitivity increased 10-fold using the LTQ instrument.

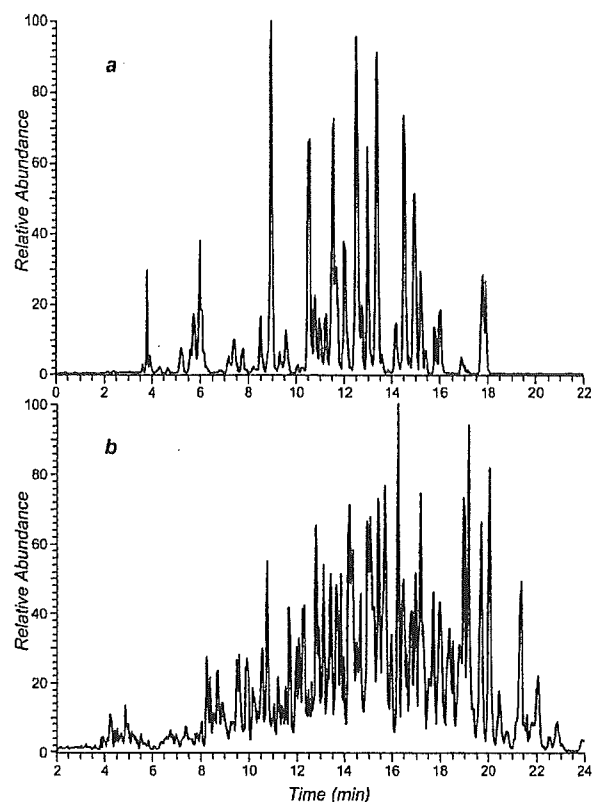


Figure 1. Base-peak chromatograms of the digested BSA (a) and 26S proteasome (b) using 1-D RP μ LC-MS/MS analysis.

A dramatic improvement is achieved in the LTQ instrument in terms of the scan speed, which is higher than that of conventional 3-D ITMS instruments. Figures 3a and 3b show the expanded mass chromatograms with stick plotting at m/z values 710.0–711.5 obtained by μ LC-MS/MS analysis of BSA digests using the LTQ and LCQ instruments, respectively. A stick in the peak represents a single full MS or MS/MS scan. The range denoted by the arrow in Fig. 3 shows that 62 events that carried out the acquisition of a full MS and an MS/MS spectra were achieved by the LTQ instrument in comparison with 12 events acquired by the LCQ instrument in 30 s. When conventional LCQ instruments are used, we usually apply three and two microscans for full MS (50 ms trapping time) and MS/MS (200 ms) accumulations, respectively, in order to obtain a better quality spectrum from a single scan. On the other hand, both spectra for peptide sequencing in protein identification were effectively acquired by one microscan of both full MS (50 ms) and MS/MS (100 ms) accumulations by LTQ instrument. As a result, using the LTQ instrument, it is possible to obtain an approximately five-fold higher number of MS/MS spectra in the same analytical run time.

In general, it is necessary to acquire a greater number of MS/MS spectra for the identification of a greater number of proteins using peptide sequencing. In studies where LC-MS/

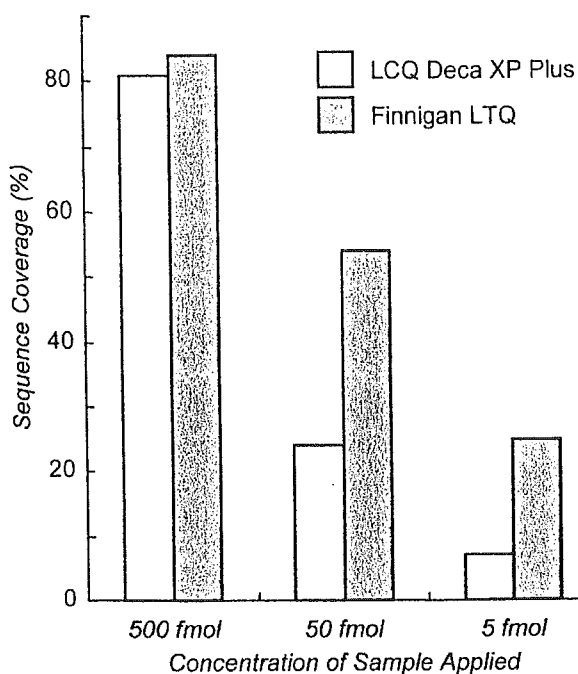


Figure 2. Comparison of sequence coverage of BSA digests (5–500 fmol) by μ LC-MS/MS analysis using LCQ Deca XP plus 3-D ion-trap and Finnigan LTQ linear ion-trap MS instruments.

MS was used for comprehensive proteome analysis, several researchers investigated various methods to data-dependently amass MS/MS spectra for a single analysis. These methods include employing a longer analytical time, triple and more MS/MS acquisition against a single full MS spectrum, multiple analyses of the same sample using common conditions or fractionated mass range, etc. While obtaining the MS/MS spectra, the scan speed is a mechanical limitation and a data-dependent scan misses many of the peptide sequences for low abundance peaks that are behind large peaks. Therefore, it is necessary to choose the applications of these techniques for comprehensive proteome analysis of highly complex protein mixtures such as human plasma and whole cell lysates. The drawbacks of clinical proteomics for a large number of human samples are the inability to conduct multiple analyses of the same sample and the longer running time required by the comprehensive LC-MS/MS analysis. Consequently, the performance of the LTQ instrument with a higher scan speed is better than that of the conventional 3-D ITMS instrument, because it enables more informative high-throughput LC-MS/MS analysis for highly complex clinical samples. Therefore, to verify the applicability of LTQ instruments, the human 26S proteasome, which is a highly complex protein mixture consisting of 31 components, was subjected to analysis. As shown in Fig. 1b, equivalent amounts of the digested 26S proteasome sample were analyzed using the LCQ and LTQ instruments under identical μ LC conditions. The data-dependent MS/MS

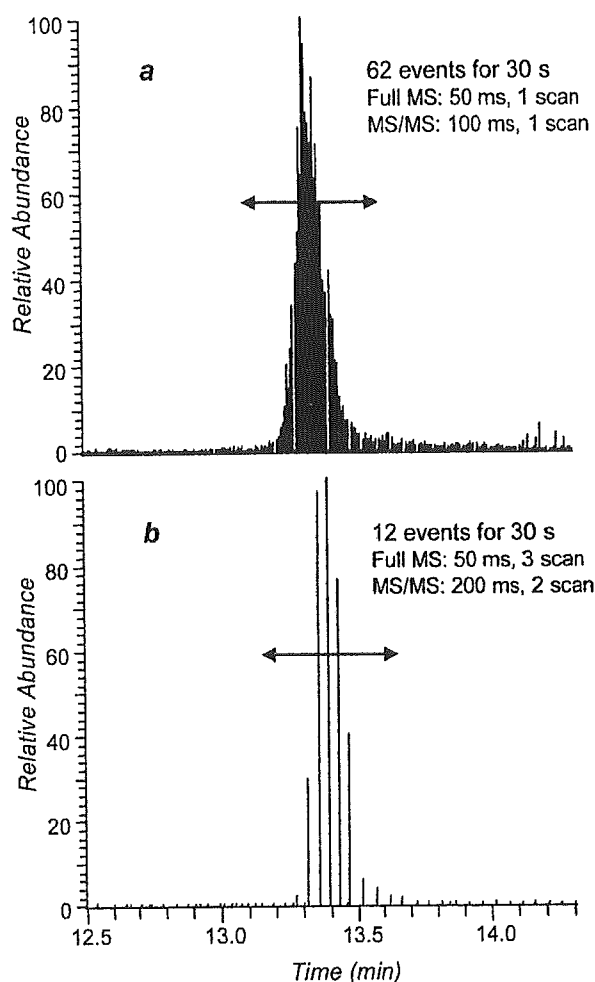


Figure 3. Mass chromatograms at m/z 710.0–711.5 by μ LC-MS/MS analysis of BSA digests using Finnigan LTQ (a) and LCQ Deca XP plus (b) instruments. A stick in the peak is a single full MS and MS/MS scan of the mass spectrometer, and 62 and 12 events (acquisition of a full MS and a MS/MS in one event) were carried out by the LTQ and LCQ instruments, respectively.

acquisition, in which the full MS acquisition is followed by a single MS/MS scan of the most intense precursor ion obtained from the full MS scan, was applied with three microscan full MS (50 ms trapping time) and two microscan MS/MS (200 ms) accumulations for the LCQ, and one microscan of both full MS (50 ms) and MS/MS (100 ms) accumulations for the LTQ instruments. During the 20 min analysis, approximately 450 and 2200 MS/MS spectra were obtained from the 1-D RP μ LC-MS/MS analyses using the LCQ and LTQ instruments, respectively. These data were evaluated using a Mascot database search against the Swiss-Prot database, and the search results obtained for the peptide MS/MS assignment were filtered based on the criterion defined as a Mascot peptide score more than 20 and ranked first, described in detail below. Table 1 (see also Supplemen-

Table 1. Protein identification results of human 26S proteasome.

Protein	LCQ Deca XP Plus			Finnigan LTQ		
	Score	Coverage	Peptide	Score	Coverage	Peptide
26S protease regulatory subunits						
PRS4	221	13	3	851	41	16
PRS6	142	9	3	757	48	16
PRS7	348	15	5	1346	55	23
PRS8	299	16	4	1304	58	20
PRSA	377	21	7	1093	53	19
PRSX	154	7	2	715	36	12
Proteasome subunit alpha types						
PSA1	494	36	8	658	48	11
PSA2	261	18	4	569	56	9
PSA3	145	13	3	584	43	11
PSA4	115	9	2	459	33	7
PSA5	158	17	3	483	47	8
PSA6	559	38	8	700	48	11
PSA7	423	35	7	772	57	12
Proteasome subunit beta types						
PSB1	275	34	5	596	53	10
PSB2	256	24	4	456	43	8
PSB3	235	24	3	379	35	5
PSB4	117	9	2	511	48	8
PSB5	528	42	7	670	58	10
PSB6	200	13	3	271	24	5
PSB7	232	14	4	381	33	7
PSB8	ND	ND	ND	180	18	4
26S proteasome non-ATPase regulatory subunits						
PSD1	227	6	3	1636	38	27
PSD2	380	10	6	1386	34	26
PSD3	690	24	10	1445	50	25
PSD4	74	5	1	358	22	6
PSD6	206	12	3	1001	46	18
PSD7	250	15	3	487	42	10
PSD8	ND	ND	ND	228	19	5
PSDB	571	22	8	1445	59	23
PSDC	359	15	5	1147	37	20
PSDD	183	10	4	966	52	17
		Total	130		Total	409

The protein identification data of 31 components consisted of human 26S proteasome including the number of the peptide fragments assigned (Peptide) and the sequence coverage according to these peptides (Coverage). The protein score is calculated by the addition of these peptide scores (Score) in comparison to 1-D μ LC-MS/MS analysis using conventional 3-D ion-trap MS (LCQ Deca XP Plus) and new linear ion-trap MS instruments (Finnigan LTQ). ND, not detected.

tary Table A) shows protein identification results including the number of peptide fragments assigned, sequence coverage with these peptides, and the protein score calculated by addition of these peptide scores with respect to the 31 components of the 26S proteasome. In the case of the LTQ instrument, 409 peptide fragments were assigned as components of the 26S proteasome, and this number was approximately three-fold higher than that obtained when the LCQ instrument was used (130 peptide fragments). The individu-

al components of the 26S proteasome were identified by 13.2 and 4.5 peptide fragments, 43.0% and 18.1% sequence coverage, and 821.9 and 292.4 protein scores on an average, using the LTQ and LCQ instruments, respectively. Twenty-nine proteins in 31 components were identified even by the LCQ instruments using our RP μ LC system with high-resolution power, as shown in Fig. 1b. However, the peptide fragments of the remaining two components were not observed from the filtered database search results. A number

of peptide fragments belonging to the 26S proteasome (>700 peptides and >60% coverage on an average) were detected by an on-line 2-D SCX/RP μ LC-MS/MS experiment with the same amount of digests using the LCQ instrument [10]. These results may simply indicate the difference in the scan speed for the limited, short analytical time between the LCQ and LTQ instruments and not a difference in the sensitivity. Accordingly, LC-MS/MS analysis using LTQ has a three-fold higher efficiency in identification in comparison with a conventional LCQ. This indicates that the LTQ has a superior protein identification capability. Thus, the introduction of LTQ into our μ LC-MS/MS system resulted in a highly improved performance, in terms of both sensitivity and protein identification efficiency, for highly complex mixtures.

3.2 Human plasma proteome analysis

The usefulness and applicability of our automated protein profiling system coupled with LTQ for clinical proteomics have been examined by analyzing human plasma samples. In the course of clinical plasma proteome studies, we investigated two types of human plasma – one from healthy donors and the other from donors with lung adenocarcinoma. All plasma samples obtained from the three healthy (H-N, H-I,

and H-S) and two adenocarcinoma (AC88 and AC94) cases were digested in a solution with trypsin after removing their HSA and IgG contents. The resultant peptide mixtures were diluted for the μ LC-MS/MS analysis. Further, equivalent mixture samples from the three healthy donors (H-NIS; H-N:H-I:H-S, 1:1:1 in volume) and the two adenocarcinoma donors (AC8894; AC88:AC94, 1:1 in volume) were also prepared as average samples for each case. The individual and mixture samples (total seven samples) were analyzed by 1-D RP μ LC-MS/MS analysis under analytical conditions for 90 min. The LTQ MS data was acquired by the double MS/MS method, in which the full MS acquisition is followed by two MS/MS scans of the two most intense precursor ions from the full MS scan. This is done to improve the protein identification results by the database search. Additionally, our established on-line 2-D SCX/RP μ LC-MS/MS system using LTQ instead of the conventional LCQ instrument was also used to analyze the mixture samples (H-NIS and AC8894) along with an additional 1-D RP analysis. In the 2-D μ LC-MS/MS analysis, six SCX separation runs were automatically carried out and analyzed by the RP μ LC-MS/MS analysis under the same analytical conditions as described earlier. The total operation time for both 1-D and 2-D μ LC-MS/MS analyses of a sample was within 11 h [10]. Figure 4 shows the base-peak chromatograms from 1-D (g) and 2-D

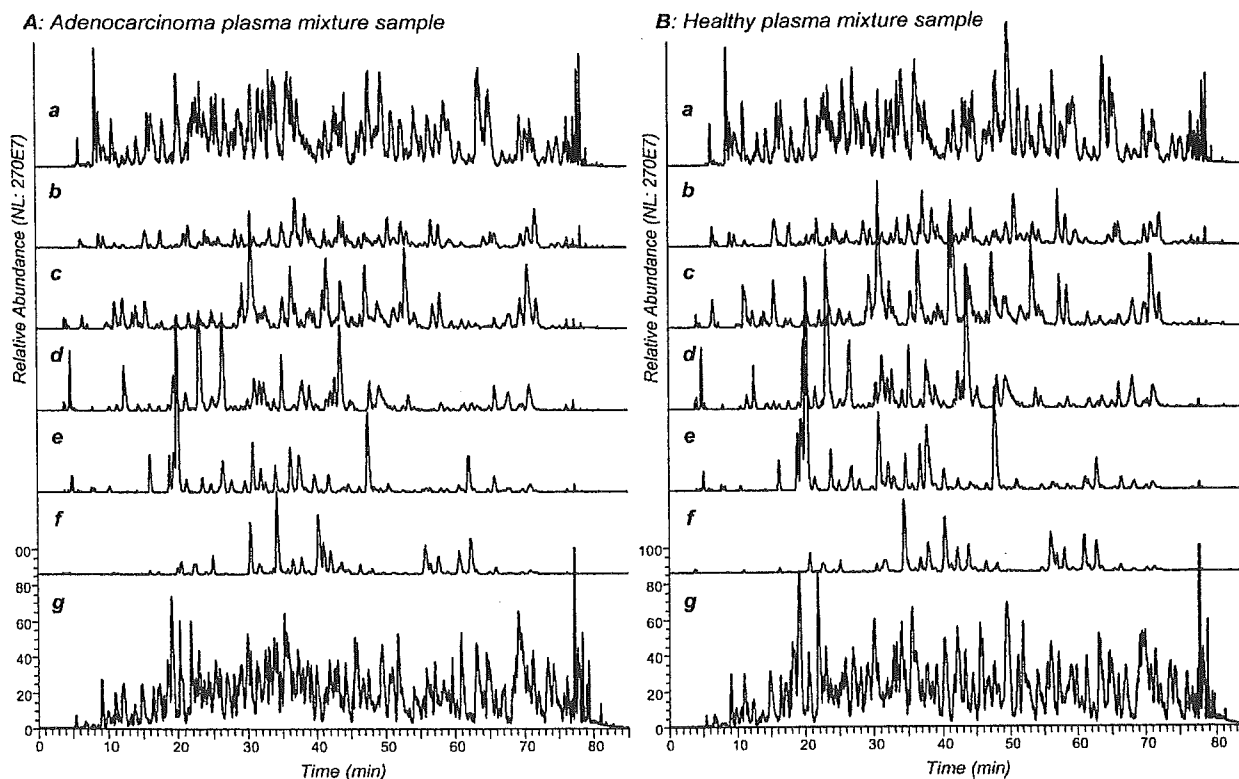


Figure 4. Base-peak chromatograms of the digested human plasma samples using 2-D SCX/RP (a–f) and 1-D RP μ LC-MS/MS analyses (g). A, mixture of plasma digests of the healthy group; B, mixture of plasma digests of the adenocarcinoma group; a, 25 mM; b, 50 mM; c, 100 mM; d, 150 mM; e, 200 mM; f, 500 mM salt concentration SCX fractions for 2-D μ LC-MS/MS analysis.

μ LC-MS/MS analyses (a–f) of approximately 2 μ g AID-HP tryptic digests corresponding to 0.4 μ L original blood plasma sample. The 1-D RP μ LC-MS/MS analysis provided approximately 10 000 MS/MS spectra for each sample, and the resultant data were evaluated using a Mascot database search against *H. sapien* subsets of the sequences in the Swiss-Prot database.

In order to achieve statistical confidence levels in identification of proteins from highly complex mixture samples, we investigated the thresholds as filters to extract data for the Mascot peptide score. We used the datasets, obtained from the 1-D μ LC-MS/MS analysis with LTQ, of the digested 26S proteasome sample within our search tolerances. Since it is possible to identify several proteins from a single MS/MS spectrum based on the hit sequence varieties, the most significant peptide sequence that was ranked first (marked with bold red in the Mascot search results), which had the highest score among the hit varieties, was extracted from the entire datasets to prevent erroneous identifications and redundancy. The resulting peptide assignments were sorted according to their Mascot peptide score and intergraded into protein identification. Figure 5 (see also Supplementary Table A) shows the relationship between the peptide score ranges and either the number of peptide fragments assigned as 26S proteasome or other proteins identified erroneously. Although peptides with a score less than 50 were assigned to both the 26S proteasome and to the other proteins, all peptide fragments with a score of more than 50 were confidently identified as belonging to the 26S proteasome. For thresholds of peptide scores higher than 20, 30, and 40, the statistical identification confidence levels of the Mascot database

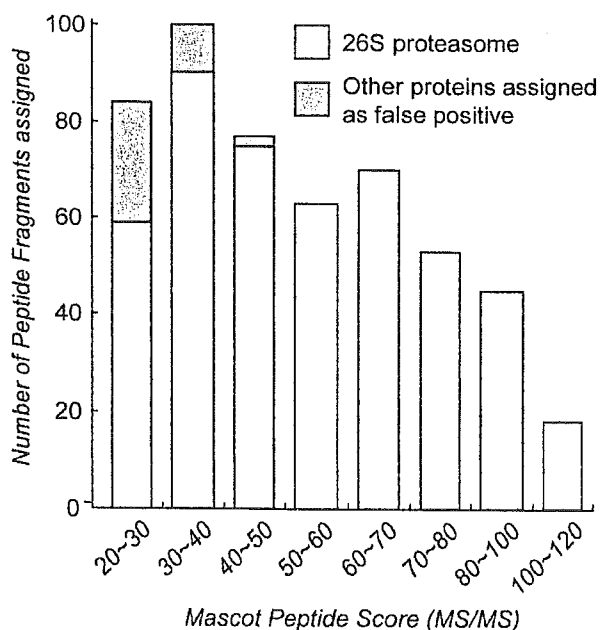


Figure 5. Mascot database search results of 1-D μ LC-MS/MS analysis of the digested 26S proteasome. See also Supplementary Table A.

search results were 70%, 90%, and 97%, respectively. Accordingly, we tentatively set peptide scores more than 30 and ranked first as the criterion for a broad protein identification index in order to integrate the datasets of the μ LC-MS/MS analysis of plasma samples. Furthermore, to extract plasma proteins with a higher confidence level, we finally tried to apply the Swiss-Prot dataset (667 proteins) in a non-redundant list of 1175 distinct proteins that Anderson *et al.* have recently developed by combining four separate sources of human plasma proteome [3, 13–16].

The Mascot database search results on plasma proteome analysis yielded data extracted under the thresholds of peptide scores higher than 30 and ranked first; an average of 108 proteins were detected from each 1-D μ LC-MS/MS analysis. From the 2-D μ LC-MS/MS analysis of the mixture sample of two groups, an average of 249 proteins was assigned as plasma proteome candidates. (Supplementary Table B). Additionally, entire datasets of these Mascot search results were integrated and processed with the data extraction. The results indicated that a total of 506 different proteins were listed, and 180 proteins were detected as common proteins from both groups (Fig. 6, in parentheses). Furthermore, plasma proteins with a high confidence level were extracted from these datasets using the Swiss-Prot dataset of the 667 plasma proteins reported. Figure 6 shows the diagrammatic representation of proteins found in the two groups comprising healthy individuals and adenocarcinoma patients, and the numbers are concordant with the proteins annotated as plasma proteins in 667 Swiss-Prot datasets. The results indicated that 84 and 85 proteins were extracted from the healthy and adenocarcinoma groups, respectively, and 69 proteins were common. In addition, 16 proteins were

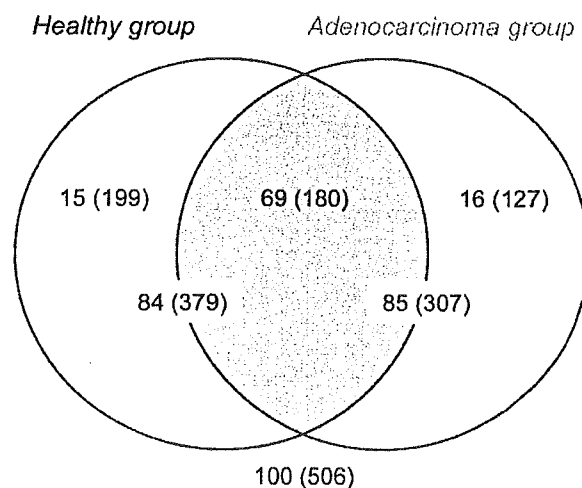


Figure 6. Diagrammatic representation of the proteins detected in the groups of healthy individuals and adenocarcinoma patients by 1-D and 2-D μ LC-MS/MS analyses of their plasma samples. Numbers that belong to the peptide score thresholds higher than 30 served as the criteria for protein extraction (expressed in parentheses), and the numbers are concordant with the plasma proteins reported in the 667 Swiss-Prot datasets.

Table 2. Selected specific protein list of the healthy and lung adenocarcinoma groups in human blood plasma.

Accession No.	Protein name	Adenocarcinoma plasma samples					Healthy plasma samples				
		2-D analysis		1-D μ LC-MS/MS analysis			2-D analysis		1-D μ LC-MS/MS analysis		
		AC8894	AC88	AC94	AC8894-1	AC8894-2	H-NIS	H-N	H-I	H-S	H-NIS-1
P02649	Apolipoprotein E	A	C	A	A	A			C		
Q14624	Inter-alpha-trypsin inhibitor heavy chain H4	A	C	B	C		C	C	C	C	C
P04196	Histidine-rich glycoprotein	A									
P00748	Coagulation factor XII	B									
P00488	Coagulation factor XIII A chain	B					C				
P02570	Actin, cytoplasmic 1	C					A	A	C		C
P27169	Serum paraoxonase/arylesterase 1						A				
P29312	14-3-3 protein zeta/delta							A			
P54108	Cysteine-rich secretory protein-3	C					B				
Q14791	Apolipoprotein L1						B				
P02751	Fibronectin						B				
P06396	Gelsolin, plasma						B				

Two adenocarcinoma plasma samples (AC88 and AC94) and three healthy plasma samples (H-N, H-I, and H-S) were analyzed by 1-D μ LC-MS/MS analysis. The mixture samples separated into both groups (AC8894 and H-NIS) were analyzed twice by 1-D and once by 2-D μ LC-MS/MS analysis (2-D analysis). A–C indicated the presence of peptide fragment(s) assigned to the listed protein. A, mascot peptide score higher than 50; B, score 40 to 50; C, score 20 to 40. See also Supplementary Table B.

detected as specific significant proteins of the adenocarcinoma group, and 15 proteins were not detected in the adenocarcinoma group. Table 2 (see also Supplementary Table B) shows a list of the specific proteins assigned by peptide fragment(s) with scores higher than 40. These specific proteins detected from only one group might be candidate biomarkers of lung adenocarcinoma in human blood plasma. However, further statistical verification of our results through data accumulation of more disease plasma samples and the investigation concerning the reproducibility of protein identifications for each sample are necessary. Additionally, validation of these protein identifications by several biochemical approaches would be required. In the present study, we could indicate that several significant protein candidates in the plasma proteome are possibly associated with the pathological differences in lung adenocarcinoma. Functions of specific proteins and their correlations with adenocarcinoma along with the other proteins are not listed in this paper and will be reported elsewhere. These experimental achievements suggest that our automated 1-D and 2-D μ LC-MS/MS protein profiling systems, in which the LTQ was incorporated, are powerful in identifying low-abundance proteins of great clinical importance, because these molecules directly report the occurrence and progress of various diseases.

4 Concluding remarks

In the course of the Human Plasma Organization Plasma Proteome Project, several research groups have prepared contrast reference specimens of human plasma using various technology platforms [17]. It is extremely important to catalog the plasma proteome as a protein database for clinical plasma proteomics. Applied technology platforms are very powerful, particularly for comprehensive broad protein identification of highly complex samples such as blood plasma. However, it seems difficult to stably and reproducibly apply these to routine clinical investigations that require a large number of proteome analysis runs for a large number of human samples. We have recently established a fully automated, high-throughput 2-D SCX/RP μ LC-MS/MS protein profiling system, which can perform large-scale analysis in clinical proteomics [10]. In this study, the LTQ, which is superior to a conventional 3-D ITMS instrument in sensitivity and scan speed, was utilized in our high-throughput system, and it was evaluated by analyzing BSA and human 26S proteasome. Furthermore, the system was applied to plasma proteome analysis in a few cases of both healthy individuals and lung adenocarcinoma patients. The results confirmed that a 10-fold increase in terms of sensitivity was achieved in our system using the LTQ instrument for protein

identification. Further, in comparison with the conventional 3-D ITMS instrument, a three-fold higher number of peptide fragments was identified as belonging to the 26S proteasome, indicating significant improvement in resolution for the analytical time point. Additionally, approximately 250 and 100 different proteins were detected, based on the investigation criterion for a 90% confidence level of protein identification, from only 0.4 μ L human plasma using 2-D and 1-D μ LC-MS/MS analyses, respectively. The entire operation was automatically carried out within 11 h for both single 1-D and 2-D μ LC-MS/MS analyses. From the protein identification datasets of both healthy and adenocarcinoma plasma samples, several disease-specific proteins were found in the human plasma based on the plasma proteome database reported earlier. Consequently, it was demonstrated that our μ LC-MS/MS protein profiling system is feasible for large-scale analyses such as clinical plasma proteomics studies. Although plasma proteome analysis for clinical application still remains a great challenge due to the wide dynamic range of protein abundance, we shall continue further technological development of the large-scale proteome analysis based on the high-throughput μ LC-MS/MS system reported in this paper. Such high-throughput and large-scale analysis of human plasma would lead to the discovery of new disease-associated protein markers with high sensitivity and high specificity in early disease detection and diagnosis, and this would revolutionize current therapeutics.

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FROM THE ASCO-JSCO JOINT SYMPOSIUM

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Postoperative adjuvant therapy for completely resected early-stage non-small cell lung cancer

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Abstract Consensus on adjuvant therapy for completely resected non-small cell lung cancer until 2002 was as follows. (1) There was no significant impact of postoperative adjuvant chemotherapy based on meta-analysis and previous clinical trials. (2) Confirmatory studies are necessary in large-scale prospective clinical trials. However, recent mega trials have introduced epoch-making changes for postoperative adjuvant chemotherapy in clinical practice since ASCO 2003. The effectiveness of UFT in N0 patients was confirmed. Patients with completely resected stage I non-small cell lung cancer, especially T2N0 adenocarcinoma, will benefit from adjuvant chemotherapy with UFT. The results of the International Adjuvant Lung Trial (IALT) have confirmed the meta-analysis in 1995. Also, both the JBR10 and Cancer and Leukemia Group B (CALGB) 9633 studies have also confirmed positive IALT results of the benefit for postoperative platinum-based chemotherapy in completely resected non-small cell lung cancer. Adjuvant chemotherapy for pathological stage IB to II, completely resected non-small cell lung cancer is standard care based on clinical trials. UFT showed the strongest evidence for IB in Japan. Platinum doublet chemotherapy with third-generation anticancer agents is also recommended. Adjuvant chemotherapy should be offered as standard care to patients after completely resected early stage non-small cell

lung cancer. However, there is no evidence of the feasibility and efficacy for adjuvant chemotherapy with the platinum-based regimen in Japan. Careful management should be necessary in such treatment.

Key words Adjuvant therapy · Chemotherapy · Surgery · Non-small cell lung cancer · Early-stage lung cancer

Introduction

The 5-year survival rate after surgical treatment in the United States and Japan in each stage of non-small cell lung cancer is shown in Table 1.^{1,2} Although these surgical outcomes reveal the slight difference among two groups, we are not satisfied with the results, particularly in stage IB and II, which are so-called early stage. Surgery is still the best therapeutic modality for the potential cure of the patient with non-small cell lung cancer, especially in stage I. However, in the patient with pathological stage IB, the 5-year survival rate is only about 60%. The recurrence pattern is frequently due to distant metastasis.³ Therefore, perioperative adjuvant therapy is required for improvement of survival after surgical resection.

Meta-analysis of the randomized trials of adjuvant therapy of non-small cell lung cancer in 1995 suggested the survival benefit of cisplatin-based chemotherapy after surgery.⁴ However, there are no statistical differences between postoperative adjuvant group and surgery alone,⁴ and this includes a number of small trials and trials with the following disability criteria and chemotherapy regimens.

Consensus on adjuvant therapy for completely resected non-small cell lung cancer up to 2002 was as follows: (1) there was no significant impact of postoperative adjuvant chemotherapy based on meta-analysis and previous clinical trials. (2) Confirmatory studies are necessary in large-scale prospective clinical trials.^{5–10} However, recent mega trials have introduced epoch-making changes for postoperative adjuvant chemotherapy in clinical practice since ASCO 2003.^{11–19}

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