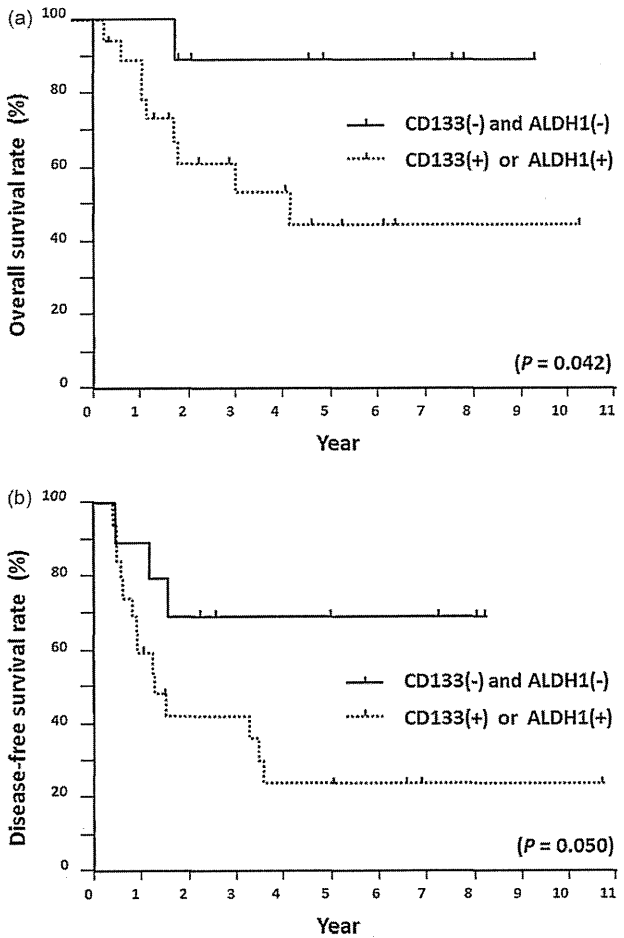

Fig. 2. Kaplan–Meier curves showing the (a) OS and (b) DFS rates according to cancer stem cell related markers expression. The OS and DFS rates in the CD133-positive or ALDH1-positive patients were significantly poorer than that of the patients who were negative for both CD133 and ALDH1 ($P = 0.042$ and $P = 0.050$, respectively).

to give rise to spheres and to act as tumor-initiating cells [28]. It has been also reported that CD133 positive endothelial progenitor cells are found in previously untreated frozen NSCLC tissue obtained by surgery and seem to be related to vasculogenesis [29]. On the other hand, Salnikov et al. reported that CD133 was indicative of a resistance phenotype but was not a prognostic marker for survival in surgically resected specimens of previously untreated NSCLC [30]. The authors showed a significant association between the expression of resistance-related proteins, such as glutathione S-transferase, thymidylate synthase, catalase, O^6-methylguanine-DNA methyltransferase, and p170, and CD133. In addition, Bertolini et al. reported that the cisplatin treatment of lung cancer cells in vitro resulted in enrichment of the CD133-positive fraction after both acute cytotoxicity and in cells with a stable cisplatin-resistant phenotype [23]. These findings are consistent with our result, in which the expression of CD133 in surgically resected specimens after induction CRT was related to an unfavorable outcome. ABCG2, a member of the ATP binding cassette (ABC) transporter superfamily, is an important determinant of the side population (SP) phenotype [31]. SP cells with stem cell-like capabilities marked by ABCG2 have been found in a variety of hematologic and solid malignancies, as well as NSCLC [32,33]. Bmi-1 is a member of the Polycomb group family of proteins, which act as epigenetic chromatin modifiers [34,35]. Bmi-1 is known to be a key regulator in the self-renewal of stem cells [36], and a recent report has shown that the expression of Bmi-1 in surgically resected specimens was a significant prognostic factor of a poor outcome in lung adenocarcinoma [20]. In our study, no obvious relation was seen between the expression of ABCG2 or Bmi-1 and patient outcome. This discrepancy may be due to differences in the kinds of samples that were examined, since our specimens had been treated with CRT. In addition to the markers examined in the present study, other CSC-related markers have also been reported. Further study examining the impact of such markers on trimodality therapy is necessary.

Surgically resected specimens that had been treated with CRT were used in this study. Regarding the clinical relevance, the expression status of CSC-related markers in post CRT specimens may be useful as biomarkers for the selection of adjuvant therapies after surgery. Since appropriate strategies for the treatment of CD133 or ALDH1-positive cases remain unknown at present, further investigation to establish appropriate adjuvant strategies is warranted. As other strategies, CSC-related markers could also be examined in enough amounts of specimens obtained before or after induction therapy to determine the contents of induction therapy or to determine the necessity of the further treatment, including surgery. However, based on the concept of CSC, the population of CSC-related marker positive cells may be very limited, and the possibility of misclassification, particularly the possibility of a false-negative diagnosis, is a concern. Indeed, the expression of CD133 was observed in a very limited portion of the surgically resected specimens in the present study.

What is the appropriate therapeutic strategy for tumors with positive CSC-related makers? In our knowledge, there is no relevant therapeutic strategy to specifically target cells with CSC-like phenotype in lung cancer. Of interest, histone deacetylase inhibitors are effective in chronic myelogenous leukemia stem cells appeared after acquisition of imatinib mesylate resistance [37].

In conclusion, the expression of CD133 or ALDH1 was significantly associated with an unfavorable prognosis. Although a study involving a large number of patients is required before a definite conclusion can be made, our results suggest that the development of a therapeutic strategy that considers the expression of CSC-related markers may be a key to further improvements in the prognosis of patients undergoing trimodality therapy.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.lungcan.2012.02.006.

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INSTITUTIONAL REPORT

Long-term outcome of induction chemoradiotherapy with docetaxel and cisplatin followed by surgery for non-small-cell lung cancer with mediastinal lymph node metastasis

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THORACIC ONCOLOGIC

Abstract

The purpose of this study was to show the long-term outcome of induction chemoradiotherapy, using docetaxel and cisplatin with concurrent radiotherapy followed by surgery for non-small-cell lung cancer (NSCLC) with mediastinal nodal metastasis. Between January 2000 and July 2006, 22 consecutive NSCLC patients with pathologically proven mediastinal nodal metastasis were treated with tri-modality therapy. The regimen consisted of docetaxel and cisplatin plus concurrent radiation at a dose of 40–46 Gy. The induction therapy was followed by surgery 4–6 weeks later. The pulmonary resections were composed of a lobectomy in 19 patients, including 3 with a sleeve lobectomy, a bilobectomy in 2 patients and a left pneumonectomy in 1 patient. With a median follow-up duration of 8.7 years, the 3-year and 7-year overall survival (OS) rates for the entire population were 72.7 and 63.6%, respectively. Our results suggest that tri-modality therapy is promising for NSCLC patients with mediastinal nodal metastasis.

Keywords: Non-small-cell lung cancer • Induction chemoradiotherapy • N2

INTRODUCTION

Mediastinal lymph node metastasis is significantly associated with a poor outcome in non-small-cell lung cancer (NSCLC). The standard treatment of N2 or N3 disease with good performance status (PS) is concomitant chemoradiotherapy with or without consolidation chemotherapy [1]. On the other hand, although surgical resection after the induction therapy is not currently considered as an established standard approach, surgery after the induction therapy is mainly performed by experienced institutions worldwide.

Two recent, large, randomized phase III trials (the Lung Intergroup trial 0139 and the European Organization for Research and Treatment of Cancer (EORTC) trial 08941) investigated the prognostic impact of surgery on patients with pN2 stage IIIA [2, 3]. Although the study designs and patient populations of each study differed, the two studies failed to demonstrate a benefit from the addition of surgery in the entire population. However, in the subset analysis of the Lung Intergroup trial 0139 for patients who underwent a lobectomy versus a matched subset undergoing chemoradiotherapy, a significant difference in the 5-year survival rate was found [2]. This result strongly suggests the possible advantage of surgical resection after induction chemoradiotherapy for a select population of patients with N2 disease.

Various kinds of chemotherapeutic regimes have been reported for the first-line treatment in patients with advanced NSCLC. We reported the feasibility and favourable prognosis of concomitant chemoradiotherapy using docetaxel and cisplatin in patients with unresectable locally advanced NSCLC with moderate, but acceptable toxicities [4, 5]. Given the success of this regimen, we selected this treatment for induction chemoradiotherapy followed by surgery, and reported the feasibility of the treatment and promising outcomes in patients with locally advanced NSCLC [6]. Here, we present the long-term survival data of tri-modality therapy for NSCLC patients with mediastinal nodal metastasis.

MATERIALS AND METHODS

Patient selection and evaluation

Previously untreated NSCLC patients with pathologically confirmed mediastinal nodal metastasis were eligible for enrolment in the study. Patients with mediastinal lymph node longer than 10 mm along the short axis as viewed on a CT scan underwent a cervical mediastinoscopy to evaluate stations 2, 4 and 7. An

anterior mediastinoscopy was combined when metastasis was suspected at stations 5 or 6 [7].

The inclusion criteria were age ≤75 years, with an Eastern Cooperative Oncology Group (ECOG) PS of 0–1 [8] and adequate functional reserves of major organs as described previously. Written informed consent was obtained from all patients. This protocol was approved and amended in 2000 by the Institutional Review Board/Ethical Committee of Okayama University. Disease stage was evaluated using chest radiography, enhanced chest and abdominal CT scans, including adrenal glands, enhanced brain magnetic resonance imaging (MRI) and radionuclide bone scan, or [18-fluoro-2-deoxyglucose positron emission tomography (PET-CT) scan] and bronchoscopy [7]. After completion of tri-modality treatment, chest and abdominal CT (or PET-CT) and enhanced brain MRI were repeated every 3 months at least 2 years after completion of the tri-modality therapy. Between 3 and 5 years after the completion, chest and abdominal CT (or PET-CT) and enhanced brain MRI were repeated every 6 months. After 5 years, chest X-ray was repeated every year, and further image analyses were conducted if necessary.

Treatment plan

Docetaxel (40 mg/m^2) was administered intravenously on days 1 and 8 over 1 h followed by 1-h infusion of cisplatin (40 mg/m^2) before the radiation therapy [6]. Chemotherapy was repeated at 3- or 4-week intervals. Chemotherapy dose and schedule modification were as reported previously [6]. Radiotherapy was started on the first day of chemotherapy, using a linear accelerator (6–10 MV). A total radiation dose of 46 Gy was planned, in principle, using a conventional fractionation regimen (2 Gy/day). The original volume included the site of the primary tumour and mediastinum, as described previously [6].

Following induction chemoradiotherapy, patients were evaluated for response based on a chest radiograph and CT scans. Patients without progressive disease (PD) were scheduled to undergo surgery within 6 weeks of completing the induction therapy. The surgical procedure was determined, based on the disease extent, before induction treatment. While a posterolateral thoracotomy was used as the basic approach, a median sternotomy was applied for patients with contralateral mediastinal lymph node metastasis or when great vessels, such as the main pulmonary artery, needed to be secured for a safe resection. A lobectomy with mediastinal lymph nodal dissection was basically the resection of first choice; however, a bilobectomy or pneumonectomy was performed in cases requiring these procedures because of disease extension. A sleeve resection was preferred to avoid a pneumonectomy, if appropriate. A complete ipsilateral superior mediastinal and subcarinal lymphadenectomy was performed in all the cases. For patients with primary lower lobe lesions, lymph nodes from stations 8 and 9 were also resected. Patients with primary left pulmonary lesions also underwent the resection of lymph node stations 5 and 6. A sharp resection with division of the azygous vein on the right-hand side and the Botallo ligament on the left-hand side was applied to enable a complete lymph node dissection in the mediastinum, since a 'frozen' mediastinum was sometimes observed because of chemoradiotherapy and pre-treatment mediastinoscopy. The bronchial stump was covered with the pericardial fat tissue or the pedicled intercostal muscle. When a sleeve resection was performed, the greater omentum was basically used to wrap the anastomosis. Postoperative adjuvant treatment was left to the physician's discretion.

Table 1: Patient characteristics

Characteristics	No. of patients
Median and range of age (years)	60 (31–74)
Sex	
Male	16
Female	6
ECOG performance status	
0	12
1	10
Histological subtypes	
Squamous cell carcinoma	11
Adenocarcinoma	9
Adenosquamous carcinoma	1
Large cell carcinoma	1
c-Stage	
IIIA	19
T1N2M0	2
T2N2M0	14
T3N2M0	3
IIIB	3
T2N3M0	1
T4N2M0	2
Lymph node status	
pN2	21
pN3	1

Table 2: Toxicity of induction chemoradiotherapy

Toxicity	Grade of toxicity (*n*)					% of Toxicities ≥grade 3
	0	1	2	3	4	
Leucopenia	2	2	8	8	2	45.5
Neutropenia	3	2	6	5	6	50.0
Anemia	0	9	11	2	0	9.1
Thrombocytopenia	13	9	0	0	0	0
Nausea/vomiting	2	8	8	2	2	18.2
Diarrhoea	6	15	1	0	0	0
Constipation	18	3	0	0	1	4.5
Hepatic	10	9	2	1	0	4.5
Renal	19	3	0	0	0	0
Cardiac	19	2	0	0	1	4.5
Pulmonary	0	4	1	1	0	4.5
Oesophagitis	7	2	11	2	0	9.1
Allergy	20	1	0	0	1	4.5

Toxicity was assessed by the National Cancer Institute Common Terminology Criteria for Adverse Events, v3.0.

Response, survival and toxicity assessments

Radiological response was basically assessed using the ECOG criteria with some modification and classified into four categories: complete response (CR), partial response (PR), stable disease (SD) and PD [6, 8]. The pathological response of the induction therapy was classified into three categories: pathological CR; pathological major response, pathological minor response [9].

The overall survival (OS) and the event-free survival (EFS) were calculated from the date of initiation of chemoradiotherapy until the date of death or the last follow-up for OS, and until confirmed death of any cause or recurrence at local or distant site for EFS. The survival curve was calculated by the Kaplan–Meier method, and difference between groups was compared with the log-rank test. Toxicity was assessed by the National Cancer Institute Common Terminology Criteria for Adverse Events, v3.0. All data were analysed using JMP® 9.0.0 Program for Windows (SAS Institute, Inc., Cary, NC, USA). All statistical tests were two-sided and probability values <0.05 were defined as being statistically significant.

RESULTS

Patient characteristics and the induction therapy

Between January 2000 and July 2006, a total of 22 NSCLC patients with pathologically proven mediastinal lymph node metastasis underwent tri-modality treatment. The patient characteristics are shown in Table 1. Among the 22 patients, 7 completed the planned induction treatment. Administration of chemotherapeutic agents was modified due to toxicity in 15 patients. The total radiation dose was 40 Gy in three patients and 46 Gy in 19 patients. The radiological response to the induction therapy was PR in 8 patients (36.4%) and SD in 14 patients (63.6%). The toxicities experienced during the induction therapy are listed in Table 2. The toxicity was manageable using standard approaches.

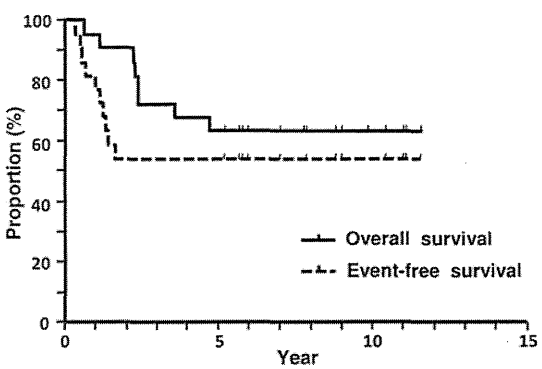

Figure 1: Overall and event-free survival curves for 22 patients who were treated with tri-modality therapy.

Surgery and pathological response

The median time from the end of the induction therapy until surgery was 37 days (range: 30–59 days). The surgical procedures included a lobectomy in 16 patients, a sleeve lobectomy in 3 patients, a bilobectomy in 2 patients and a left pneumonectomy in 1 patient. Twenty-one patients had a complete tumour resection with microscopically negative margins and the complete resection rate was 95.4%.

The pathological responsiveness of the resected specimens was estimated. Three (13.6%) patients exhibited a complete pathological response, 13 (59.1%) exhibited a major pathological response and 6 (27.3%) exhibited a minor pathological response. Mediastinal lymph node downstaging was obtained in eight (36.4%) patients: seven patients to pN0 and one patient to pN1.

Postoperative complications

The major postoperative complication was pulmonary toxicity. Four patients experienced radiation pneumonitis (grade 2 in three patients, and grade 3 in one patient). These four patients were successfully treated with the steroid therapy. One patient had acute pneumonia. No treatment-related deaths occurred in this series.

Table 3: OS and EFS rates according to clinicopathological factors

Subsets (*n*)	OS (%)		*P*-value	EFS (%)		*P*-value
	3 years	7 years		1 year	2 years	
Sex						
Male (16)	68.6	62.5	0.71	75.0	56.3	0.77
Female (6)	83.3	66.7		66.7	50.0	
Histology						
Non-adenocarcinoma (13)	69.2	69.2	0.77	69.2	61.5	0.60
Adenocarcinoma (9)	77.8	55.6		77.8	44.4	
c-Stage						
IIIA (19)	73.7	68.4	0.20	63.2	57.9	0.69
IIIB (3)	33.3	33.3		66.7	33.3	
Radiological response						
CR/PR (8)	75.0	75.0	0.41	75.0	62.5	0.62
SD (14)	71.4	57.1		71.4	50.0	
Pathological response						
Complete/major (16)	75.0	75.0	0.12	81.3	68.9	0.026
Minor (6)	66.7	33.3		50.0	16.7	
Mediastinal downstaging						
Downstaged (8)	100	100	0.012	87.5	87.5	0.036
Non-downstaged (14)	57.1	42.9		57.1	35.7	

OS: overall survival; EFS: event-free survival; CR: complete response; PR: partial response; SD: stable disease; *P*-value was calculated by log-rank test.

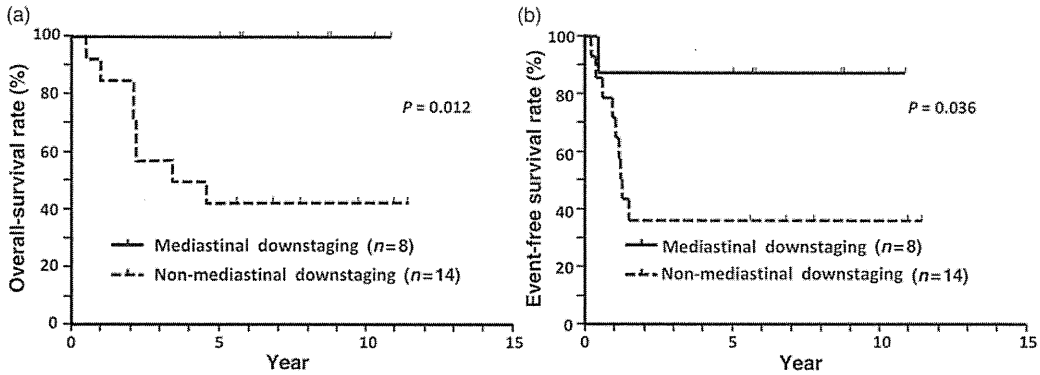

Figure 2: Overall and event-free survival curves stratified by mediastinal downstaging. Overall (a) and event-free survival (b)

Postoperative treatment

Of the 22 patients, nine received postoperative adjuvant chemotherapy: four patients received docetaxel and cisplatin; three received irinotecan and cisplatin; one received gemcitabine and cisplatin; one received gemcitabine and carboplatin.

Survival and pattern of relapse

At the final data analysis in July 2011, the median follow-up period was 8.7 years, ranging from 5.1 to 11.4 years. Fourteen patients (63.6%) were alive, including one with recurrent disease. Of the 21 patients who underwent a complete resection, disease relapse was observed in five patients as only distant, two patients as both distant and locoregional and one patient as only locoregional metastases. One patient with incomplete resection had dissemination.

The survival curves for the entire population are shown in Fig. 1. The 3-, 5- and 7-year OS rates were 72.7, 63.6 and 63.6%, respectively. The 1- and 2-year EFS rates were 72.7 and 54.5%, respectively. Subset analyses for survival were conducted to identify factors that were related to a favourable prognosis. The results are summarized in Table 3. Mediastinal downstaging was identified as a favourable factor related to prolonged OS ($P = 0.012$) and EFS ($P = 0.036$) times (Fig. 2).

DISCUSSION

The rationale for induction treatment in patients with locally advanced disease is to facilitate a complete surgical resection by reducing the number of cancer cells in the primary tumour and metastatic regional nodes and to eradicate possible micrometastases [10], resulting in a high cure rate. Thus, a powerful treatment for local and distant sites is crucial. The majority of the reported tri-modality therapies used a combination of second-generation agents, including etoposide or vinblastine plus cisplatin [2, 11, 12]. The ECOG study showed that paclitaxel, a third-generation agent, plus cisplatin, was superior to etoposide plus cisplatin with regard to the outcomes of patients with advanced NSCLC [13]. Of note, Stupp *et al.* also reported the excellent prognosis (40% of the 5-year OS rate after a median follow-up of 58 months) of stage IIIB patients who were treated with docetaxel and cisplatin followed by accelerated radiotherapy (44Gy) and surgery [14].

Needless to say, surgery is also crucial for the tri-modality therapy. A significantly high incidence of postoperative complications after induction treatment has been reported, particularly, after a pneumonectomy [2]. In our series, we tried to avoid a pneumonectomy by using a sleeve resection whenever possible, as the safety of this procedure after the induction therapy has been confirmed despite the negative effect of chemoradiotherapy on the healing of the suture line. Among surgically related complications, bronchopleural and bronchopulmonary artery fistula are critical complications, sometimes resulting in treatment-related death. To minimize the risk of these bronchial fistulas, the bronchial suture lines were wrapped and separated from the pulmonary artery using the pericardial fat tissue, the pedicled intercostal muscle or the greater omentum in cases with a sleeve lobectomy.

To assist the proper interpretation of our study, limitations need to be considered. Some of the inherent limitations of our study are the small number of patients and the lack of a randomized design, suggesting that a significant selection bias may be present.

In conclusion, concomitant chemoradiotherapy using docetaxel and cisplatin followed by surgery is a feasible therapeutic option for NSCLC patients with mediastinal nodal metastasis.

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Strong anti-tumor effect of NVP-AUY922, a novel Hsp90 inhibitor, on non-small cell lung cancer

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ABSTRACT

The anti-tumor activity of a newly developed Hsp90 inhibitor, NVP-AUY922 (AUY922), against non-small cell lung cancer (NSCLC) was examined. Twenty-one NSCLC cell lines were used, the somatic alterations of which were characterized. Cell proliferation was analyzed using a modified MTS assay. Expression of the client proteins was assessed using Western blotting. The cell cycle was analyzed using flow cytometry. The IC_{50} value of AUY922 for the NSCLC cell lines ranged from 5.2 to 860 nM (median, 20.4 nM). Based on previous data, cells with an IC_{50} of less than 50 nM were classified as sensitive cells and 19 of the 21 NSCLC cell lines were judged to be sensitive. The IC_{50} of five malignant pleural mesothelioma (MPM) cell lines revealed that the MPM cells had a significantly higher IC_{50} value (median, 89.2 nM; range, 22.2–24,100 nM) than the NSCLC cells ($p = 0.015$). There was significant depletion of both the total and phosphorylated client proteins – EGFR, MET, HER2 and AKT – at low drug concentrations (50–100 nM) in drug-sensitive cell lines. Cell-cycle analysis was performed for two sensitive cell lines, H1975 and H838. Following AUY922 treatment, an increase in the sub-G_0-G_1 cell population, as well as appearance of cleaved PARP expression, indicated the induction of apoptosis. In conclusion, AUY922 was effective against most NSCLC cell lines, independent of the type of known molecular alteration, and appears to be a promising new drug for the treatment of NSCLC.

1. Introduction

Lung cancer is associated with various types of molecular alteration, including epidermal growth factor receptor (EGFR) mutation, *K-ras* mutation, *HER2* amplification and, as recently found, *EMK4-ALK* gene fusion [1–3]. Improvements in our understanding of the molecular alterations involved in lung cancer have brought significant advancements in molecular-targeted therapy [4]. Among these alterations, *EGFR* mutations, which are frequent alterations in lung adenocarcinoma, are a predictive factor for the efficacy of EGFR-tyrosine kinase inhibitors (EGFR-TKIs), such as gefitinib and erlotinib [1,2]. These EGFR-TKIs have a marked anti-tumor effect on NSCLCs with common *EGFR* mutations. However, acquired resistance from, for example, a secondary *EGFR* T790M mutation or *MET* amplification is a major problem that is responsible for treatment failure [5–7].

The heat-shock protein 90 (Hsp90) complex is a chaperone protein that facilitates the refolding of unfolded or misfolded proteins. It plays a pivotal role in cancer cell survival, as it stabilizes a large set of proteins, so-called client proteins, many of which are essential for apoptosis, cell-cycle regulation, proliferation, and other characteristic properties of cancer cells [8,9]. In NSCLC, Hsp90 stabilizes oncogenic proteins such as EGFR, MET, HER2 and AKT [9,10]. We and some other studies have shown that geldanamycin (GM) and its analogues, the benzoquinone ansamycin class (17 allylamino-17demethoxygeldanamycin [17-AAG] and 17 dimethylaminoethylamino-17-demethoxygeldanamycin [17-DMAG]), are effective against *EGFR*-mutated cell lines, even those that contain the *EGFR* T790M mutation that causes resistance to EGFR-TKI [11–14]. However, the results of clinical trials for 17-AAG and 17-DMAG were somewhat disappointing [15–19] and new potent Hsp90 inhibitors have therefore been pharmacologically designed and synthesized to offer improved efficacy and acceptable toxicity. NVP-AUY922 (AUY922) is one of these newly designed small-molecule Hsp90 inhibitors based on the 4,5-diarylisoxazole scaffold; it has a much higher affinity for Hsp90 than previous GM

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analogues [20]. AUY922 is bound to the ATP binding site of Hsp90α at the N-terminal domain, and its X-ray crystal structure confirms a crucial network of hydrogen bonding interactions. It exhibits the tightest binding of any small-molecule Hsp90 ligand because the entropy of binding to Hsp90 is almost negligible. Indeed, preclinical data from various types of human cancer have shown an anti-proliferative effect of AUY922, with low nanomolar potency both *in vivo* and *in vitro*, with no major adverse effects being observed in mice [20–24]. In these studies, AUY922 suppressed the client proteins (EGFR, MET, HER2 and AKT) that participate in the progression of various cancer cells, and AUY922 is considered to be a promising agent for NSCLC. However, to our knowledge, the efficacy of AUY992 has been reported in only one NSCLC cell line (A549) to date [25], although Phase II clinical trials for patients with advanced NSCLC have recently started.

In this study, we examined the anti-tumor effect of AUY922 against NSCLC cell lines containing several known genetic alterations, including *EGFR* mutations.

2. Materials and methods

2.1. Drugs and cell lines

AUY922 was obtained from Novartis (Nuremberg, Germany) and dissolved in dimethyl sulfide (DMSO) at stocked concentrations of 10 mM and stored at −20 °C. Working dilutions were always freshly prepared. Most of NSCLC and MPM cell lines used in this study were established at two institutions. The prefix NCI-H- (abbreviated as H-) indicates cell lines established at the National Cancer Institute-Navy Medical Oncology Branch, National Naval Medical Center, Bethesda, MD, and the prefix HCC- indicates lines established at the Hamon Center for Therapeutic Oncology Research, the University of Texas Southwestern Medical Center at Dallas, Dallas, TX. These cell lines were kindly provided by Dr. Adi F. Gazdar (University of Texas Southwestern Medical Center at Dallas, Dallas, TX, USA). A549 was purchased from American Type Culture Collection (Manassas, VA). NCI-H3255 was provided from Dr. Bruce Johnson (Lowe Center for Thoracic Oncology, Dana-Farber Cancer Institute, Boston, MA). PC-9 was provided from Immuno-Biological Laboratories (Takasaki, Gunma, Japan). Gefitinib-resistant PC-9 cell line (RPC-9) was provided from the Department of Hematology, Oncology, and Respiratory Medicine, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Japan [26]. All the cancer cell lines were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum. All cell lines were incubated at 37 °C in a humidified atmosphere with 5% CO_2.

2.2. Determination of cell proliferation

Cell proliferation was determined by a modified MTS assay with CellTiter 96® AQueous One Solution Reagent (Promega, Madison, WI). Cells were seeded on 96-well flat-bottomed tissue culture plates (Becton Dickinson, San Jose, CA) at a concentration of 3×10^3 cells/well with complete culture medium and allowed to adhere to the plate for 24 h. Then the cells were incubated in the presence of the drug of each concentration ranging from 0 (control) to 10 μM of for another 72 h at 37 °C in a humidified atmosphere of 5% CO_2 in air. After the treatment, 20 μL of CellTiter 96® AQueous One Solution Reagent was dropped into each well of plates. After the incubation of another 60 min, the optical densities (ODs) of these samples were directly measured using an Immuno Mini NJ-2300 (Nalge Nunc International, Rochester, NY). A reference wavelength at 490 nm was used to subtract background contributed by excess cell debris, fingerprints and other nonspecific absorbance. The OD of control samples was regarded as 100 and others were compared to the control. Each drug concentration was distributed in 4-replicate wells and each experiment was repeated thrice. The anti-proliferative activity of AUY922 was shown as IC_{50}, which is the concentration of the drug required to inhibit cell proliferation by 50%.

2.3. Western blot analysis and immunoprecipitation

Protein expression analysis was assessed by Western blotting. The lysate was extracted and 20 μg of total protein was then separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane. The membranes were incubated with anti-EGFR, anti-phospho-EGFR (Ty1068), anti-Met (25H2), anti-phospho-Met (3D7, Tyr1234/1235), anti-HER2, anti-phospho-HER2 (Tyr877), anti-Akt, anti-phosphor-Akt (Ser473), anti-p44/42 mitogen-activated protein kinases (MAPK), anti-phosphor-MAPK (Thr202/Tyr204), anti-Cyclin D1, anti-cdc2 and anti-cleaved poly (ADP-ribose) polymerase (PARP) (Asp214) (19F4) antibodies (Cell Signaling Technology, Beverly, MA), anti-Hsp90 (Novocastra, Newcastle, UK), anti-Hsp70 (Stressgen Bioreagents, Ann Arbor, MI), anti-CDK4 (C-22) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Actin (used as loading control, Millipore, Billerica, MA) and then with goat anti-rabbit and goat anti-mouse IgG-HRP coupled to horseradish peroxidase conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). After the incubation with antibodies, the membranes were developed by ECL Plus Western Blotting Detection Reagents (Amersham Biosciences UK Limited, Buckinghamshire, UK).

2.4. Flow cytometric analysis

Cells were harvested and resuspended in PBS containing 0.2% Triton X-100 and 1 mg/mL RNase for 5 min at room temperature and then stained with propidium iodide at 50 μg/mL to determine subdiploid DNA content using a FACScan. Doublets, cell debris, and fixation artifacts were gated out, and cell cycle analysis was done using CellQuest version 3.3 software.

3. Results

3.1. Anti-proliferative effect of AUY922 in NSCLC cell lines

The concentrations of AUY922 at IC_{50} in each cell line are shown in Table 1 and Fig. 1. The molecular characteristics of NSCLC cell lines are also described (Table 1). The IC_{50} values in the NSCLC cell lines ranged from 5.2 to 860 nM, whereas those in the MPM cell lines ranged from 22.2 to 24,100 nM ($p = 0.015$), indicating a significant difference in AUY922 sensitivity between NSCLC and MPM cell lines. For NSCLC, AUY922 exhibited a strong anti-proliferative effect in cell lines with *EGFR* mutations that were either sensitive to EGFR-TKI or that had acquired resistance to EGFR-TKI, similar to the effects of GM analogues. Furthermore, AUY922 also exhibited anti-proliferative effects on cell lines with wild-type *EGFR*, a *K-ras* mutation, *EML4-ALK* fusion gene, or other genetic alterations.

We also determined the IC_{50} value of the SKBR3 breast cancer cell line to validate the IC_{50} value determined with our MTS assay by comparing it with published data [22]. Our and previously published IC_{50} values were 9.7 ± 3.5 nM and 3.3 ± 0.9 nM, respectively, suggesting that the IC_{50} value measured using our system was not remarkably different from the published data [22]. Thus, in accordance with the published criteria, an IC_{50} value of less than 50 nM was regarded as being a sensitive cell line [22]. On the basis of this criteria, 19 of the 21 NSCLC cell lines and two of the five MPM cell lines were classified as being sensitive ($p = 0.034$).

Table 1
IC_{50} inhibition values for NVP-AUY922 in NSCLC and MPM cell lines.

Cancer type	Cell lines	Histological subtypes	AUY922		Genetic alterations	
			Sensitivity[a]	IC_{50} (nM)		
NSCLC	PC-9	AD	Sensitive	8.6 ± 0.5	*EGFR* mutation	Exon19 del.
	HCC2935	AD	Sensitive	9.2 ± 0.1		Exon19 del.
	HCC827	AD	Sensitive	16.9 ± 0.4		Exon19 del.
	HCC2279	AD	Sensitive	26.3 ± 3.6		Exon19 del.
	HCC4011	AD	Sensitive	17.9 ± 0.1		L858R
	H3255	AD	Sensitive	29.5 ± 5.8		L858R
	RPC-9	AD	Sensitive	20.4 ± 1.4		Exon19 del. ± T790M
	H1975	AD	Sensitive	5.2 ± 0.3		L858R ± T790M
	H1650	AD	Sensitive	23.5 ± 2.9		Exon19 del. ± PTEN del.
	H1299	LC	Sensitive	32.4 ± 0.1	*N-ras* mutation	
	A549	AD	Sensitive	16.3 ± 0.6	*K-ras* mutation	
	H2009	AD	Sensitive	21.4 ± 0.8	*K-ras* mutation	
	H358	AD	Sensitive	28.1 ± 4.1	*K-ras* mutation	
	H2170	SQ	Sensitive	9.1 ± 0.3	*HER2* amplification	
	H1648	AD	Sensitive	9.6 ± 0.1	*HER2* amplification	
	H1819	AD	Sensitive	23.9 ± 1.0	*HER2* amplification	
	Calu3	AD	Resistant	248 ± 8.5	*HER2* amplification	
	H1993	AD	Sensitive	7.7 ± 0.2	*MET* amplification	
	H1395	AD	Resistant	860 ± 7.1	*B-raf* mutation	
	H2228	AD	Sensitive	20.4 ± 6.5	*EML4-ALK* fusion gene variant E6a/b;A20	
	H838	AD	Sensitive	17.1 ± 0.6	None	
MPM	H211	Biphasic	Sensitive	22.2 ± 3.8		
	H290	Epithelial	Sensitive	27.3 ± 3.8		
	H28	Sarcomatoid	Resistant	89.2 ± 8.2		
	HP1	Biphasic	Resistant	1070 ± 10		
	H2052	Epithelial	Resistant	24,100 ± 4900		
BC	SKBR3		Sensitive	9.7 ± 3.5		

NSCLC, non-small cell lung cancer; MPM, malignant pleural mesothelioma; BC, breast cancer; AD, adenocarcinoma; LC, large cell carcinoma; SQ, squamous cell carcinoma.
[a] Sensitivity: sensitive cell lines, IC_{50} value ≤ 50 nM; resistant cell lines, IC_{50} value > 50 nM; del, deletion; NVP-AUY922 exhibited strong effects to most NSCLC cell lines with *EGFR* and *K-ras* mutation or *HER2* and *MET* amplification.

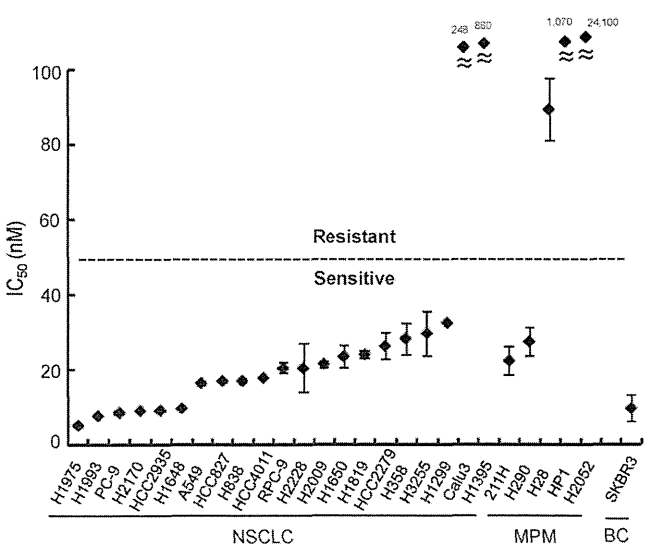

Fig. 1. IC_{50} values of non-small cell lung cancer and malignant pleural mesothelioma cell lines.

Two cell lines, H1395 and Calu3, were considered to be resistant. H1395 contains a *B-raf* mutation as a known molecular alteration, while Calu3 has a strong amplification of *HER2* and increased copy numbers of *EGFR* and *PIK3CA*. However, the H2170 cell line, which also exhibited strong *HER2* amplification and an increased copy number of *EGFR*, was classified as a sensitive cell line (IC_{50} = 9.1 ± 0.3), suggesting that amplification of *HER2* or *EGFR* is not the factor that causes resistance to AUY922.

3.2. Effects of AUY922 on molecular signature in NSCLC cell lines

Subsequent experiments focused on NSCLC. The effect of AUY922 on protein expression was examined according to concentration and exposure time in three sensitive cell lines (H1975, A549, and H838) and two resistant cell lines (H1395 and Calu3). Cells were harvested 24 h after drug treatment in a concentration gradient experiment (Fig. 2 and Supplementary Fig. 1). In sensitive cell lines, the depletion of both the total and the phosphorylated client proteins, such as EGFR, MET, HER2, AKT, and Cyclin D1 (CCND1), was observed after treatment with 50 nM of AUY922. Suppression of phospho-MAPK (p-MAPK) but not total-MAPK (t-MAPK) may be caused by down-regulation of its upstream molecules, which are the client proteins of Hsp90. Although inhibition of Hsp90 activity with drugs is generally correlated with Hsp70 protein levels after treatment [22,27], Hsp70 expression increased in both sensitive and resistant cell lines. In terms of the resistant cell lines, although expression of the client proteins was not depleted after treatment with a high concentration of AUY922 in Calu3 (IC_{50} = 248 nM), H1395 – another resistant cell line (IC_{50} = 850 nM) – showed depletion of client proteins after treatment with AUY922 at a low concentration (Fig. 2 and Supplementary Fig. 1).

For exposure time analysis, each cell line except H1395 was treated with the AUY922 concentration, which was five times as high as each IC_{50}. H1395, the IC_{50} of which was 850 nM, was exposed to 100 nM of AUY922. Although variation of protein depletion and recovery was observed according to proteins or cell lines, decreased expression of the majority of proteins was observed from 12 to 72 h (Fig. 3 and Supplementary Fig. 2). Of note, there was no major difference in the pattern of the protein expression profile time course between sensitive cell lines and H1395-resistant cell lines.

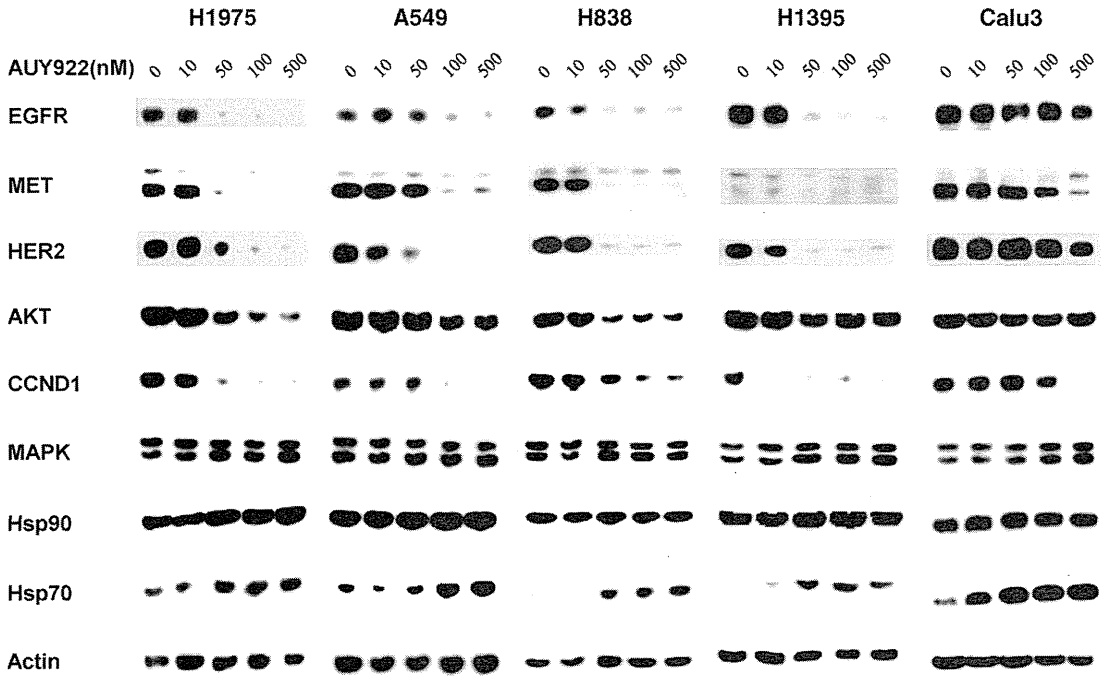

Fig. 2. The profiles of protein expression under the treatment of different AUY922 concentrations for 24 h.

3.3. *Effects of AUY922 on cell cycle and apoptosis*

We analyzed the cell cycle in two sensitive cell lines (H1975 and H838) to examine the impact of AUY922 on cell-cycle distribution, especially induction of apoptosis. Whereas the pattern of cell-cycle distribution after treatment of AUY922 was different between two cell lines, sub-G_0-G_1 DNA content increased in a time-dependent manner for both cell lines. Cleaved PARP also increased with AUY922 treatment, indicating that AUY922 induced apoptosis in these two cell lines (Fig. 4).

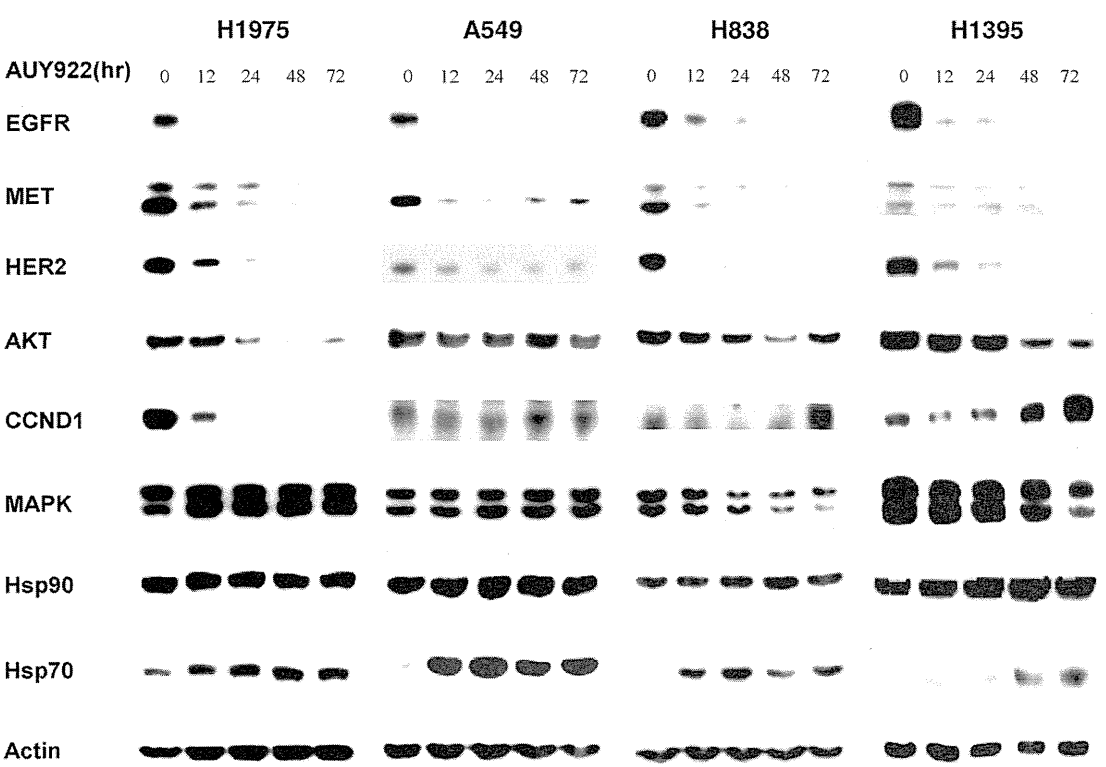

Fig. 3. The profiles of protein expression according to exposure time with AUY922. Each NSCLC cell line (H1975, A549, and H838) was treated with AUY922 of which concentration was five times as high as each IC_{50}. H1395 was exposed to 100 nM of AUY922.

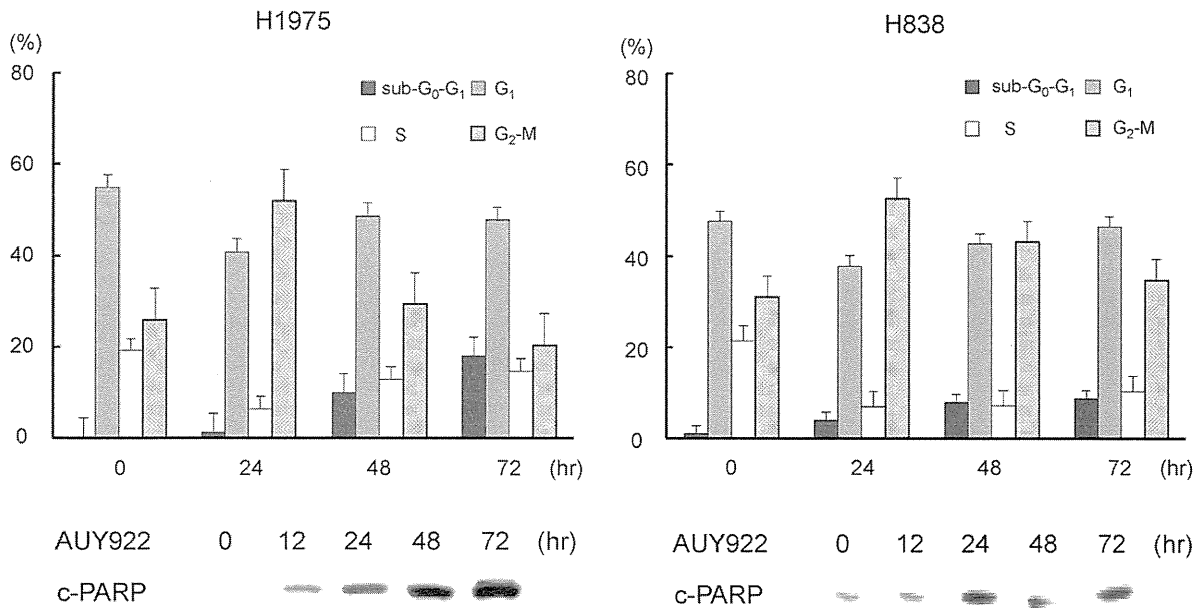

Fig. 4. The impact of AUY922 on cell cycle distribution and induction of apoptosis. Using two sensitive cell lines, cell cycle distribution was analyzed using flow cytometry and cleaved PARP expression was examined using Western blotting. After treatment of AUY922, sub-G_0-G_1 DNA content increased in a time-dependent manner and cleaved PARP also increased with AUY922 treatment.

4. Discussion

In this study, we found that AUY922 had a strong anti-proliferative effect on most NSCLC cell lines. Previous studies have indicated that GM analogue Hsp90 inhibitors have an anti-tumor effect on *EGFR* mutant NSCLC cell lines, including acquired TKI-resistant NSCLC. This suggests that Hsp90 inhibitors are promising agents for resistance to EGFR-TKI in the treatment of NSCLC [12]. However, a recent clinical trial for IPI-504, an analogue of 17-AAG, failed to show its significant effectiveness for *EGFR* mutant NSCLC patients [17]. On the other hand, IPI-504 showed response to 2 of 3 NSCLC patients with *EML4-ALK* fusion gene. One of the reasons is that enrolled patients with *EGFR* mutation had been treated at least two prior EGFR-TKI agents, suggesting that the biological features of these *EGFR* mutant tumors might be different from those of untreated tumors with single oncogene addicted status. In addition, cancer cell lines with *EML4-ALK* might be more sensitive for 17-AAG than those with *EGFR* mutation [17]. Unlike GM analogues including 17-AAG, AUY992 exhibited similar anti-tumor effect not only in *EGFR* mutant tumors, but also in wild-type *EGFR* tumors with various molecular alterations including *K-ras* mutation, *EML4-ALK* fusion gene, or *MET* or *HER2* amplification. One reason is that AUY922 has a much higher affinity for the N-terminal nucleotide-binding site of human Hsp90 than other Hsp90 inhibitors and can strongly suppress the expression of many client proteins at low concentrations [20].

Cell-cycle distribution was examined in two cell lines to assess the induction of apoptosis, but the pattern of distributions was not identical. Many client proteins of Hsp90 are thought to be involved in the pathogenesis of cancers. The degree and manner of involvement of each client protein should vary according to the cancer type, resulting in variation of the cellular response, such as cell-cycle distribution and degree of apoptosis. This would account for the difference in pattern of cell distribution and degree of apoptosis even in the sensitive cell lines.

In our series, the two cell lines Calu3 and H1395 were regarded as being resistant to AUY922. The client proteins in Calu3 were not depleted with AUY922 treatment as much. The fact that expression of Hsp70 was induced in Calu3 confirmed the inhibition of Hsp90 with AUY922, which suggested that drug transporters or metabolic activity might not be responsible for the resistance of Calu3. The cause of preserved expression of client proteins is unclear. In contrast, H1395 showed decreased expression of the client proteins at a low concentration of AUY922, which was similar to the response in sensitive cell lines. As early recovery of client proteins under AUY922 treatment was related to drug resistance in glioblastoma [28], we examined whether there was a difference in the recovery time of depleted proteins between sensitive and resistant cell lines. However, there was no difference between them in NSCLC and the mechanism of resistance was unclear. One possible explanation for the observed resistance is that although Hsp90 has many client proteins that are generally essential for tumor proliferation and survival in the majority of cancers, when cancer cells do not depend on these client proteins for survival, the inhibition of Hsp90 may not be effective. Of clinical relevance, this point may suggest that the selection of patients suited to AUY922 treatment based on molecular properties is difficult. Further investigation to identify the factors that can predict sensitivity or resistance to AUY922 is necessary.

Our results suggest that AUY922 is not effective in MPM compared to NSCLC. Although the precise mechanism of resistance is not clear, the molecular characteristics of MPM are different from those of NSCLC [29,30]. Regarding the clinical use of AUY922, Phase I/II trials of intravenously administered AUY922 are currently ongoing (http://clinicaltrials.gov/) for patients with various types of cancer. From February 2011 to present, two interesting clinical trials have begun for advanced NSCLC. The NCT01124864 trial is for patients who have received at least two lines of prior chemotherapy, and the patients are stratified according to *K-ras* and *EGFR* mutation status. The NCT01259089 trial is for patients with lung adenocarcinoma with "acquired resistance" to EGFR-TKI. It is noteworthy that our data strongly support the use of AUY922 for the treatment of NSCLC patients with various somatic alterations or with acquired resistance to EGFR-TKI.

In conclusion, our study suggests that AUY922 is a potent candidate for the treatment of the majority of NSCLCs, independent of the major known genetic alterations.

Conflict of interest statement

None.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.lungcan.2011.09.011.

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ORIGINAL ARTICLE

Clinicopathological characteristics of surgically resected pulmonary pleomorphic carcinoma

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Abstract

OBJECTIVES: Since few large-scale studies of patients with pleomorphic carcinoma have been documented, factors affecting survival after pulmonary resection for pleomorphic carcinoma, as well as its clinicopathological characteristics, are still unknown. For a better understanding of the patients undergoing resection of pulmonary pleomorphic carcinoma, we reviewed our experience with these patients.

METHODS: Between 2002 and 2010, 26 patients with pulmonary pleomorphic carcinoma underwent macroscopically complete pulmonary resections. Various perioperative variables were investigated retrospectively to confirm a role for pulmonary resection and to analyse prognostic factors for overall survival and disease-free survival after lung resection.

RESULTS: Twenty-four patients (92%) were male. Twenty-one patients (81%) were smokers and all of them smoked more than 30 pack-years. In 25 patients (96%), the tumour was located peripherally. Twenty-three of these 25 patients revealed the tumour touching the visceral pleura widely in the preoperative chest computed tomography. In all 26 patients, the tumour was completely resected macroscopically; however, three patients (12%) had microscopically positive surgical margins. Among them, additional irradiation was conducted in two patients and additional surgical resection was performed in one patient. Combined resections were performed in 11 patients (42%), including chest wall resections in 7 patients. Overall survival rate after pulmonary resection was 48% at 5 years. Disease-free survival rate after pulmonary resection was 33% at 5 years. Patients with tumours invading the visceral pleural surface and microscopically positive surgical margin had significantly worse overall survivals ($P = 0.048$ and 0.037, respectively). However, there were no significant prognostic factors for disease-free survival.

CONCLUSIONS: Despite small number of cases, we found that pleural invasion suggested a worse prognosis for resection of pulmonary pleomorphic carcinoma. Surgical strategy might be constructed to achieve not only macroscopically, but also microscopically complete resection for such large tumours with aggressive nature and peripheral preference.

Keywords: Lung cancer • Pleomorphic carcinoma • Pleural invasion • Prognostic factor • Surgery

THORACIC

INTRODUCTION

Pulmonary pleomorphic carcinoma is a rare epithelial malignant tumour and its incidence has been reported to range from 0.1 to 0.4% of all lung cancers [1]. The 1999 World Health Organization (WHO) classification identified pulmonary pleomorphic carcinoma as a specific type of lung cancer with pleomorphic, sarcomatoid or sarcomatous elements [2]. Pulmonary pleomorphic carcinoma has a more aggressive clinical course than other non-small-cell lung cancers (NSCLCs) [3–5], and recent reports also described that pleomorphic carcinoma had a worse outcome than other NSCLCs [3, 6, 7]. Although there are several reports about the survival and prognostic factors for the patients undergoing surgical resection of pulmonary pleomorphic carcinomas, the clinicopathological characteristics of pleomorphic carcinoma are not well known, and the reliable predictors of long-term survival are still controversial [3, 6, 8, 9].

We reviewed the clinical data of patients with pulmonary pleomorphic carcinoma who underwent pulmonary resection at our hospital to better understand the clinical behaviour and the prognostic predictors of survival in this histotype of pulmonary carcinoma.

PATIENTS AND METHODS

From January 2002 to December 2010, 28 patients had undergone pulmonary resection for pleomorphic carcinoma according to our medical records. Two patients were excluded from this study because the tumour was not completely resected surgically due to its invasion of the thoracic aorta in one patient and small-cell carcinoma was also detected in the resected lung in the other patient. The patients consisted of 24 males and 2 females with a median age of 69 years (range, 49–83 years).

Medical records provided information on age, gender, presenting symptoms, smoking habits, tumour markers [preoperative carcinoembryonic antigen (CEA) levels], radiological findings, surgical procedures and pathological results (Tables 1–3). Basic bronchoscopies were performed preoperatively in order to diagnose the malignancy and to confirm the extent of tumour invasion. Preoperative evaluations included physical examinations, chest roentgenographies and tumour marker analyses. In addition, computed tomographic scans of the chest and abdomen were obtained routinely. Either computed tomography or magnetic resonance imaging of the brain was performed in order to rule out brain metastasis. Either bone scintigraphy or positron emission tomography was also conducted routinely. Staging was made based on the new International Staging System for Lung Cancer [10]. Standard surgical techniques were used and accompanied by routine systemic dissection or sampling of the hilar and mediastinal lymph nodes in every case. Intraoperative frozen tissue examinations were performed in order to determine the extent of resection and to assess nodal status. After surgical interventions, generally, the patients were examined in the outpatient clinic at 3–6-month intervals for at least 5 years. Pleomorphic carcinoma was diagnosed according to the 2004 WHO classification [11]. A diagnosis was made on the basis of the light microscopic findings and it was confirmed with immunohistochemical examinations, if needed. Pleomorphic carcinoma was defined as an NSCLC containing 10% sarcomatoid components. Sarcomatoid components were classified as follows: spindle cell type, giant cell type or the combination of spindle and giant cell types. Epithelial elements were also described. Pleural invasion was described using PI factors as follows: PI 0 (tumour within the subpleural parenchyma or invading superficially into the pleural connective tissue below the elastic layer), PI 1 (tumour invading beyond the elastic layer), PI 2 (tumour invading into the visceral pleural surface) and PI 3 (tumour invading the parietal pleura). An institutional review board approved this retrospective study, and written informed consent for the surgical intervention was obtained from each patient.

The endpoint assessment consisted of overall survival (OS) and disease-free survival (DFS) after pulmonary resection. OS was defined as the length of time between the date of the pulmonary resection and the date of the last follow-up or death by any cause. DFS was defined as the length of time between the date of the pulmonary resection and the date of proven detection of recurrence or metastases.

Statistical analysis

Statistical analyses were performed with the StatView (version 4.5) software package (Abacus Concepts, Berkeley, CA, USA). The postoperative survival rate was analysed by the Kaplan–Meier method. The prognostic influence of variables on survival was analysed using the log-rank test for univariate analyses. Differences were considered significant when $P < 0.05$.

RESULTS

Twenty-six patients with pulmonary pleomorphic carcinoma underwent a macroscopically complete pulmonary resection. All the detailed data of each patient were shown in Tables 1

Table 1: The characteristics of all patients: preoperative and pathological data

Pt. No.	Age/sex	Presenting symptoms	CEA levels	Smoking (pack-years)	Tumour location	Tumour size (mm)	p-TNM
1	49/M	Haemosputum	Normal	42	LUL	100	T3N0M0
2	57/M	Cough	Normal	84	LUL	63	T3N0M0
3	72/M	None	High	0	LUL	27	T2N1M0
4	67/M	None	High	40	LUL	25	T2N1M0
5	68/M	Haemosputum	High	120	RUL	40	T2N0M0
6	69/F	None	Normal	0	RUL	21	T2N0M0
7	68/M	Haemosputum	Normal	80	RUL	37	T2N0M0
8	71/M	None	Normal	75	RLL	20	T2N0M0
9	80/M	Cough	Normal	0	RUL	14	T2N0M0
10	75/M	Haemosputum	Normal	83	LUL	50	T2N2M0
11	48/M	Cough	Normal	90	RUL	80	T4N1M0
12	65/M	None	Normal	105	LUL	25	T2N0M0
13	78/M	None	High	31	LUL	38	T2N0M0
14	65/M	Cough	Normal	45	RUL	60	T2N0M0
15	74/M	Back pain	Normal	40	RUL	45	T3N1M0
16	71/M	None	High	45	RLL	70	T3N2M0
17	77/M	Haemosputum	Normal	86	LUL	48	T2N0M0
18	75/M	Haemosputum	Normal	53	RUL	84	T3N0M0
19	68/F	Haemosputum	Normal	0	RLL	26	T2N0M0
20	73/M	Haemosputum	High	50	RUL	60	T2N0M0
21	76/M	Haemosputum	Normal	59	LUL	60	T3N0M0
22	49/M	None	Normal	38	LUL	20	T3N0M0
23	83/M	None	Normal	50	RUL	25	T1N0M0
24	76/M	Back pain	Normal	28	RUL	50	T4N1M0
25	50/M	None	Normal	0	LUL	45	T2N0M0
26	52/M	None	Normal	40	LLL	20	T1N0M0

CEA levels, high (≥5 ng/ml) and low (<5 ng/ml); LLL: left lower lobe; LUL: left upper lobe; RLL: right lower lobe; RUL: right upper lobe.

Table 2: The characteristics of all patients: pathological and postoperative data

Pt. No.	PI factor	Pathological stage	Microscopically complete resection	Adjuvant chemotherapy	Follow-up period (months)	Survival
1	3	IIB	No	Yes	5	Dead
2	3	IIB	No	No	6	Dead
3	1	IIA	Yes	Yes	26	Dead
4	1	IIA	Yes	Yes	24	Alive
5	2	IB	Yes	No	27	Alive
6	1	IB	Yes	Yes	28	Alive
7	2	IB	Yes	No	28	Alive
8	1	IB	Yes	No	34	Alive
9	1	IB	Yes	No	52	Alive
10	0	IIIA	Yes	Yes	99	Alive
11	3	IIIA	Yes	Yes	35	Dead
12	2	IB	Yes	Yes	3	Dead
13	0	IB	Yes	Yes	66	Alive
14	3	IIA	Yes	No	6	Dead
15	3	IIIA	Yes	No	33	Dead
16	3	IIIA	Yes	No	1	Dead
17	1	IB	Yes	No	15	Alive
18	3	IIB	No	No	15	Alive
19	2	IB	Yes	No	14	Alive
20	0	IIA	Yes	No	9	Alive
21	3	IIB	Yes	No	56	Alive
22	3	IIB	Yes	Yes	31	Alive
23	0	IA	Yes	No	15	Alive
24	3	IIIA	Yes	No	16	Alive
25	1	IB	Yes	No	21	Alive
26	0	IA	Yes	No	1	Alive

and 2. Eleven patients were asymptomatic (Table 3). Of the 15 symptomatic patients, 9 had haemoptysis, 4 had coughs and 2 had back pain. Twenty-two of the patients (85%) were smokers, and their median smoking history was 53 pack-years. The locations of the tumours were as follows: 11 cases were in the left upper lobe, 11 were in the right upper lobe, 3 were in the right lower lobe and 1 was in the left lower lobe. In 25 patients (96%), the tumour was located peripherally. Twenty-three of these 25 patients revealed the tumour touching the visceral pleura widely in the preoperative chest computed tomography. All of the patients but one did not receive the diagnosis of pleomorphic carcinoma preoperatively or intraoperatively. In one patient, a preoperative ultrasound-guided tru-cut biopsy obtained a specimen highly suggestive of pleomorphic carcinoma. Regarding the clinical staging of the tumours, 6 cases were stage IA, 10 cases were stage IB, 2 cases were stage IIA, 6 cases were stage IIB and 2 cases were stage IIIA. In contrast, the pathological staging of the tumours revealed that 2 cases were stage IA, 10 cases were stage IB, 4 cases were stage IIA, 5 cases were stage IIB and 5 cases were stage IIIA. All 26 patients underwent macroscopically complete resections; however, in three patients, pathological examinations of the resected specimens revealed the microscopic tumour invasion beyond its margin. In two patients, additional irradiation was performed for local control. In one patient, additional resection of the chest wall was performed with negative surgical margin. Lobectomies were performed in 23 cases, whereas segmentectomies were performed in two cases and wedge resection was conducted in one case. Sublobar resection was selected because of the patient's cardiopulmonary reserve. Combined resections were performed in 11 patients, and these included the chest wall with ribs in 7 patients, the diaphragm in 1 patient, the subclavian artery in 1 patient, the azygous vein in 1 patient and the pericardium in 1 patient. Adjuvant chemotherapy was recommended if the pathological staging was higher than IB, there was no contraindication for chemotherapy and the patient was physically and mentally tolerable for the treatment. In fact, several types of adjuvant chemotherapy could be performed in only eight patients (31%).

The median length of the follow-up examinations was 23 months (range, 1–99 months). The 5-year OS rate was 48% and the 5-year DFS rate was 33% (Fig. 1). Univariate analyses showed that a better OS was observed in patients without tumour invasion to the visceral pleural surface ($P = 0.048$; Fig. 2), and patients with microscopically complete resections ($P = 0.037$, Table 4 and Fig. 3). In contrast, univariate analyses did not show any significant prognostic factors for DFS.

The first sites of relapse after the pulmonary resections were locoregional in two patients, distant in seven patients and both in two patients (Table 5). Locoregional relapse included tumour recurrence at the surgical margin and pleural dissemination. Distant relapsed sites included bone in three cases, the gastrointestinal tract in two cases, the axillary lymph nodes in two cases, the adrenal gland in two cases, the liver in one case and the tonsils in one case. As shown in Table 4, 4 of 12 patients (33%) with PI < 2 showed recurrence after surgery, whereas 7 of 14 patients (50%) with PI ≥ 2 presented tumour recurrence. On the other hand, distant relapse was seen in 4 of 12 patients with PI < 2 and 5 of 14 patients with PI ≥ 2, but there was no locoregional relapse in patients with PI < 2. Furthermore, two of four patients with PI < 2 showing

Table 3: Patient characteristics

Age	49–83 years (median 69 years)
Gender	
Male	24
Female	2
Presenting symptoms	
Yes	15
No	11
CEA levels	
High (≥5 ng/ml)	6
Low (<5 ng/ml)	20
Smoking habits	
Yes	22
No	4
Tumour location	
Upper lobe	22
Lower lobe	4
Tumour size	14–100 mm (median 45 mm)
P-T stage	
1	2
2	15
3	7
4	2
PI factor	
0	5
1	7
2	4
3	10
P-N stage	
0	19
1	5
2	2
P-staging	
I	12
II	9
III	5
Microscopically complete resection	
Yes	23
No	3
Adjuvant chemotherapy	
Yes	9
No	17

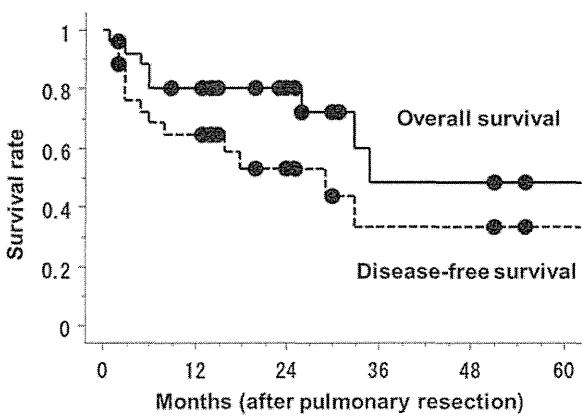

Figure 1: OS and DFS for patients after pulmonary resection ($n = 26$).

distant relapse had pN2 disease, whereas one of five patients with PI ≥ 2 presented with pN2 disease. In two relapsed patients with PI 3, who were both pN0, tumour relapse was detected very early both locoregionally and distantly at the same time (3 and 5 months) after surgery.

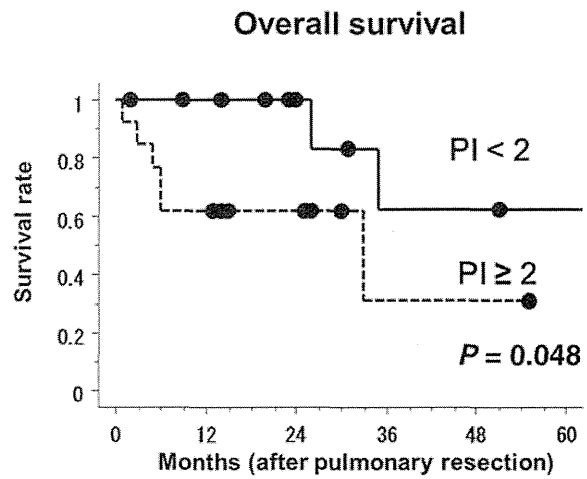

Figure 2: OS for patients with or without tumours invading the visceral pleural surface (PI ≥ 2 or PI < 2). A better OS was observed for patients without tumours invading the visceral pleural surface (PI < 2, $P = 0.048$).

DISCUSSION

In 1999, pleomorphic carcinoma was recognized as a neoplasm with pleomorphic, sarcomatoid or sarcomatous elements in the category of carcinomas by the WHO classification [2]. Before 1999, pleomorphic carcinoma was considered as a variant of other well-known lung carcinomas because of its biphasic appearance and its frequent association with other histologic types [12]. Diagnosis of this histotype was problematic and confusing because of the lack of uniform diagnostic criteria. Thus, few studies of a large series of patients with pulmonary pleomorphic carcinoma have been described in the international literature [3, 6, 8, 9], and we therefore decided to report our experience with pulmonary resections in patients with pleomorphic carcinoma in order to evaluate the role of resection in their treatment with a focus on OS and DFS. The 5-year OS rate for patients who underwent a pulmonary resection for pleomorphic carcinoma was ~40% in studies with a relatively large number of patients [3, 9], which was consistent with our results.

Several favourable prognostic factors have been reported, such as no nodal involvement [3, 6, 8, 9], complete resection [6], lower pathologic stages [9] and massive necrosis [9]. According to Yamamoto *et al.* [6], pleural involvement was not a significant adverse prognostic factor, but they did suggest that patients with no pleural involvement had a tendency for a better OS. In our study, we confirmed that patients with tumours that did not invade the visceral pleura had a better OS. This result suggests the aggressive feature of this malignant tumour, which favours peripheral location. In NSCLCs, visceral pleural invasion was also significantly associated with a higher frequency of lymph node involvement [13, 14] and was observed significantly more frequently in tumours with factors indicative of tumour aggressiveness and invasiveness [13]. In addition, microscopically incomplete resection, which was seen in only patients with PI 3, was also an adverse prognostic factor. This also implied that pleural invasion might jeopardize the patients due to the incomplete local control of the disease.

In terms of the relationship between the first relapsed site and tumour pleural invasion, two of the four distantly relapsed patients with PI < 2 presented with nodal metastasis, whereas

Table 4: Univariate analysis for OS

Variables	Number of patients	2-year OS (%)	5-year OS (%)	*P*-value
Age				
<70 years	13	66.7	0	0.14
≥70 years	13	92.3	63.3	
Gender				
Male	24	78.4	47.0	-
Female	2	100	-	
Presenting symptoms				
Yes	15	80.0	48.0	0.78
No	11	80.8	60.6	
CEA levels				
High (≥5 ng/ml)	6	83.3	55.6	0.85
Low (<5 ng/ml)	20	78.9	47.4	
Smoking habits				
Yes	22	76.4	45.8	0.69
No	4	100	-	
Tumour size				
<49 mm	16	93.3	53.3	0.21
≥50 mm	10	60.0	40.0	
P-T stage				
1, 2	17	87.5	72.9	0.13
3, 4	9	66.7	-	
PI factor				
<2 (0, 1)	13	100	62.5	0.048
≥2 (2, 3)	13	61.5	-	
P-N stage				
0	19	77.8	77.8	0.29
1, 2	7	85.7	21.4	
P-stage				
I, II	21	80.0	68.6	0.45
III	5	80.0	26.7	
Microscopically complete resection				
Yes	23	86.5	51.9	0.037
No	3	-	-	
Adjuvant chemotherapy				
Yes	9	77.8	41.5	0.61
No	17	81.6	-	

OS: overall survival. –, no data were applicable since any patients in a group did not survive longer than 5 years.

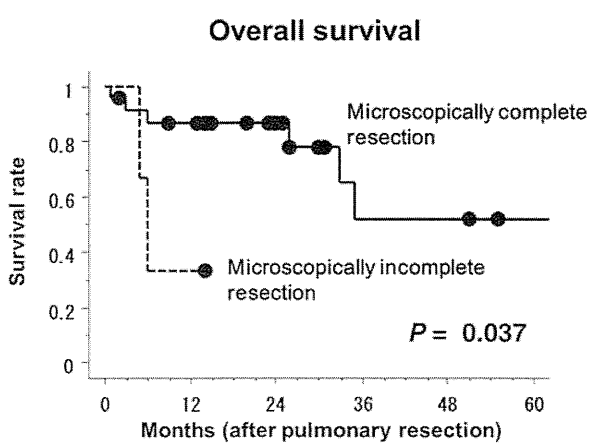

Figure 3: OS for patients with or without microscopically complete resection. A better OS was observed for patients with microscopically complete resection ($P = 0.037$).

Table 5: The first relapsed sites according to the tumour pleural invasion

PI factor	Total patients	Relapsed patients	Locoregional relapse	Distant relapse
0	5	2	0	2
1	7	2	0	2
2	4	2	1	1
3	10	5	3[a]	4[a]

[a]In two patients, locoregional relapse and distant metastasis were detected at the same time.

one of the five distantly relapsed patients with PI ≥ 2 had nodal metastasis. In addition, two relapsed patients with PI ≥ 2, who developed simultaneous locoregional and distant metastasis relatively soon after surgery, did not show nodal metastasis. Despite the small number of cases, these facts partly support the idea that pleural invasion might significantly affect OS. In the current study, nodal involvement was not a significant adverse prognostic factor, which might be because there were only two patients with pN2 disease in our series of patients. Furthermore, one patient with pN2 (patient 10) survived 99 months after surgery, probably because he presented only one station pN2 disease.

Pulmonary pleomorphic carcinoma often presents in symptomatic male smokers as a large peripheral lesion [9]. It is of note that our series of patients also exhibited this feature, which was seen as part of the presenting symptoms of 58% of the patients, 92% of the males, 85% of the smokers, 38% of patients with tumours more than 50 mm and there was a peripheral location in all patients but one.

In terms of adjuvant therapies, there has yet been no consensus on the treatment of pleomorphic carcinoma even among specialists. Since few studies have reported on the use of chemotherapy for pulmonary pleomorphic carcinoma, treatment regimens remain controversial [4, 9]. Therefore, we have not designed any fixed criteria or regimen as an adjuvant setting in this series of patients. In fact, there was no significant effect on outcomes of adjuvant chemotherapy in our study. However, Kaira *et al.* [15] described that a mutation of the epidermal growth factor receptor was recognized in ~20% of patients with pleomorphic carcinoma, and the use of a molecular targeting drug might improve the outcomes of the patients with pleomorphic carcinoma. The use of radiation therapy for pleomorphic carcinoma of the lung also remains controversial [6]. In our series, we performed adjuvant radiation therapy on two patients with microscopically incomplete resections. One patient relapsed with a tumour soon after the irradiation, whereas the other patient was still alive without an evident relapse 15 months after surgery.

There are several limitations to our analysis. The retrospective design is the most practical way of addressing our question because of the low incidence of pleomorphic carcinoma, but the results should be interpreted with caution. That is, the adverse prognostic factors suggested in our study should be reconfirmed by a prospective study. Furthermore, our results were based on a small number of patients at one institution. We recognize that this is the biggest limitation of our study and, therefore, a prospective, large-scale study at multiple institutions is needed in the future to confirm the current results.

In conclusion, despite small number of cases, we found that pleural invasion of the visceral pleural surface suggested a worse prognosis after resection of pulmonary pleomorphic carcinoma. Furthermore, microscopically incomplete resection was also an adverse prognostic factor. Therefore, surgical strategy might be constructed to achieve not only macroscopically, but also microscopically complete resection for such large tumours with aggressive nature and peripheral preference; however, an accumulation of cases is necessary to accurately evaluate prognostic factors and to determine the selection criteria for resection.

Conflict of interest: none declared.

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Higher expression of EphA2 and ephrin-A1 is related to favorable clinicopathological features in pathological stage I non-small cell lung carcinoma

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ABSTRACT

Background: The overexpression of receptor tyrosine kinase EphA2 has been reported in various cancers. In non-small cell lung cancer (NSCLC), a positive correlation has been reported between high EphA2 immunohistochemical staining level and poor prognosis. However, its ligand, ephrin-A1, is supposed to act as a tumor suppressor via the kinase activity of EphA2. Thus, the biphasic roles of this system are not fully elucidated. We retrospectively evaluated the expression levels of EphA2 and ephrin-A1 in surgically treated pathological (p-) stage I NSCLC tumor samples, and their relation to clinicopathologic features or postoperative prognoses.

Methods: The levels of EphA2 and ephrin-A1 mRNA expression were quantified by real-time reverse-transcription polymerase chain reaction in tissue samples from p-stage I NSCLC patients who had undergone complete resection in our facility ($n=195$). They were divided into two (EphA2/ephrin-A1-Low and -High) groups based on the median expression level, and their respective clinicopathologic features and prognoses were analyzed. Furthermore, samples were stained immunohistochemically and classified into four groups according to their staining levels, and their prognoses analyzed.

Results: Marked demographic differences were found between EphA2/ephrin-A1-Low and -High groups. Both EphA2-High and ephrin-A1-High groups had more females, no smoking history, adenocarcinoma histology, well-differentiated carcinomas, p-stage IA patients, and patients with EGFR gene mutations. Five-year overall survival rates of the EphA2-Low and the EphA2-High patient groups were 68.9% and 86.1%, respectively ($P=0.017$), and five-year disease-free survival rates were 69.9% and 83.2%, respectively ($P=0.035$). There were no statistical differences between ephrin-A1-Low and ephrin-A1-High groups concerning postoperative survival. Although showing smaller differences, the findings from the immunohistochemical analyses supported the above results.

Conclusions: Higher expression of EphA2 and ephrin-A1 was more related to the female sex, reduced smoking status, adenocarcinoma, well differentiated carcinomas, p-stage IA, and EGFR gene mutations. Higher EphA2 mRNA expression in p-stage I NSCLC patients was positively related to improved prognoses. These results may reflect a tumor suppressive role for the EphA2/ephrin-A1 system in a population of patients restricted to p-stage I NSCLC.

1. Introduction

Non-small cell lung cancer (NSCLC), which accounts for 80–85% of all primary lung cancers, is rapidly increasing and is the leading cause of cancer-related deaths throughout the world [1]. Although any possibility of a cure rests almost entirely with complete surgical resection, postoperative recurrence rate is still high and the survival rate remains low compared with other types of cancers [2]. Even in pathological (p-) stage I NSCLC, the overall five-year survival rate after complete surgical resection is 50–75% [3,4]. Developing and deploying more effective therapeutic modalities are vital for improving its curability.

Like other types of cancers, recent strategies for NSCLC treatment are focused on inhibiting oncogenic pathways or molecules that are involved. Receptor tyrosine kinases (RTKs) are the most common class of molecules investigated for that purpose, as the advent of small-molecule RTK inhibitors like erlotinib and gefitinib has already changed the treatment strategies of NSCLC dramatically [5]. Further investigations on other RTKs will provide us with novel treatment options.

The Eph families, which were first identified in 1990, are considered the best candidates because they comprise the largest group of

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