Table 2—*Multivariate Analysis of Risk Factors for Recurrence*

Factors	Unfavorable	Favorable	Hazard Ratio	95% CI	*P* Value
Age, y	≥ 65	< 65	1.449	0.988-2.125	.057
Gender	Female	Male	1.033	0.587-1.815	.911
Smoking habits	Current or former smoker	Nonsmoker	1.02	0.563-1.847	.948
FEV_1/FVC, %	< 70	≥ 70	1.373	0.889-2.121	.153
CEA[a]	Elevated	Within normal range	1.129	0.758-1.683	.550
Tumor size, mm	> 20	≤ 20	1.005	0.686-1.473	.978
Histologic differentiation	Poorly/moderately differentiated	Well differentiated	2.310	1.449-3.683	< .001[b]
Intratumoral vessel invasion	Present	Absent	2.913	1.915-4.432	< .001[b]
Visceral pleural invasion	Present	Absent	1.829	1.200-2.787	.005[b]

See Table 1 for expansion of abbreviation.

[a]Normal upper limit at 5 ng/mL.

[b]Indicates significance.

number of T1a patients develop recurrence, which results in cancer death. In our study of stage I NSCLC patients with tumors of 3 cm or less, multivariate analysis found that tumor size was not a significant risk factor for recurrence. Patients with a 2- to 3-cm tumor may include a subgroup with good prognosis, whereas patients with a tumor of 2 cm or less may include a subgroup with poor prognosis.

By multivariate analyses, we identified three independently significant predictors for recurrence: histologic differentiation (hazard ratio, 2.310), presence of intratumoral vessel invasion (hazard ratio, 2.913), and presence of pleural invasion (hazard ratio, 1.829). Of these three risk factors, VPI had already been adopted as a specific description in the TNM classification of the UICC staging system in the mid-1970s, and has remained unchanged: a tumor 3 cm in maximum dimension, if it is associated with VPI, is upgraded to T2.[13] In the UICC's seventh edition of TNM for lung and pleural tumors, VPI is clearly defined, and T1 tumors continue to be upgraded to T2 when the visceral pleura elastic layer is invaded.[1] Our results supported the concept of upgrading due to VPI.

Ichinose et al[4] reported that poorly differentiated histology was an independent prognostic factor for poor survival in stage I NSCLC patients. The 10-year recurrence-free probability was significantly lower in patients with moderately/poorly differentiated tumors (67.8%) than in patients with well-differentiated tumors (90.1%). These results indicate that tumor differentiation has a significant impact on clinical outcome. In the current study, the histologic grade was determined by a single pathologist (G. I.) throughout the trial, which should have contributed to diagnostic consistency. However, no objective criteria have been established for standardized differentiation grade diagnoses. The World Health Organization's *Histologic Typing of Lung and Pleural Tumors*[2] merely makes brief reference to the histologic grade in adenocarcinoma and squamous cell carcinoma. Objective differentiation-grading criteria need to be established for reproducible assessment.

In most studies that included this factor in analyses, intratumoral vessel invasion has also been reported to be a strong independent recurrence predictor in

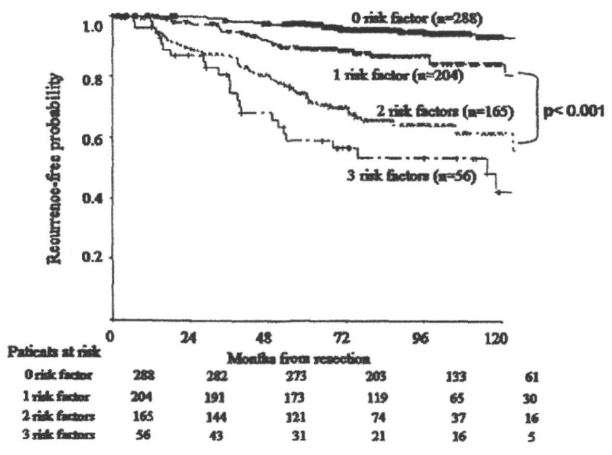

FIGURE 1. Recurrence-free probability curves according to the number of risk factors.

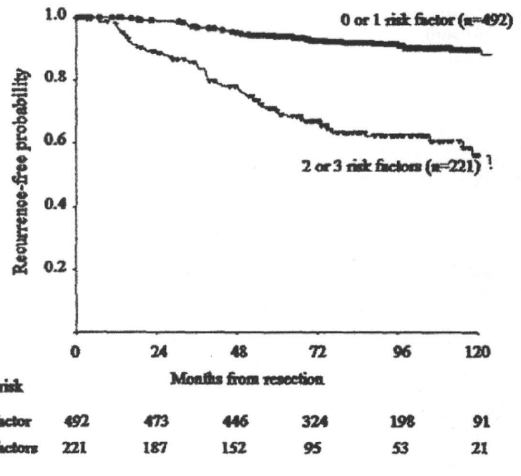

FIGURE 2. Recurrence-free probability curves according to the combined number of prognostic factors: zero or one, and two or three.

Table 3—*Multivariate Analysis of Risk Factors for Recurrence in Stage IA Patients*

Factors	Unfavorable	Favorable	Hazard Ratio	95% CI	*P* Value
Age, y	≥ 65	< 65	1.558	0.999-2.526	.051
Gender	Female	Male	1.100	0.544-2.225	.791
Smoking habits	Current or former smoker	Nonsmoker	1.198	0.562-2.555	.64
FEV_1/FVC, %	< 70	≥ 70	1.500	0.897-2.511	.122
CEA	Elevated	Within normal range	1.303	0.801-2.117	.286
Tumor size, mm	> 20	≤ 20	1.015	0.636-1.621	.949
Histologic differentiation	Poorly/moderately differentiated	Well differentiated	2.622	1.458-4.717	.001[b]
Intratumoral vessel invasion	Present	Absent	3.100	1.902-5.054	< .001[b]

See Table 1 for expansion of abbreviation.

[a]Normal upper limit at 5 ng/mL.

[b]Indicates significance.

pathologic stage I disease,[3-6,9] with some exceptions.[7,8] The current study also suggested that intratumoral vessel invasion status is a significant risk factor for recurrence in stage I NSCLC patients with tumors up to 3 cm in maximum dimension.

Although T1 tumors are upgraded to T2 due to VPI in the seventh edition of TNM classification, neither histologic differentiation grade nor intratumoral vessel invasion have been incorporated into the TMN classifications, including the seventh edition. In the present study, however, multivariate analysis demonstrated that these two factors were significantly stronger recurrence predictors than was VPI (hazard ratio, 1.829) in the new TNM staging system in Japanese patients. We therefore propose that, in addition to VPI, histologic differentiation (hazard ratio, 2.310) and intratumoral vessel invasion (hazard ratio, 2.913) should be examined and data concerning them collected for the next revision of the TNM staging system.

On multivariate analysis using the Cox regression model, histologic differentiation and presence of intratumoral vessel invasion were also statistically significant independent predictors for recurrence in 605 stage IA patients without VPI. Recent randomized controlled trials have shown a survival benefit from platinum-based adjuvant chemotherapy in stage II or higher NSCLC patients.[14,15] For stage IB adenocarcinoma patients, based on a large adjuvant trial and metaanalyses on oral uracil-tegafur (UFT), UFT adjuvant chemotherapy is recommended as the standard treatment in Japan.[16] Although surgery alone remains the standard treatment of stage IA patients, recent Japanese studies[16,17] showed that oral UFT may improve stage IA-T1b patient survival. High-risk small tumor N0 patients, identified by factors other than tumor size and VPI, such as differentiation and vessel involvement, may also benefit from adjuvant chemotherapy in improving survival. Improved quantification of recurrence risk should improve clinical decision making and help design future trials. This study highlighted considerable outcome disparity in stage IA NSCLC patients based on the TNM classification of the UICC, seventh edition. When we divided stage IA patients into zero-, one-, or two-risk-factor groups, we found 10-year recurrence-free probabilities of 93.2%, 85.0%, and 58.9%, respectively. The two-risk-factor patients accounted for 15% of T1a patients and 30% of T1b patients, and

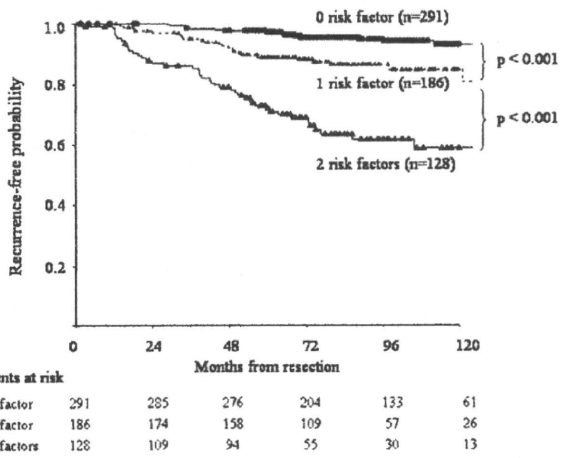

FIGURE 3. Recurrence-free probability curves in stage IA patients according to the number of risk factors.

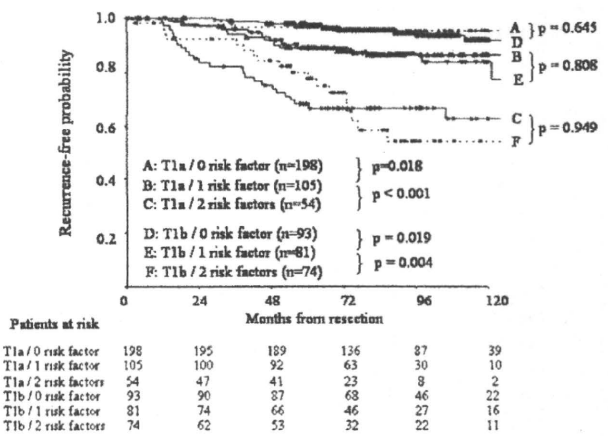

FIGURE 4. Recurrence-free probability curves of subgroups stratified according to T subclassification (T1a or T1b) and combined number of risk factors.

these patients may be good candidates for adjuvant chemotherapy.

Conclusions

In 713 stage I NSCLC patients with tumors up to 3 cm in maximum dimension, we identified three risk factors for recurrence that independently increase risk of recurrence: poor or moderate histologic differentiation, presence of intratumoral vessel invasion, and presence of VPI. Poor or moderate histologic differentiation and presence of intratumoral vessel invasion were significantly better recurrence predictors than was VPI. These factors should be examined and their data collected for the next revision of the TNM staging system. Poor or moderate histologic differentiation and presence of intratumoral vessel invasion were also shown to be independently significant recurrence risk factors in stage IA patients without VPI. When two of these factors are combined, a high-risk subgroup of stage IA NSCLC patients can be identified, and this group may benefit from adjuvant chemotherapy.

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Clinical response of large cell neuroendocrine carcinoma of the lung to perioperative adjuvant chemotherapy

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Patients with large cell neuroendocrine carcinoma (LCNEC) of the lung are considered to have poor prognosis. However, the benefit of adjuvant chemotherapy for these patients has not been established. In this study, we retrospectively evaluated the efficacy of perioperative chemotherapy for patients with completely resected LCNEC in a single-center setting. From 1999 through 2007, 45 patients with surgically resected LCNEC or mixed LCNEC containing at least one portion of the neuroendocrine differentiation or morphology in non-small cell lung carcinoma were enrolled as participants of this study. Survival rates were calculated by the Kaplan–Meier method. Differences between survival curves were computed with the log-rank test. For multivariate analysis, the Cox's proportional hazards regression model was used to evaluate variables that were significant predictors of survival. Of 1397 patients undergoing surgical resection for primary lung cancer from 1999 to 2007, 45 (3.2%) were classified as LCNEC. Thirty-six (80%) patients were men, and nine (20%) were women. Twenty-four (92%) of 26 patients were present or past smokers. Twenty-three (41%) of 45 patients received perioperative chemotherapy, including seven induction chemotherapies and 16 adjuvant chemotherapies. Survival of patients who underwent perioperative adjuvant chemotherapy was significantly higher than that of those who received surgery alone ($P=0.04$). The 5-year survival rate of patients who underwent perioperative adjuvant chemotherapy was 87.5%, whereas that of patients who underwent surgery alone was 58.5%. Even in stage I cases, perioperative adjuvant chemotherapy still favors survival compared with surgery alone. In the Cox proportional hazard multivariate analysis, surgery with or without chemotherapy showed an independent prognostic influence on overall survival ($P=0.0457$). Patients who received surgery alone were 9.5 times more likely to die than patients who underwent surgery plus chemotherapy. In conclusion, perioperative chemotherapy will be needed to improve survival in patients with LCNEC. As the population of LCNEC is small, it has been difficult to conduct randomized controlled trials to show the survival benefit of adjuvant chemotherapy. This should be, therefore, evaluated further in prospective multi-institutional phase II trials. *Anti-Cancer Drugs* 21:89–93 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Keywords: large cell neuroendocrine carcinoma, perioperative adjuvant chemotherapy, prognostic factor

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Introduction

Pulmonary neuroendocrine tumors include a spectrum of four clinicopathological entities classified on the basis of the morphological and biological features: typical carcinoid and atypical carcinoid, which are tumors of low-grade and intermediate-grade malignancy, respectively, and large cell neuroendocrine carcinoma (LCNEC) and small cell lung carcinoma (SCLC), which are considered to be high-grade malignancy tumors. Travis *et al.* [1] were the first to propose the term LCNEC in 1991. In 1999, the World Health Organization proposed a classification with rigorous histologic criteria for each subtype of LCNEC: (i) neuroendocrine morphologic features; (ii) high mitotic rate; (iii) necrosis; (iv) cytologic features of non-small cell lung carcinoma (NSCLC); and (v) positive immunohistochemical staining for one or more neuroendocrine markers [2].

LCNEC of the lung represents approximately 2–3% of lung malignancies and is known for its poor prognosis [3–10]. A recent large sample study from Japan, which was conducted in a retrospective, multi-institutional setting, including a critical review of histology by an expert panel, apparently showed that no prognostic difference was noted between LCNEC and SCLC [11]. Now, it is recognized that high-grade neuroendocrine histology uniformly indicated poor prognosis regardless of its histologic type. Considering the necessity of adjuvant

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therapy for LCNEC, this preliminary study was conducted to evaluate the efficacy of perioperative adjuvant chemotherapy in a retrospective single-center setting.

Patients and methods

Patients and pathological review

Of 1397 patients who underwent surgical resection for primary lung cancer from 1999 to 2007 at Tokyo Medical University, 45 (3.2%) patients with the histological characteristics of LCNEC were enrolled as participants of this study. According to the histological typing of lung and pleural tumors in the World Health Organization International Histological Classification of Tumors, 3rd edition [2], LCNEC is classified as a variant of large cell carcinoma (LCC). In this schema, LCCs are classified into four types: pure LCNEC, LCC with neuroendocrine differentiation, LCC with neuroendocrine morphology, and classic LCC. As earlier studies reported that NSCLC with neuroendocrine features have similar prognosis to that of pure LCNEC [3,12–14], in this study, LCNEC, including pure LCNEC and mixed LCNEC, in which at least one portion of the neuroendocrine differentiation or morphology in NSCLC, were enrolled.

Neuroendocrine morphology includes the following features: (i) neuroendocrine morphology, such as organoid nesting, nuclear palisading, rosettes, and trabecular pattern; (ii) a high mitotic rate of at least 11 per 2 mm^2 (10 high-power fields); (iii) necrosis (often large zone); and (iv) cytologic features of NSCLC, including large cell size, low nuclear-to-cytoplasm volume ratio, vesicular or fine chromatin, or frequent nucleoli, or a combination of these. Immunohistochemical analysis was performed to confirm the neuroendocrine differentiation of the tumors. For this purpose, formalin-fixed paraffin sections were stained for a panel of neuroendocrine markers, including chromogranin A (1 : 1500; Dako, Tokyo, Japan), synaptophysin (1 : 100; Dako), and neural cell adhesion molecule (1 : 50; Dako), using standard methods. Immunohistochemically, the tumor was considered as positive if the tumor cells exhibited focal, patchy, or diffuse staining in the intracellular locations for each antigen. Histological specimens were diagnosed by experienced pathologists (K.M. and J.M.) at the Department of Pathology, Tokyo Medical University.

Clinical findings and statistical analysis

Clinical information about the cases was obtained from the medical records. The final pathological staging was assigned according to the International Union Against Cancer TNM classification system [15]. Follow-up information was completely acquired within the last 6 months for all the patients. The survival time was measured from the date of first treatment, including operation or induction chemotherapy. All statistical analyses were performed with the StatView software package (StatView 5.0; SAS Institute Inc., Cary, North Carolina, USA). Survival rates were calculated by the Kaplan–Meier method. Differences between survival curves were computed with the log-rank test. For multivariate analysis, the Cox's proportional hazards regression model was used to evaluate variables that were significant predictors of survival. In all statistical analyses, significance was defined as a P value of less than 0.05.

Results

Patient demographics

Of 1397 patients undergoing surgical resection for primary lung cancer from 1999 to 2007, 45 (3.2%) were classified as LCNEC, including pure LCNEC, LCC with neuroendocrine differentiation, LCC with neuroendocrine morphology, and mixed LCNEC. Of these, only 7 (15%) were diagnosed as LCNEC before surgery, nine as poorly differentiated adenocarcinoma, two as poorly differentiated squamous cell carcinoma, one as LCC, four as NSCLC, three as SCLC, and one as adenosquamous cell carcinoma.

The clinicopathological profiles of the cases are summarized in Table 1. The median age of the patients was 65 years (range, 40–83 years). Thirty-six (80%) patients were men, and nine (20%) were women. Twenty-four (92%) of 26 patients were present or past smokers in the clinical chart. Operative procedures performed included 40 (90%) lobectomies, three (6%) wedge resections, and two (4%) pneumonectomies. The distribution of pathological stage was 11 (25%) stage IA, 16 (36%) stage IB, 5 (11%) stage IIA, 3 (6%) stage IIB, 7 (16%) stage IIIA, and 2 (4%) stage IIIB.

Twenty-three (41%) of 45 patients received perioperative chemotherapy, including seven induction chemotherapies and 16 adjuvant chemotherapies. The regimens of these chemotherapies are listed in Table 2. Of these, 21 (91%) patients had received platinum-based chemotherapy before or after surgery.

Survival in all stages

Survival data were collected for each patient from the data of operation or first chemotherapy, with a median

Table 1 Clinicopathological profiles of 45 surgically resected LCNEC cases

Mean age, range (years)	66.4 (40–83)
Sex, *n* (%)	
Men	36 (80)
Women	9 (20)
Present and past smoking, *n* (%)	24/26 (92)
Pathological staging (%)	
IA	11 (25)
IB	16 (36)
IIA	5 (11)
IIB	3 (6)
IIIA	7 (16)
IIIB	3 (6)
Surgical procedure, *n* (%)	
Wedge resection	3 (6)
Lobectomy	40 (90) (bilobectomy, three cases)
Pneumonectomy	2 (4)

LCNEC, large cell neuroendocrine carcinoma.

Table 2 Regimens of perioperative chemotherapy

Induction chemotherapy, *n* (%)	7 (13)
CBDCA + PTX	4
CDDP + CPT-11	3
Adjuvant chemotherapy, *n* (%)	16 (28)
CBDCA + PTX	5
UFT	2
CDDP + VP-16	2
CDDP + CPT-11	2
CBDCA + CPT-11	4
CBDCA + DTX	1
Surgery alone, *n* (%)	22 (59)

CBDCA, carboplatin; CDDP, cisplatin; CPT-11, topotecan; DTX, docetaxel; PTX, paclitaxel; UFT, uracil and tegafur; VP-16, etopocide.

follow-up of 31 months, because recent cases were enrolled and analyzed. As shown in Fig. 1, the 2 and 5-year overall survival for the entire group was 89.2 and 69.4%, respectively (40.3% 5-year survival reported by Asamura *et al.* [11]) (Table 3). Survival of patients who underwent perioperative adjuvant chemotherapy was significantly higher than that of those who underwent surgery alone ($P = 0.04$) (Fig. 2). The 5-year survival rate of patients who underwent perioperative adjuvant chemotherapy was 87.5%, whereas that of patients who underwent surgery alone was 58.5% (88.9% 5-year survival reported by Iyoda *et al.* [16]) (Table 3). There was no significant difference in clinicopathological factor, including age, sex, pathological staging, and surgical procedure between perioperative adjuvant chemotherapy group, and surgery alone group (data not shown).

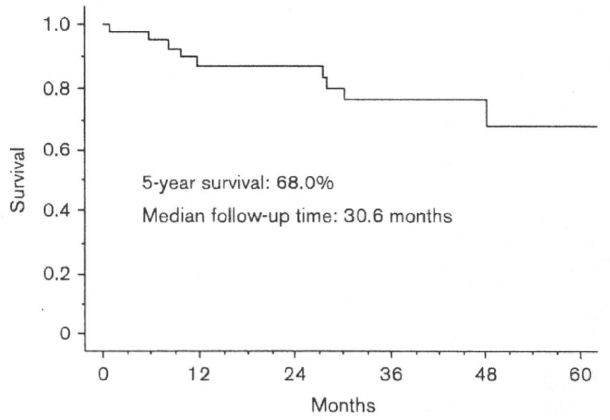
Survival in stage I

Figure 3 shows a comparison of survival between LCENC ($n = 27$) and NSCLC ($n = 774$) in early-stage lung cancer patients who underwent surgery during the same period. Prognosis is significantly worse in stage I patients with LCNEC than in those with NSCLC (65.4 vs. 84.5%, $P = 0.0067$). Interestingly, survival benefit of perioperative adjuvant chemotherapy can be observed even in the stage I LCNEC cases as well as in all stage cases (Fig. 4).

Multivariate analysis of survival benefit

The association of sex, age, pathological staging, and surgery with or without chemotherapy with survival was analyzed by the Cox proportional hazard multivariate analysis. As shown in Table 4, surgery with or without chemotherapy showed an independent prognostic influence on overall survival ($P = 0.0457$). Patients who underwent surgery alone were 9.5 times more likely to die during the follow-up period than were patients who underwent surgery plus chemotherapy.

Fig. 1



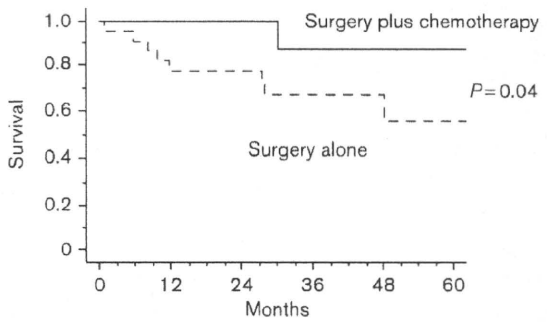
Overall survival of 45 surgically resected large cell neuroendocrine carcinoma cases.

Table 3 Five-year survival rate of LCNEC in the literature

Study	Number of cases	5-year survival rate (%)
Iyoda *et al.* [16]	15	88.9 (adjuvant chemotherapy)
Veronesi *et al.* [10]	144	42.5
Asamura *et al.* [11]	141	40.3
Battafarano *et al.* [9]	45	30.2
Doddoli *et al.* [7]	123	36.0
Paci *et al.* [6]	48	21.2
Zacharias *et al.* [14]	20	47.0
Takei *et al.* [5]	87	57.0
Iyoda *et al.* [16]	50	Approximately 35[a]
Garcia-Yuste *et al.* [17]	22	21
Travis *et al.* [4]	37	27.0

LCNEC, large cell neuroendocrine carcinoma.
[a]Number estimated from survival curve.

Fig. 2

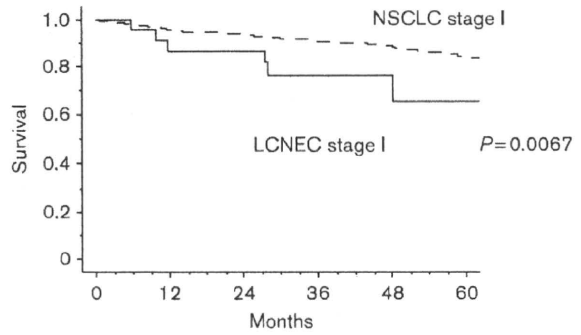


Overall survival of patients in surgery plus chemotherapy [$n=23$ (87.5%)] and surgery-alone groups [$n=22$ (55.8%)].

Discussion

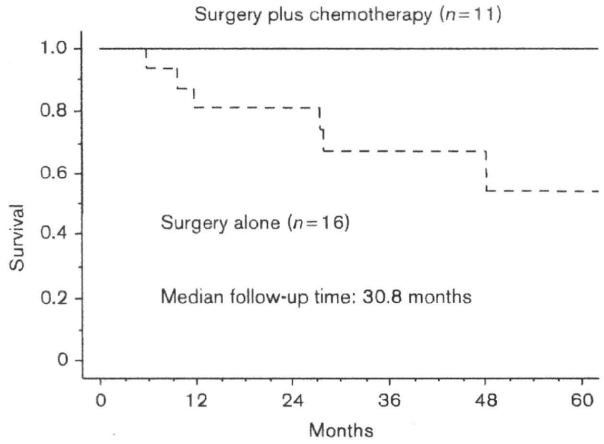
LCNEC of the lung was initially characterized by Travis *et al.* [1] in 1991, forming a separate category of neuroendocrine tumors of the lung. In 1999, the WHO proposed a new classification of pulmonary neuroendocrine tumors. As surgical resection of LCNEC in many series has been described with 5-year actuarial survival that is far worse than that reported for other histologic variants of NSCLC [4–7,9–12,14,17], considerable debate has emerged as to whether these tumors should be classified and treated as NSCLC or SCLC. Efforts to

Fig. 3



Overall survival of non-small cell lung carcinoma (NSCLC) [$n=774$ (84.5%)] and large cell neuroendocrine carcinoma (LCNEC) [$n=27$ (65.4%)] in stage I cases.

Fig. 4



Overall survival of surgery plus chemotherapy ($n=11$) and surgery-alone groups ($n=16$) in stage I large cell neuroendocrine carcinoma cases.

Table 4 Results of multivariate analysis of prognostic factors influencing survival of patients with LCNEC after treatment

Clinical factors	HR (95% CI)	*P* value
Treatment		
Surgery alone/surgery plus chemotherapy	9.472 (1.050–85.478)	0.0457[a]
Sex		
Women/men	1.400 (0.272–7.203)	0.7668
Age		
70 years and more/less than 70 years	0.491 (0.135–1.789)	0.2812
Staging		
I/II, III	0.807 (0.196–3.325)	0.7668

CI, confidence interval; HR, hazard ratio; LCNEC, large cell neuroendocrine carcinoma.
[a]Statistically significant.

identify effective adjuvant therapies might be useful in improving treatment outcomes with this aggressive type of lung cancer.

The incidence of LCNEC in our series of resected lung cancers was 2.3%, and this figure is in agreement with other series reported earlier. In addition, the mean age of patients treated for LCNEC ranged from 40 to 83 years of age, with a mean of 65 years and the predominant patients with LCNEC were men (80%) and/or smokers (92%). The overall 5-year survival for patients with LCNEC treated with surgical resection with or without chemotherapy was 68.0%, which was relatively better than that reported in earlier studies except for one study reported by Iyoda *et al.* [16] as shown in Table 3. The reason of this relatively good prognosis is that recent cases were enrolled in our study and nearly 40% of our cases received chemotherapy before or after surgery.

In an effort to improve cure rates of LCNEC patients, postoperative adjuvant chemotherapy or radiotherapy has been used in several studies. Unfortunately, no study has yet reported a definitive survival benefit of postoperative adjuvant therapy in LCNEC patients. Rossi *et al.* recently reported that adjuvant chemotherapy based on CDDP plus VP-16 was effective for patients with LCNEC [18]. As reported earlier, we also show the survival benefit of perioperative adjuvant chemotherapy for resected LCNEC in our current series. Moreover, to the best of our knowledge, this is the first report to show that the survival benefit can be observed even in stage I cases. In general, it is already known that adjuvant chemotherapy is indicated and its survival benefit is proved in stage II or III NSCLC [19–23]. However, there is no clinical trial that has shown survival benefit of adjuvant chemotherapy in stage I NSCLC. We showed the possibility of survival benefit in stage I LCNEC in this series. Our studies were conducted in retrospective cohort and were not followed in enough periods. Therefore, prospective pooling experience from multicenter to reach a large number of cases is warranted to give additional information on the impact of perioperative chemotherapy on stage I LCNEC.

As a result of high expression of the multidrug resistance gene (*MDR1*) in LCNEC, it has been suspected that LCNEC is resistant to conventional chemotherapy for NSCLC. Yamazaki *et al.* [24] reported on the clinical response of a series of 20 cases of LCNEC to chemotherapy suggesting that the response rate of LCNEC to cisplatin-based chemotherapy was comparable with that of SCLC, with a response rate of 50% for one complete response. Hiroshima *et al.* [25] postulated that chemotherapy might be as effective for LCNEC as for SCLC, because the genetic profile of LCNEC is similar to that of SCLC. The expression of p53, Ki-67, K-ras, and C-raf-1 in LCNEC is genetically and immunohistochemically more similar to that in SCLC

than in NSCLC [26,27]. Although Iyoda *et al.* [16] reported a small-sized phase II study of 15 patients with LCNEC, they were the first to report and establish the benefit of adjuvant chemotherapy for patients with completely resected LCNEC in a prospective phase II study setting, and a regimen of adjuvant chemotherapy consisting of cisplatin and VP-16, which is one of the standard regimens for SCLC, which seems promising for the improvement of the prognosis of patients with completely resected LCNEC. In addition, in our series, all the patients who underwent surgery plus perioperative chemotherapy received platinum-based chemotherapy except for two receiving UFT (a combination of uracil and tegafur) as adjuvant chemotherapy. Moreover, 14 of 23 cases (60%) in this group received platinum-based chemotherapy, including VP-16 or CPT-11, which is one of the standard regimens for SCLC. Survival analysis, including the Kaplan–Meier method and multivariate analysis using the Cox's proportional hazards regression model, showed that perioperative chemotherapy could improve the survival for patients with resected LCNEC. Moreover, even in our series with stage I disease, prognosis is significantly worse in LCNEC than in NSCLC. Therefore, adjuvant chemotherapy for stage I LCNEC might also be considered.

Conclusion

In conclusion, LCNEC of the lung is an uncommon but aggressive lung cancer associated with a poor prognosis, even in patients with stage I disease. Perioperative chemotherapy, in particular using a regimen for SCLC, such as platinum plus VP-16 or CPT-11, will be needed to improve survival in patients with resected LCNEC. As the population of LCNEC is small, it has been difficult to conduct randomized controlled trials to show the survival benefit of adjuvant chemotherapy. This should, therefore, be evaluated further in multi-institutional phase II trials.

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CASE REPORT

Extrathoracic protrusion of a chronic expanding hematoma in the chest mimicking a soft tissue tumor

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Abstract We report an uncommon clinical case of extrathoracic protrusion of a chronic expanding hematoma in the chest, mimicking a soft tissue sarcoma. A 77-year-old Japanese man was successfully treated by chest wall resection and partial decortication of the lung. The postoperative pathology examination confirmed a diagnosis of a granular cell reaction. Details of the clinical and radiographic features are presented.

Key words Chest wall · Positron emission tomography · Sarcoma

Introduction

Chronic expanding hematoma (CEH) of the chest, which is a specific form of chronic empyema or chronic pleurisy, is a rare clinical entity.[1] A slowly expanding mass sometimes develops in patients with a history of thoracoplasty, tuberculous pleurisy, or trauma.[2] CEHs can be misdiagnosed as malignant tumors because of their size and slowly progressive enlargement.[3–5] We report a rare case of extrathoracic protrusion of a CEH mimicking a soft tissue sarcoma originating from the chest wall.

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Case

A 77-year-old man was referred to our institution with a 4-month history of an expanding right chest wall mass. The patient denied having associated symptoms except intermittent pain in his right chest wall. The patient had received conservative treatment for tuberculous pleurisy with antituberculous agents at the age of 25 years.

Physical examination revealed a soft mass in the right anterior chest wall that was well circumscribed and measured 45 × 22 mm. Repeated needle biopsy at the referral hospital revealed necrotic tissue only, with an absence of viable cells. Laboratory studies revealed chronic hypochromic anemia, hemoglobin 12.1 g/dl, hematocrit 38.0%, and a normal serum level of soluble interleukin-2 receptor.

Enhanced chest computed tomography (CT) showed a large homogenous mass filling most of the right hemithorax along with central attenuation and a thick wall containing flecks of calcification; there was a small extrathoracic mass extending to the chest wall accompanied by destruction of the fifth and sixth ribs (Fig. 1). Contrast-enhanced T1-weighted images showed a homogeneous, enhanced mass with a low-intensity signal; and contrast-enhanced T2-weighted images showed a homogeneous, enhanced mass with an isointensity signal along with rib destruction. 2-Deoxy-[^{18}F]fluoro-D-glucose positron emission tomography (FDG-PET) images revealed positive uptake in the chest wall mass (Fig. 2). The maximum standardized uptake value (SUV max) of the lesion was 5.5 in the early phase and 7.1 in the late phase. These findings were interpreted as suspicious for malignant lymphoma associated with chronic empyema.

After obtaining informed consent, exploratory thoracotomy was performed to obtain a definitive diagnosis

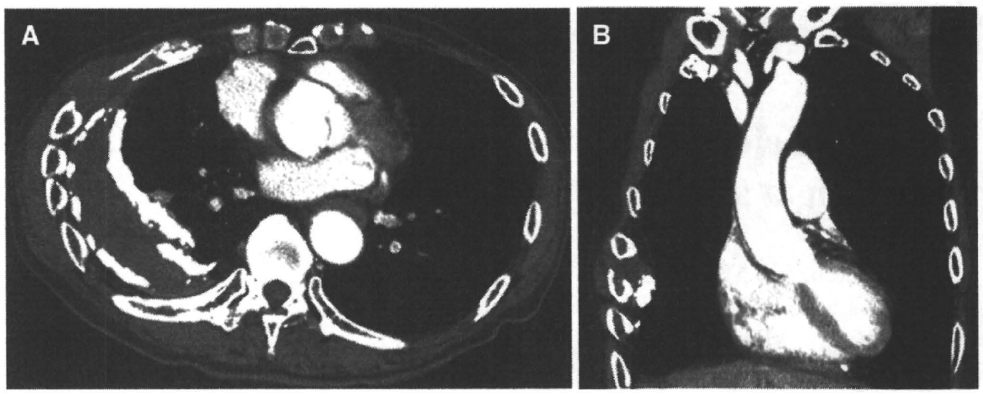

Fig. 1 Contrast-enhanced computed tomography of the chest. **A** Axial image. **B** Sagittal image. A large homogeneous mass with central attenuation and a thick wall containing flecks of calcification filled most of the right hemithorax, and a small extrathoracic mass extended to the chest wall. It was accompanied by destruction of the fifth and sixth ribs

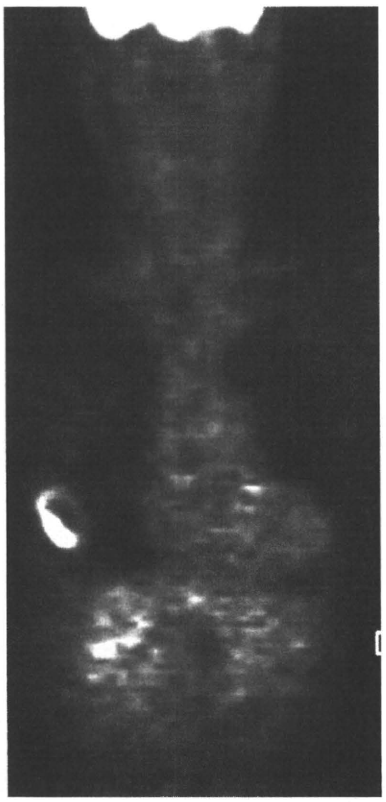

Fig. 2 Fluorodeoxyglucose (FDG) positron emission tomography image shows moderate focal FDG uptake in the extrathoracic protruding mass. Abnormal accumulation, suggesting a malignant lesion, is not visible in adjacent lesions

and to treat the expanding chest wall mass. Tumor extirpation combined with partial resection of the fifth and sixth anterior ribs was performed. Incision of the mass revealed reddish brown fluid accompanied by destruction of rib trabecula. The intraoperative rapid histological diagnosis of the mass indicated mostly necrotic tissues. The microbiological examination of the samples revealed a positive culture of methicillin-susceptible *Staphylococcus epidermidis*. The inferior aspect of the mass was associated with a larger calcified mass in the right hemithorax, seen when it was separated from the larger mass by its calcified wall. We opened the large mass carefully after puncturing it with an 18-gauge needle and found that it was composed of blood clots. Approximately 600 ml of the clots were curetted and removed, and partial decortication of the lung was performed. The duration of the operation was 118 min, and total amount of blood loss was 640 ml.

Histopathologically, the samples in the chest wall mass consisted of dense collagen tissue, a large amount of small eosinophilic amorphous necrotic tissue, and cholesterin deposition in the xanthogranulomatous reactive tissue with positive staining for CD68. These findings confirmed that the chest wall mass was a granular cell reaction with no malignant components.

The postoperative course was uneventful, and the patient was discharged without complications. At 12 months postoperatively, the patient remains well with no evidence of recurrence of the chest wall mass.

Discussion

Chronic expanding hematoma in the chest is recognized as a specific type of chronic empyema, called an organizing empyema.[3–5] Labadie and Glover, when proposing a mechanism for the expansion seen in CEHs,[6] theorized that the breakdown of leukocytes, hemoglobin, platelets, and fibrin results in an inflammatory process that effectively damages the capillaries of the capsule, increasing the permeability of the vascular wall and causing bleeding from dilated microvessels underneath the fibrous capsule. Despite the calcification of the capsule, it seems that a small degree of elasticity exists, which is the reason hematomas usually expand over a long period of time. These lesions are difficult to differentiate from soft tissue

sarcomas or other malignancies because sarcomas can also exhibit hemorrhagic and cystic changes radiologically.[5–7]

FDG-PET imaging is increasingly used in clinical oncology because it allows functional imaging of a variety of tumors. Generally, high-grade sarcomas have higher SUVs than benign lesions.[8] However, the use of FDG-PET imaging for tumor diagnosis is limited by the fact that FDG is taken up not only by tumor cells but also by macrophages and granulation and inflammation tissue. In our case, an increased FDG uptake was observed only in the extrathoracic protruding portion of the mass. The maximum SUV of the lesion was 5.5 in the early phase and 7.1 in the late phase. These findings were suggestive of a malignant lesion.[4] However, because methicillin-susceptible *Staphylococcus epidermidis* was detected in the sample from the extrathoracic protruding mass, it was thought that this inflammatory reaction likely caused the positive uptake of FDG seen in this lesion.

Surgical resection at an early stage is the preferred treatment to prevent compressing the mediastinum and contralateral lung, extrathoracic protrusion, and rupture.[2,5] Hanagiri et al. reported that the surgical procedure should be complete resection because incomplete resection might result in massive bleeding from the hypervascular subcapsular lesion.[2] However, complete resection would not have been possible without pleuropneumonectomy, which the 77-year-old patient would not have survived. Therefore, we performed careful removal of the hematoma along with a partial decortication of the lung as a palliative procedure.

Conclusion

Chronic expanding hematoma in the chest is a rare condition but should be considered in the differential diagnoses in cases in which an expanding mass is observed in the thoracic cavity. This is especially true when the patient has a history of tuberculous pleurisy, thoracotomy, or thoracic trauma. To our knowledge, this is the first report of the FDG-PET findings of CEH in the chest with SUV values suggestive of a soft tissue sarcoma.

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MT1-MMP plays an important role in an invasive activity of malignant pleural mesothelioma cell☆

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ABSTRACT

Malignant pleural mesothelioma (MPM) has a poor prognosis and is a treatment resistant tumor, which is increasing in frequency throughout the world. The poor prognosis is due to the aggressive local invasiveness rather than distant metastasis. In this study, we established a cell line of malignant mesothelioma from a clinical specimen and assessed the relationship between the expression of MT1-MMP and the invasion ability of that line, as well as the cultured cells of several other lines, using the simple method that we created previously. We established a cell line from a clinical specimen from a patient with malignant mesothelioma. We assessed the invasive activities of MPM cells in an easy-to-prepare double-layered collagen gel hemisphere (DL-CGH) system that enabled us to visualize cell movements during invasion. To assess the role of MT1-MMP in the invasive activity of MPM cells, we knocked down its expression by RNA interference (RNAi). The invasion assay with DL-CGH revealed that a high expression of MT1-MMP in MPM cells was associated with aggressive invasive activity. The RNAi of MT1-MMP indicated that the expression of MT1-MMP might have a crucial role in the invasiveness of MPM cells. The MT1-MMP expression in MPM cells is related to their capacity for locally aggressive spreading into the pleura and the surrounding tissues, and MT1-MMP should be a suitable molecular target for the suppression of the invasiveness of MPM.

Introduction

Malignant pleural mesothelioma (MPM) has a poor prognosis and is a treatment resistant tumor, which is increasing in frequency throughout the world (Robinson et al., 2005). MPM is not likely to metastasize distantly to other organs; its malignancy is due to its locally aggressive spreading into the pleura and surrounding tissues (Zhong et al., 2006; Pistolesi and Rusthoven, 2004).

It is said that the microenvironment (both cellular and extracellular elements) of the local host tissue plays an important role in the process of tumor cell invasion and that interaction between the ECM and tumor cells is essential for the degradation of ECM by the tumor cells (Liotta and Kohn, 2001). Matrix metalloproteinases (MMPs) are proteins that play an important role in this process (Curran and Murray, 2000).

The MMP family consists of more than 25 structurally related, zinc-dependent endopeptidases that are capable of degrading the basement membrane and the ECM (Konstantinopoulos et al., 2008). Among the members of this family, MMP-2 and MMP-14 play important roles in the MPM, and some epithelial malignant tumors show the overexpression of MMP-14 (Atkinson et al., 2007; Edwards et al., 2003). MMP-14, which is known as a membrane-type matrix metalloproteinase (MT-MMP), is mainly concentrated at the surface of the cells (Sato et al., 1994; Takino et al., 2007), so it is possible that MT1-MMP directly contributes to the degradation of the ECM. Because of these characteristics we focused on MMP-14 (MT1-MMP) as one of the potentially important factors that help MPM spread directly into other organs. Moreover, various methods for *in vitro* 3-D studies of cell invasion using a collagen gel have been described (Albini et al., 1987; Nyström et al., 2005; Duong et al., 2005; Takata et al., 2007), and we believe that these methods are very useful for the assessment of the invasion ability of MPM.

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In this study, we established a cell line of malignant mesothelioma from a clinical specimen. We then assessed the relationship between the expression of MT1-MMP and the invasion ability of this established cell line and other cell lines using the simple method that we created previously (Takata et al., 2007).

Materials and methods

Cell lines

The A549 (bronchiolo-alveolar carcinoma of lung) cell line was obtained from the Health Science Research Resources Bank (Osaka, Japan); the WI-38 cell line was obtained from the RIKEN Bioresource Center (Tsukuba, Japan). NCI-H28 (pleural effusion), NCI-H2452 (epithelial mesothelioma) and MSTO-211H (biphasic mesothelioma) were obtained from the American Type Culture Collection (Manassas, USA). Cells were maintained in RPMI-1640 medium supplemented with penicillin (100 U/mL), streptomycin (100 U/mL), and 10% bovine calf serum.

Establishment of a cell line of malignant mesothelioma

A clinical specimen from a patient with malignant mesothelioma was minced finely using scalpel or razor blade and digested in a cell dispersion enzyme solution (EZ; Nitta Gelatin Inc., Osaka, Japan) for 2 h. The dispersed cancer cells were treated with ethylene-glycol-tetra-acetic acid (EGTA)-trypsin and filtered through a 200-μm nylon mesh. The cells were then incubated in a collagen-gel-coated flask (CG-flask; Nitta Gelatin Inc., Osaka, Japan) containing a preculture medium with 10% fetal bovine serum (FBS; PCM-1; Nitta Gelatin Inc., Osaka, Japan) at 37 °C in 5% CO_2 overnight. We collected the viable cancer cells that adhered to the collagen gel and performed repeated subculturing until fibroblasts and other normal cells had disappeared.

Immunohistochemistry

Immunohistochemistry was performed to detect the MT1-MMP expression in paraffin sections, and tissue microarray samples were analyzed immunohistochemically. The MT1-MMP primary antibody (MAB3328, Chemicon International a Serologicals Company) was diluted 1:100 in a blocking solution before use. This diluted primary antibody was added to the tissue sections and incubated overnight at 4 °C. Antigen–antibody complexes were detected by the avidin–biotin peroxidase method (Vectastain Elite ABC Kit, Vector Laboratories, Inc., Burlingame, CA) and diaminobenzidine tetrahydrochloride reagents (DAKO EnVision™/HRP, Dako, Japan). Sections were counterstained with hematoxylin.

Western blotting

Cultured cells washed with PBS^- were lysed with 100-μl Laemmli sample buffer, and 10 μl of these samples were analyzed by SDS-PAGE. Then, the separated bands were transferred to nitrocellulose membranes (Amersham Biosciences Corp.). After washing the membranes with PBS-T, they were blocked for 30 minutes (5% skim milk, diluted by PBS-T). Following 2 rinses with PBS-T, membranes were incubated (1 hour, room temperature) with the primary antibody for MT1-MMP (MAB3328, Chemicon International a Serologicals Company), which was diluted 1:500 with 5% BSA/PBS-T. After washing with PBS-T, membranes were incubated (30 minutes, room temperature) with the secondary peroxidase-labeled sheep anti-mouse Ig whole antibody (Amersham Biosciences Corp.), which was diluted 1:5000 with PBS-T. Membranes were then washed with PBS-T and visualized using the luminoimage analyzer LAS-3000(Fuji film Inc., Tokyo, Japan) treated with a detection kit (Amersham Biosciences Corp.).

As a control assay, we performed Western blotting using the same membranes. The primary antibody was directed against β-actin (#AB6276, Abcam, Cambridge, UK), and the secondary antibody was peroxidase-labeled sheep anti-mouse Ig whole antibody (Amersham Biosciences Corp.).

Preparation of double-layered collagen gel hemispheres

Acid-soluble collagen I (Nitta Gelatin Inc., Osaka, Japan), tenfold concentrated Ham's F-12 medium, and reconstruction buffer (2.2-g $NaHCO_3$ + 4.77-g HEPES in 100 ml of 0.05-N NaOH) were mixed at a volume ratio of 8:1:1 and then seeded with cultured cells at a density of 3.0×10^6 cells/ml. Five microliters of the mixture, containing 3.0×10^4 cells, were dropped onto a plastic dish. Once the mixture had gelled, a second 30-μl drop of collagen was placed exactly on the top of the first gel drop, encapsulating it completely. The gel hemisphere was then submerged in medium and cultured. Cells were then stained with neutral red, and the gel was allowed to dry. The invasive activity of the cells was evaluated by measuring the expansion of red stain into the outer collagen layer.

RNA interference (RNAi) in WI38 and established mesothelioma cells

RNAi was performed with commercially available siRNAs (HP-validated siRNA for MT1-MMP; Qiagen GmbH, Hilden, Germany) and a non-silencing control siRNA (target sequence; AAT TCT CCG AAC GTG TCA CGT, Qiagen GmbH) according to the manufacturer's instructions. Briefly, 24 μl of transfection reagent (Hiperfect; Qiagen GmbH) was suspended in 200 μl of serum-free culture medium containing 6 μg siRNA. After a 10-minute incubation at room temperature, the mixture was added to WI38 and established mesothelioma cell culture (60-mm-round dish with 4-ml culture medium containing 10% fetal bovine serum and antibiotics mentioned above) grown to 60% confluence; the final concentration of the siRNA was 100 nM. After 24 hours (at 37 °C, 5% CO_2), these cells were suspended in phosphate buffered saline (PBS) and the cell density was calculated to prepare for the encapsulation of the cells in DL-CGH.

Time-lapse motion picture

A Moticam 2000 digital microscopy system (Shimadzu Rika Corp., Tokyo, Japan) was used to create motion pictures of cell invasion. The camera head was set at the position of the eyepiece on an inverted microscope (CKX31; Olympus Corp., Tokyo, Japan), and the entire microscope was then installed in a 37 °C, 5% CO_2 incubator without humidity (to prevent dew formation in the instruments). A DL-CGH prepared in the well of an ordinary 6-well plastic culture plate was submerged in proper medium; the residual 5 wells were filled with water to maintain humidity inside the plate. Cells were observed microscopically using a 10× objective lens, and the camera was operated from a personal computer running the Moticam 2000 software to capture and display images of living cells. Recording initiated 24 hours after DL-CGH culture continued for 96 hours. Images were captured automatically every 20 minutes, with 288 consecutive images stored as 800×600 pixel JPEG files. Using the Windows Movie Maker software (Microsoft Corp., Redmond, WA), we created a 30-second movie (saved as a WMV file) that displayed 288 consecutive images for 0.125 seconds each.

Result

Establishment of a cell line of malignant pleural mesothelioma from clinical specimen

We established an MPM cell line from a clinical specimen. To prove that these cells indeed were MPM, we sent samples of them to the

department of pathology in our hospital and requested an immunohistochemical analysis with calretinin, D2-40, CAM5.2, and AE1/AE3, which are useful markers of MPM (Mimura et al., 2007). While D2-40 was not identified, calretinin, AE1/AE3 and CAM5.2 were stained (Fig. 1). Thus, these cells were proved to be MPM immunochemically, and we had obtained a primary culture of MPM cells.

Expression of MT1-MMP in a clinical sample of MPM

To establish whether MT1-MMP was expressed in the MPM specimen we performed immunohistochemistry on the clinical samples of the MPM patient, following the technique described in Materials and methods (Fig. 2). We show the difference between normal cell structures and tumor cells in Fig. 2a. MT1-MMP was strongly expressed in the tumor cells, especially at the edge of the cells (Fig. 2b). In contrast, MT1-MMP was not expressed in the normal vascular endothelial cells.

Relationship between the MT1-MMP expression and the ability to invade, using cancer cells and normal fibroblasts

Western blotting was performed to determine if MT1-MMP was expressed in the cell lines of lung adenocarcinoma, fibroblasts and MPM (among them, the MPM cell line established in our laboratory). We detected a strong expression of MT1-MMP in WI38 and the established MPM cell line. But the expression of MT1-MMP was very weak in A549, the cell line of lung adenocarcinoma and NCI-H28, one of the acquired MPM cell lines. In the other MPM cell lines (NCI-H2452 and MSTO-211H), the expression of MT1-MMP was moderate (Fig. 3).

Then, we performed invasion assays with these cell lines using DL-CGH. A549 and NCI-H28, which showed a weak expression of MT1-MMP, showed only a minimal tendency to invade into the outer layer of collagen gel, whereas the other 4 cell lines, in which a strong expression of MP1-MMP was observed, showed a high tendency of invasion (Fig. 4). These invasive cells spread by extending their podocyte into the outer layer (time lapse).

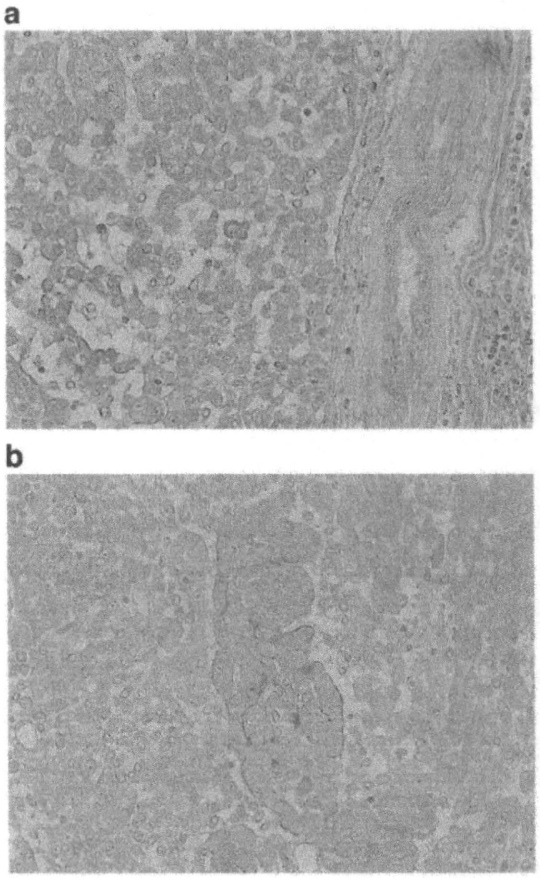

Fig. 2. Result of immunohistochemistry for MT1-MMP using surgical specimens of malignant pleural mesothelioma. Tumor cells expressed MT1-MMP strongly, but the normal vascular endothelial cells did not. (b) Especially, MT1-MMP was more expressed at the edge of the tumor cells than the inner area.

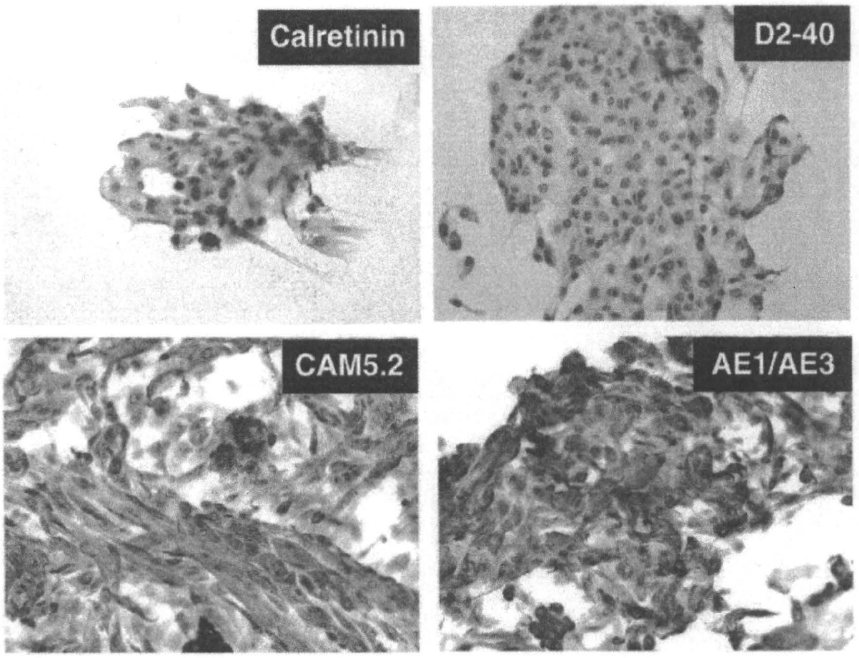

Fig. 1. Cell line established from a sample of malignant pleural mesothelioma. Calretinin, D2-40, CAM5.2, and AE1/AE3 were examined as useful markers of MPM. While D2-40 was not stained, calretinin, AE1/AE3 and CAM5.2 were stained.

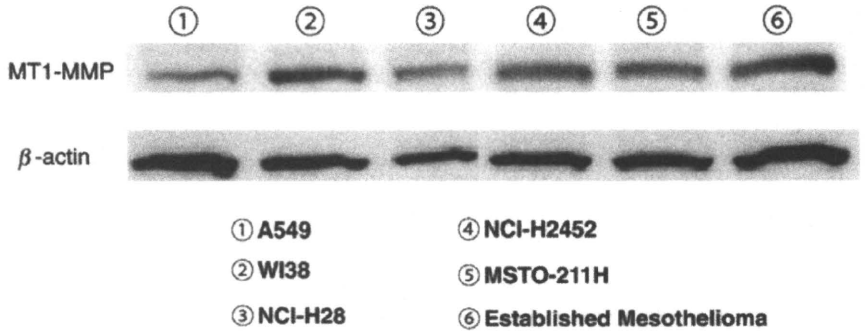

Fig. 3. Western blotting for MT1-MMP and β-actin. We detected a strong expression of MT1-MMP in WI38 and the established MPM cell line. The expression of MT1-MMP was very weak in A549, the cell line of lung adenocarcinoma, and NCI-H28, one of the acquired MPM cell lines. In the other MPM cell lines (NCI-H2452 and MSTO-211H), the expression of MT1-MMP was moderate.

Inhibition of MT1-MMP in the MPM cells and fibroblasts

We performed Western blotting to check if we could inhibit MT1-MMP in WI38 and the established MPM cell line, both of which showed wide spreading in the DL-CGH. The blotting showed about a 50% reduction in the expression of MT1-MMP protein relative to cells transfected with control siRNAs (Fig. 5).

In order to determine their invasive potential cells, transfected with inhibitory RNAs were embedded within the inner layer of DL-CGH and incubated for several days, after which we observed how the cells stained with neutral red. Cells of the established MPM cell line transfected with MT1-MMP RNAi showed only a slight invasion into the outer layer relative to the normal or control RNAi-transfected cells (Fig. 6). We also obtained similar results using WI38 cells (data not shown).

Discussion

In cell culture to establish a primary culture from a clinical specimen is one of the most difficult techniques, so many attempts result in failure. In this study, we succeeded in establishing the MPM cell line with the technique described in Materials and methods.

This is the first study to analyze the invasive activity of cell lines established from clinically resected specimens, with the aim of eventual clinical application. DL-CGH made it possible to visualize the invasive activity of the cells precisely, and the procedure would be useful for deciding a therapeutic strategy and predicting the clinical outcome. Also, the combination of DL-CGH and RNAi treatment of MT1-MMP revealed that the protein was a good candidate for a molecular target that would control the invasive activity of the cancer cells.

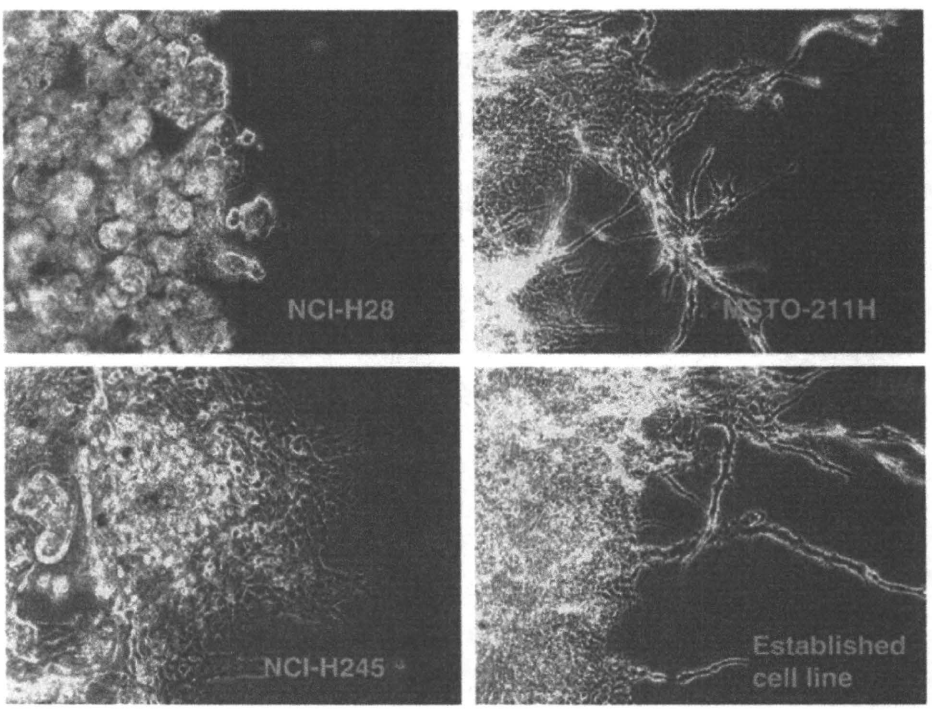

Fig. 4. Invasive activity of malignant mesothelioma cell lines assessed by DL-CGH. NCI-H28, which showed a weak expression of MT1-MMP, showed no tendency to invade the outer layer of collagen gel, whereas the other 3 cell lines showed a high tendency to invade the outer layer.

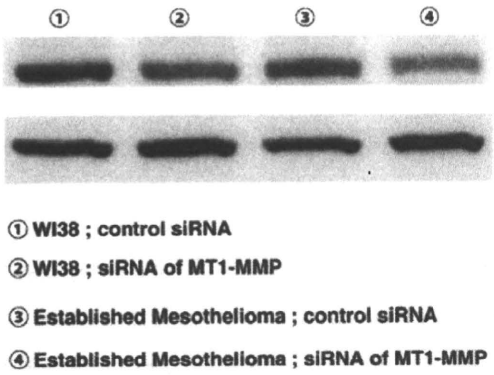

Fig. 5. Western blotting after transfection with siRNA for MT1-MMP. The blotting showed about a 50% reduction in the expression of the MT1-MMP protein relative to cells transfected with control siRNAs.

Invasion occurs within a tumor-host microenvironment, where stroma and tumor cells exchange enzymes and cytokines that modify the local extracellular matrix, stimulate migration, and promote proliferation and survival (Liotta and Kohn, 2001). It has been reported that the presence of fibroblasts is essential in cancer invasion (Olumi et al., 1999; Che et al., 2006; Gaggioli et al., 2007). The fibroblast itself is a benign mesenchymal cell that has no malignancy. Nevertheless, if fibroblasts interact with cancer cells, they play an important role in the tumor cell malignancy. In lung cancer, patients with small-sized bronchiolo-alveolar carcinoma (BAC) of the lung, in which cancer cells spread on the internal surface of alveoli but do not infiltrate interstitially, have a better prognosis than patients with BAC containing actively proliferating fibroblasts; in the latter case, cancer cells invade frequently into micro-vessels (Noguchi et al., 1995). In our study, WI38 cells (a fibroblast cell line) showed the overexpression of MT1-MMP, which indicates that fibroblasts are essential for degenerating the ECM and making tracks and scaffolding for the cancer cells. Not only fibroblasts but also some mesenchymal cells show the overexpression of MT1-MMP. Previous reports have stated that malignant mesothelioma cells produced a broad spectrum of MMPs, which might play an important role in cell invasion (Liu et al., 2001), and that the overexpression of MT1-MMP was observed in malignant mesothelioma (Sivertsen et al., 2006). In the *in vitro* experiments in this study, we observed that the level of MT1-MMP expression in established MPM cells was elevated and that these cells showed active invasion in the assay with DL-CGH.

Cancer-cell migration is typically regulated by integrins, matrix-degrading enzymes, cell–cell adhesion molecules and cell–cell communication (Friedl and Wolf, 2003). Although some tumor cells show sustained protease-independent migration resulting from a flexible amoeba-like shape change (Wolf et al., 2003), it is said that MT1-MMP is the key enzyme in the proteolytic macropatterning of collagen-rich ECM to generate space for the cell masses (Wolf et al., 2007) and that matrix degradation requires MMPs targeted to invadopodia (Sakurai-Yageta et al., 2008). In this study, we were able to establish that the invasive cells spread into the outer layer of the collagen gel by extending their podocyte (dendritic migration). Wolf et al. reported that HT1080 fibrosarcoma showed a spindle-shaped elongation of the cell body for invasion into 3-D collagen matrices (Wolf et al., 2003). We observed a similar phenomenon using the MPM cells and fibroblast. Thus, it is possible that the dendritic migration of mesenchymal cells (such as MPM) results from the overexpression of MT1-MMP.

In conclusion, the overexpression of MT1-MMP in MPM cells is associated with spreading into the surrounding matrix. Furthermore, MT1-MMP expressed in fibroblasts is involved in making a scaffold for the invasion of malignant tumor cells. Thus, we suggest that the degree of MT1-MMP expression is associated with the capacity for locally aggressive spreading into the pleura and the surrounding tissues and that MT1-MMP will be a molecular target for suppressing the invasion of MPM.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.yexmp.2010.10.008.

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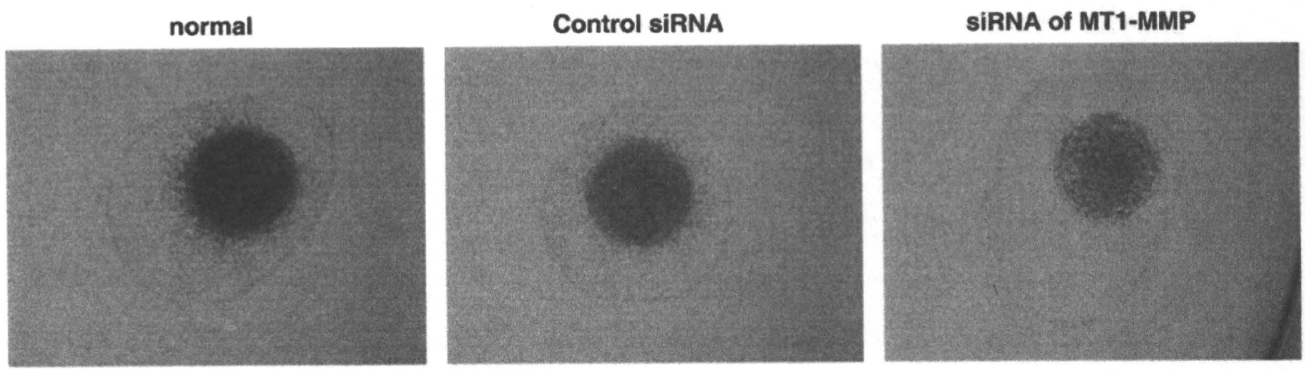

Fig. 6. Result of DL-CGH using MPM cell lines stained with neutral red. In the established MPM cell line, cells transfected with MT1-MMP RNAi showed only a slight invasion into the outer layer relative to that of the normal cells and control-treated cells.

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