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ORIGINAL PAPER

WT1 peptide immunotherapy for gynecologic malignancies resistant to conventional therapies: a phase II trial

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Abstract
Objective The aim of the present study was to analyze the long-term survival effects of WT1 peptide vaccine, in addition to its anti-tumor effects and toxicity.
Methods A phase II clinical trial was conducted during the period of 2004–2010 at Osaka University Hospital, Osaka, Japan. The patients who had gynecologic malignancies progressing against previous treatments received WT1 peptide vaccine intradermally at 1-week intervals for 12 weeks. The vaccination was allowed to further continue, unless the patient's condition became significantly worse due to the disease progression.
Results Forty out of 42 patients, who met all the inclusion criteria, underwent WT1 peptide vaccine. Among these 40 patients, stable disease was observed in 16 cases (40 %). Skin toxicity of a grade 1, 2 and 3 occurred in 25 cases (63 %), 9 cases (23 %) and a single case (3 %), respectively, and liver toxicity of grade 1 in a single case (3 %). The overall survival period was significantly longer in cases positive for the WT1 peptide-specific delayed-type hypersensitivity (DTH) reaction after the vaccination, compared to those negative for the DTH reaction ($p = 0.023$). Multivariate Cox proportional hazards analysis demonstrated that the adjusted hazard ratio for the

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negative DTH reaction was 2.73 (95 % CI 1.04–7.19, $p = 0.043$).

Conclusion WT1 peptide vaccine may be a potential treatment, with limited toxicity, for gynecologic malignancies that have become resistant to conventional therapies. Larger scale of clinical studies is required to establish the efficacy of the WT1 peptide vaccine for gynecologic malignancies.

Keywords WT1 peptide immunotherapy · Gynecologic malignancy · Anti-tumor effect · Survival · Stable disease · Toxicity

Abbreviations

CR	Complete response
CT	Computed tomography
HLA	Human leukocyte antigen
HPV	Human papillomavirus
OS	Overall survival
PD	Progressive disease
PFS	Progression-free survival
PR	Partial response
PS	Performance status
RECIST	Response evaluation criteria in solid tumor
RR	Responsive rate
SD	Stable disease
TC	Paclitaxel and carboplatin

Introduction

The Wilms tumor gene, *WT1*, was first identified as a tumor suppressor gene responsible for Wilms tumors of the kidney. However, a series of investigations demonstrated that *WT1* possesses an oncogenic, rather than a tumor-suppressive, function, and WT1 protein is expressed in various kinds of hematological and solid malignancies, indicating that immunotherapy targeting WT1 could potentially be used for treatment of a variety of such malignancies (Oka and Sugiyama 2010). In fact, WT1 has been regarded as one of the most promising target antigens for cancer immunotherapy by an American National Cancer Institute pilot project (Cheever et al. 2009). It has already been demonstrated that WT1 vaccination is safe, and encouraging reports that showed its efficacy for several kinds of tumors have been accumulated (Oka and Sugiyama 2010; Hashii et al. 2010; Oji et al. 2010; Izumoto et al. 2008). A previous phase I study empirically determined a safe dose of the WT1 peptide, which was intradermally injected with Montanide ISA 51 adjuvant for patients with solid tumors, as 3 mg per injection, and this dose was shown to have little toxicity except skin reaction of the vaccination sites (Morita et al. 2006).

Ovarian carcinoma accounts for 5 % of all cancers among women and will eventually develop in 1 of every 58 women. It has an extremely high mortality rate; consequently, aggressive cytoreductive surgery followed by chemotherapy with taxane and platinum is the gold standard for its therapy. Endometrial carcinoma is an even more common malignant neoplasm of the female pelvis and is the fourth most common cancer of women today. Endometrial carcinoma is usually confined to the uterus or pelvis, has a lower mortality rate than ovarian cancer and is commonly treated by resection of the uterus and adnexae, with or without co-resection of the regional lymph nodes. Another common gynecological tumor, uterine cervical carcinoma, is mostly associated with a human papilloma virus (HPV) infection, and its incidence appears to vary from one locality to another. It is important to note that in some Asian and South American countries, cervical carcinoma accounts for the largest percentage of cancer deaths in women. Cervical carcinoma is usually treated by radical surgery and/or radiation therapy. And lastly, yet another kind of uterine tumor, the leiomyosarcoma, although rare, has an extremely poor prognosis (DiSaia and Creasman 2002).

Tumors in the early stage of all these diseases are usually treated relatively successfully, while the advanced and recurrent forms of these diseases are often very difficult to treat. Salvage, second-line and third-line chemotherapies are effective in only a fraction of the cases, and the best available supportive care is usually proposed to the patients whose tumors have become resistant to prior therapies.

An immunotherapeutic approach that is less toxic than available chemotherapies might be a more promising option for those whose gynecologic malignancies continue to progress despite conventional chemotherapy and radiation treatments. A previous small study showed that disease stabilization was achieved in 3 (25 %) of 12 gynecologic malignancies by vaccination with an antigenic WT peptide (Ohno et al. 2009). There is only one case report on the effect of WT1 peptide for the survival elongation in a ovarian cancer case (Dohi et al. 2011). In the present phase II trial, we have analyzed for the first time the long-term survival effect of the WT1 peptide vaccine, as well as its anti-tumor effects, evaluated by the usual response evaluation criteria in solid tumor (RECIST) and toxicity.

Materials and methods

Eligibility

This phase II trial was conducted at Osaka University Hospital, Osaka, Japan, during the period of 2004–2010. Major inclusion criteria were as follows: having a gynecologic malignancy progressing despite previous treatments;

WT1 protein expression in the primary or metastatic tumor tissue using anti-WT1 rabbit polyclonal antibody C-19 (Santa Cruz Biotechnology) or anti-WT1 mouse monoclonal antibody 6F-H2 (Dako Cytometry); positive status for human leukocyte antigen (HLA)-A*2402; performance status (PS) of 0–2; and life expectancy >3 months.

Vaccination schedule

The HLA-A*2402-restricted, 9-mer modified WT1 peptide (amino acids 235–243: CYTWNQMNL) emulsified with Montanide ISA 51 adjuvant, was used for the vaccination, as previously described (Hashii et al. 2010). The dose of WT1 peptide injected was 3 mg per body. The WT1 vaccination was scheduled to be performed intradermally every week for 12 weeks but was allowed to continue even after 12 weeks, unless the patient's condition became significantly worse due to the disease progression.

Evaluation of the WT1 vaccine effects

The primary endpoints of the WT1 vaccine study were its anti-tumor effect and its toxicity. Computed tomography (CT) was performed every 4 weeks to evaluate tumor size. The anti-tumor effect was evaluated by the RECIST (version 1.1) (Eisenhauer et al. 2009) after the vaccination during 12 weeks. Adverse effects were graded based on the National Cancer Institute's Common Toxicity Criteria (version 2.0). A test for delayed-type hypersensitivity (DTH) reaction specific to the WT1 peptide used for vaccination was performed at week 4 and 8. We regarded the patient as DTH positive, if the DTH reaction of the patient was positive either at week 4 or at week 8.

Secondary endpoints were progression-free survival (PFS) and overall survival (OS). PFS was defined as the period from the date of the start of WT1 vaccination to the date of the radiologic or pathologic relapse, or to the date of the last follow-up. OS was defined as the period from the start of the vaccination to the patient's death or to the date of the last follow-up. OS was analyzed for its association with DTH.

Cancellation or termination of WT1 vaccination

If grade 3 toxicity was observed, the next injection of the WT1 vaccine was postponed until the toxicity returned to grade 2 or less. The vaccination was permanently terminated if grade 4 toxicity was detected or if a performance status of 3 or worse was observed.

Statistical analysis

MedCalc (MedCalc Software, Mariakerke, Belgium) was used for statistical analysis. The association between DTH induction and anti-tumor effect, including RECIST evaluation, PFS and OS, was analyzed by Fisher's exact test. OS curves were constructed using the Kaplan–Meier method and evaluated for statistical significance by the log-rank test. Multivariate Cox proportional hazards model (stepwise method) for the factors including age, origin of the disease, histology, evaluation of the previous therapy and number of recurrence was calculated to evaluate whether DTH was a significantly important factor on OS. Results were considered to be significant when the p value was <0.05.

Statements of ethics

This study was approved by the Institutional Review Board and the Ethics Committee of the Osaka University Hospital. All patients provided written informed consent. (Approval of this analysis: #10302, approved on March 11, 2011).

Results

Clinical characteristics of the patients and completion rate of the study schedule

During the study period, 42 patients entered the study. Among these, 2 patients were excluded from the present analysis due to protocol violation. The clinicopathological characteristics of these patients are shown in Table 1. The median age was 56 (35–75). The histological diagnosis was obtained as ovarian carcinoma in 24 cases, cervical carcinoma in 11 cases, uterine sarcoma (leiomyosarcoma and carcinosarcoma) in 5 cases. These patients had already received 1–11 (median: 3) kinds of treatments prior to the WT1 vaccination and were considered to have disease

Table 1 Clinical characteristics of patients enrolled in the phase II study

Characteristics	
Number (cases)	40
Median age (years) (range)	56 (35–75)
Type of malignancy	
Ovarian carcinoma	24 (60 %)
Cervical carcinoma	11 (28 %)
Uterine leiomyosarcoma/carcinosarcoma	5 (13 %)
Performance status	
0	35 (88 %)
1	4 (10 %)
2	1 (3 %)
Median number of previous treatment regimens (range)	3 (1–11)

resistant to conventional therapies such as chemotherapy and radiotherapy.

Injection of the WT1 vaccine was performed weekly for 1–50 (median: 14.5) times. The 12 injections prescheduled upon entry to this trial were completed in 32 of the 40 cases (80 %). Vaccination was terminated prior to the 12th injection due to progression of the disease including worsening of PS in 8 cases (20 %).

Anti-tumor effect of the WT1 peptide vaccine evaluated by RECIST

Among the 40 patients who received the WT1 vaccination, neither complete response (CR) nor partial response (PR) was obtained. Encouragingly, however, stable disease (SD) of 3 months or more was observed in 16 cases (40 %), including 10 cases of ovarian carcinoma, 5 cases of cervical carcinoma and a single case of uterine leiomyosarcoma, respectively.

The WT1 peptide-specific DTH reaction appeared after the vaccination in 27 cases (68 %); however, the vaccine's anti-tumor effect evaluated by RECIST was not correlated to the appearance of DTH (data not shown).

Toxicity of the WT1 vaccination

An adverse effect was observed in 36 cases (90 %): grade 1, 2 and 3 of skin reaction in 25 cases (63 %), 9 cases (23 %) and a single case (3 %), respectively, and grade 1 liver toxicity in a single case (3 %). The skin reactions had definite relationship with WT1 injection because the reactions were observed only in WT1 injected area. The liver toxicity occurred after first injection of WT1, and the relationship between WT1 vaccine and liver toxicity was probable. Postponement of the next injection due to adverse effects occurred in one case with grade 3 of skin reaction. However, termination of the WT1 vaccine injection due to adverse effects was never required.

Prognosis of the patients treated with WT1 peptide vaccine: the vaccines' survival effect

The PFS was 84 days (11–497). Surprisingly, among these WT1-vaccinated cases, which had been already resistant to conventional therapies and the disease had exhibited continuous progression against various other treatments for 40–1,198 days (median: 185 days), progression-free survival for a range of 67–427 days (median: longer than 160 days) was achieved in 16 SD cases (Table 2). The median OS of all the patients was 193 days (29–941).

Although an association between an anti-tumor effect evaluated RECIST and an appearance of DTH reaction was not observed, the PFS tended to be longer in DTH-positive cases than DTH-negative ones ($p = 0.23$ by the log-rank test), and the OS was significantly longer in DTH-positive cases than DTH-negative ones ($p = 0.023$ by the log-rank test) (Fig. 1).

Table 2 Duration of disease progression before WT1 vaccination was begun and progression-free period afterward in stable disease (SD) cases

Case number	Duration of disease progression before WT1 vaccine (days)	Progression-free survival after WT1 vaccine (days)
1	40	105[a]
2	55	67
3	61	427[a]
4	81	320
5	97	126
6	142	145
7	155	92
8	178	273
9	192	140[a]
10	324	84
11	405	175
12	434	196
13	439	84[a]
14	655	196
15	737	219
16	1,198	180[a]
Median	185	160[a]

The duration of disease progression before the WT1 vaccine, and the progression-free period after the start of WT1 vaccination in 16 SD cases, is demonstrated

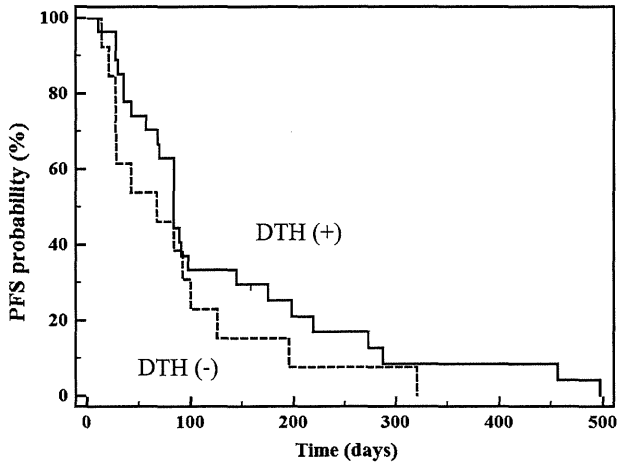
[a] The cases in which the disease was stable after WT1 vaccination without progression

Multivariate Cox proportional hazards analysis

We utilized the multivariate Cox proportional hazards model in order to find evidence to further support our belief that the DTH reaction was significantly associated with the survival. The DTH reaction was demonstrated to be an independent factor for overall survival of the patients (Table 3). The adjusted hazard ratio (HR) for the DTH reaction (− vs. +) was 2.73 (95 % CI 1.04–7.19, $p = 0.043$).

Discussion

A National Cancer Institute pilot project recently suggested that WT1 was one of the most promising targets for cancer immunotherapy (Cheever et al. 2009), and it has been demonstrated that WT1 vaccination is safe and has therapeutic potential for at least several kinds of tumors (Oka and Sugiyama 2010; Hashii et al. 2010; Oji et al. 2010;



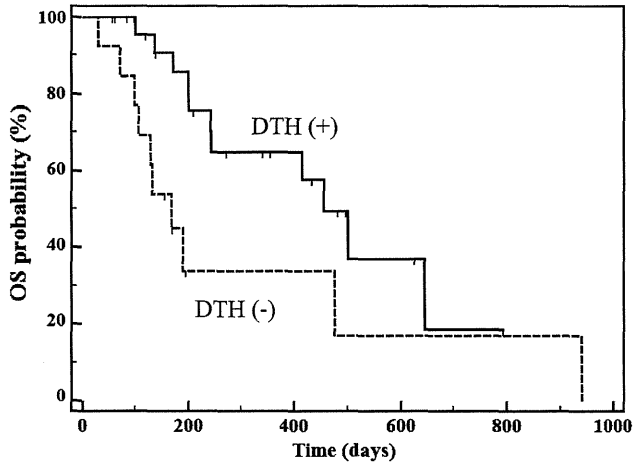


Fig. 1 PFS and OS association with DTH after WT1 vaccination. **a** The PFS tended to be longer in positive DTH cases than in DTH-negative cases ($p = 0.23$ by the log-rank test). **b** The OS was significantly longer in positive DTH cases than in DTH-negative cases ($p = 0.023$ by the log-rank test). *Solid line*: DTH (*plus*), *broken line*: DTH (*minus*)

Izumoto et al. 2008; Ohno et al. 2009). In this current phase II trial, we have tested the efficacy and safety of WT1 immunotherapy for gynecologic malignancies that were progressing, that is, resistant against conventional therapies.

In general, gynecologic tumors, including ovarian, endometrial and cervical carcinomas and uterine sarcomas, are very difficult to further treat, once the disease become resistant to conventional therapies such as chemotherapy or radiotherapy. For example, when ovarian carcinoma is first treated with cytoreductive surgery, the surgery is immediately followed by combination chemotherapy with paclitaxel and carboplatin (TC). If there is a failure of this first-line treatment, a single drug or combination chemotherapy for the recurrent disease, chosen based on the patient's treatment-free interval, can still be performed effectively in some cases (Koensgen et al. 2008; Markman et al. 2003; Harries and Gore 2002; Dizon et al. 2003). However, even though some third-line regimens have been reported to be occasionally effective for second relapses of some of these advanced stage diseases (Vergote et al. 2009; Chiyoda et al. 2010); the efficacy of each attempt becomes progressively lower as the number of previous treatment failures increases.

In the present study, the median number of the previous treatment regimens was 3 (range 1–11 treatments). Since all of the patients in the present trial had exhibited resistance to previous therapies, normally supportive care would have been considered as the only remaining option for them; however, the experimental WT1 vaccination immunotherapy was offered to them as an alternative.

A previous small study showed that stable disease was achieved by WT vaccination in 3 (25 %) of 12 gynecologic malignancies (Ohno et al. 2009). However, that study was so small that a survival effect was not analyzed. The response rate (CR + PR/all) in our study was 0 % (0 of 40 cases). However, the disease control rate (CR + PR + SD/all), which corresponds to disease stabilization lasting at least 3 months from the start of the vaccination, was 40 % (16 of 40 cases). The median PFS was 84 days (11–497), and the median OS of all the patients was 193 days (29–941). Considering that these cases were resistant to various kinds of therapies, and the diseases were progressing prior to the vaccination, these results of disease control rate and PFS time may be favorable, and were consistent with results of the previous smaller study that suggested the therapeutic potential of WT1 vaccine for gynecologic malignancies. Furthermore, surprisingly, in these SD cases, whose tumors had continuously progressed against previous therapies during the median of 185 days of treatments (range 40–1,198 days), the disease was durably controlled, without significant progression of the disease, for the median of longer than 160 days (range 67–427 days) after starting the WT1 immunotherapy (Table 2), implying an improved survival effect of the WT1 peptide vaccine. The adverse effect by the WT1 peptide-based immunotherapy with the dosage and schedule adopted here was limited and largely tolerable.

We next investigated the association of DTH and the efficacy of the WT1 immunotherapy. The OS of the patients with a positive DTH reaction was significantly better than that of those with a negative DTH reaction ($p = 0.023$ by the log-rank test) (Fig. 1). Moreover, the DTH reaction was demonstrated to be an independent factor for overall survival of the patients by multivariate Cox proportional hazards analysis (Table 3). These findings suggested that the induction of WT1-specific immune response, that is, the peptide-specific DTH, is a potential

Table 3 Multivariate Cox proportional hazards analysis on overall survival

Variable	Number of cases	Adjusted HR	95 % CI	*p* value
Age (years)				0.44
<60	24	1		
≥60	16	0.64	0.21–1.96	
Origin of the disease				0.75
Uterus	16	1		
Ovary	24	1.17	0.44–3.14	
Histology				0.98
Carcinoma	35	1		
Sarcoma, carcinosarcoma	5	0.99	0.28–3.42	
Evaluation of the previous therapy				0.39
SD	4	1		
PD	36	1.88	0.46–7.71	
Number of previous therapy regimens				0.034
<3	12	1		
≥3	28	4.28	1.12–16.37	
DTH				0.043
+	27	1		
−	13	2.73	1.04–7.19	

Multivariate Cox proportional hazards analysis (stepwise method) for the factors including age, origin of the disease, histology, evaluation of the previous therapy, number of previous therapy regimens and DTH was performed to evaluate whether DTH was an independently significant factor on OS

SD stable disease, *PD* progressive disease

predictor for the induction of clinical response, leading to a better prognosis.

The number of previous treatment regimens was also demonstrated to be an independent factor for survival prognosis after WT1 immunotherapy. The response rate of the first-line chemotherapy was quite high for ovarian carcinoma, however, that of second-line and the third-line chemotherapy was 34.5 and 27.5 %, respectively (Nishio et al. 2006). Effectiveness of WT1 was demonstrated to be associated with the number of previous treatment regimens, which was similar to that of the cell toxic chemotherapy. As the number of chemotherapy regimen increases, the tumor cells are considered to become resistant to the next line therapy. Furthermore, immunological potentials of the patients treated by chemotherapy with many courses might be dampened, leading to the poor response to the administered cancer vaccine. WT1 peptide vaccination soon after the first-line therapy, including the vaccination to prevent relapse after the operation, chemotherapy or radiation therapy, may be a favorable setting for the next clinical trial.

In the present phase II prospective study with a single arm, we have, for the first time, analyzed the survival effect of the WT1 vaccine for gynecologic malignancies, in addition to its anti-tumor effect conventionally evaluated by RECIST and toxicity, which had previously been reported in a smaller pilot study (Ohno et al. 2009). It was strongly suggested that WT1 peptide vaccination could induce the peptide-specific immune response in patients whose gynecological tumors have become resistant to conventional therapies, leading to a better survival. Larger two-arm randomized studies will be required to confirm the efficacy and clinical usefulness of the WT1 peptide vaccine for gynecologic malignancies.

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Conflict of interest The authors have no conflict of interest.

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Experience with thoracoscopic resection for mediastinal mature teratoma: a retrospective analysis of 15 patients

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Abstract

OBJECTIVES: Although video-assisted thoracoscopic surgery (VATS) is widely used for the resection of a mediastinal mass, it is converted to an open resection in some patients with a mature teratoma because of dense adhesions. We reviewed cases with a mature teratoma removed by VATS and investigated the indications for that procedure for this tumour.

METHODS: We retrospectively investigated 15 patients with a benign mediastinal mature teratoma who underwent a thoracoscopic procedure.

RESULTS: The mean tumour diameter was 5.3 cm (range 3.2–8.5). The mean operative time was 188 min (78–430), and intraoperative blood loss was 138 ml (10–450). Thoracoscopic resection was completed in all except 3 patients with larger tumours, which presented the most difficult problems with dissection. Each of those 3 had severe preoperative chest pain and a tumour larger than 5.5 cm. No mortality or postoperative complications were recorded, except for postoperative chylothorax. Tumour recurrence did not develop in any patient during the mean follow-up period of 4.6 years.

CONCLUSIONS: For selected patients with a mediastinal teratoma, VATS may be considered standard care, as most are benign. In contrast, an open approach may be more appropriate for patients with a large tumour or preoperative symptoms.

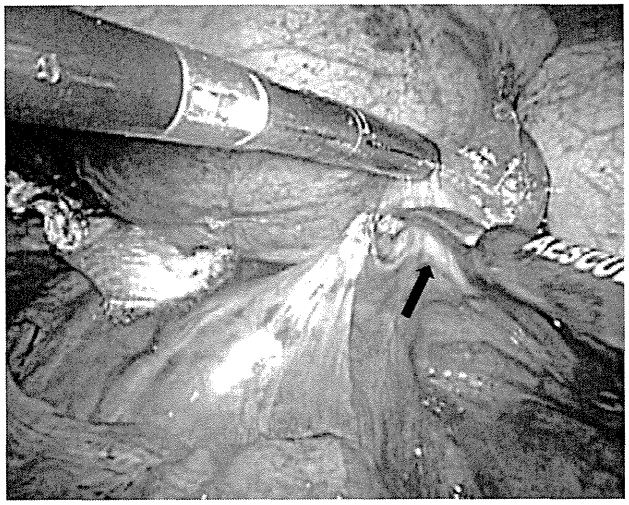
Keywords: Thoracoscopy • Mediastinal mature teratoma

INTRODUCTION

Video-assisted thoracoscopic surgery (VATS) is widely used for the resection of a mediastinal mass [1], with many successful VATS procedures to remove benign mediastinal tumours reported [2]. However, few studies have examined the outcomes of a VATS approach for mediastinal mature teratoma cases. Although such an approach for a mature teratoma is feasible and may be preferable, in some patients, VATS is converted to an open thoracotomy because of dense adhesions and the difficult dissection of the involved vessels [3]. We reviewed our series of cases with a mature teratoma removed by VATS and investigated the indications of that procedure for this type of tumour.

PATIENTS AND METHODS

From 2001 to 2012, 15 patients with a mediastinal mature teratoma underwent a thoracoscopic operation at the Department of General Thoracic Surgery at Osaka University Hospital. All received surgery under general anaesthesia with double-lumen endotracheal intubation, with each positioned in a supine position with or without use of a costal hook, as previously reported [4], except 2 patients with a tumour in the left mediastinum, who underwent a resection in a lateral decubitus position. Three ports were used in all cases: one for a 10-mm 30° thoracoscope, and two working ports for the endoscopic instruments and ultrasonic dissector. Our main concern during dissection was to maintain an accurate view of the phrenic nerve for its protection (Video 1). All resected specimens were placed into an endobag and retrieved. The operation was converted to a trans-sternal or transaxillary open procedure as necessary. Tumour size, operation time and amount of blood loss during the operation were compared between patients who underwent VATS successfully and those who required conversion to an open procedure from VATS. A chi-square test, Mann–Whitney *U*-test or Kruskal–Wallis test was used to compare the results. All statistical analyses were performed using Statview version 5.0 for Windows (Abacus Concepts, Berkeley, CA, USA). A *P*-value of <0.05 was considered to be statistically significant.



Video 1: Thoracoscopic view showing accurate display of the phrenic nerve for its protection. The right mediastinal pleura was incised just anterior to the phrenic nerve (arrow).

RESULTS

Fifteen patients (6 men, 9 women; average age 38 years, range 21–62 years) underwent thoracoscopic removal of a mediastinal teratoma. Of those, 9 were asymptomatic, and the lesions were discovered incidentally on chest X-ray images, while the others had preoperative symptoms, including chest pain, chest tightness, back pain, cough and fever. The mean tumour diameter was 5.3 cm (range 3.2–8.5). The diameter of the tumour in patients with preoperative symptoms was significantly larger than in those without symptoms (6.0 ± 1.6 vs 4.2 ± 1.6 cm, $P = 0.04$).

All patients underwent chest computed tomography (CT) and 9 also underwent magnetic resonance imaging (MRI). Those examinations revealed a capsulated and lobulated mass with watery as well as fatty components. None had radiological signs of invasion into a surrounding structure, while 5 had focally calcified lesions. No patient showed an increase in tumour markers, including carcinoembryonic antigen, α-fetoprotein, human chorionic gonadotropin-β subunit, soluble interleukin-2 receptor and acetylcholine receptor antibody. Four underwent 2-[fluorine-18]fluoro-2-deoxy-D-glucose (FDG)-positron emission tomography (PET) examinations, which showed normal or absent uptake of FDG. Based on these preoperative examination findings, the suspected clinical diagnosis was mature teratoma in all patients. Thus, none underwent a preoperative biopsy because of the non-invasive characteristic of this type of tumour.

Operation times ranged from 78 to 430 min (mean 188 min), and intraoperative blood loss was from 10 to 450 ml (mean 138 ml). When dissecting the tumour from the phrenic nerve, the contents of the tumour spilled due to breakage of the tumour capsule in 2 patients, though complications such as pleuritis were not induced, probably because of diligent washing (Fig. 1). No mortality or postoperative complications were recorded, except for postoperative chylothorax in 1 patient. Although the phrenic nerve was preserved in all patients, transient phrenic nerve palsy was observed in 1. Tumour recurrence did not develop in any cases during the mean follow-up period of 4.6 years.

Thoracoscopic resection was completed in all except 3 patients with larger tumours, which presented the most difficult problems with dissection. Two patients with lesions >5.5 cm required conversion to an open procedure to safely complete dissection of the left brachiocephalic vein, while the third had the tumour adhered to the chest wall, phrenic nerve and lung, and was converted to an open procedure to safely complete dissection of the phrenic nerve. Two of these underwent resection of the mass with the pericardium and lung. Each of these 3 patients had preoperative symptoms such as chest pain, while only 3 of the 12 patients who underwent tumour resection with VATS had preoperative symptoms (Table 1). Two patients who required conversion from VATS to open resection needed non-steroidal anti-inflammatory drugs to relieve preoperative chest or back pain. Preoperative chest X-ray images obtained before and after occurrence of severe chest pain showed changes in the shape of an abnormal mass (Fig. 2), indicating that these patients might suffer from pleuritis due to teratoma rupture. During the operation, the tumour was found adhered to surrounding structures, such as the pericardium, lung and phrenic nerve, and thus conversion from VATS to an open procedure was required (Video 2). The mean size of the tumour was smaller in the group of patients who underwent resection by VATS when compared with those who were converted to an open procedure (Table 1). Although there were no cases with a large vessel injured intraoperatively, blood loss during surgery was significantly higher in the conversion group. Tumour location is considered important for determination of conversion; thus, we compared the positional relationship between the tumour and left brachiocephalic vein. However, there were no significant differences between patients with or without conversion.

DISCUSSION

Mature mediastinal teratomas are commonly seen in young women, thus VATS is considered to be a feasible technique for resection and better than an open approach in regard to aesthetic issues. However, resection can be surgically challenging because of potential adhesions to major organs, including major vessels in the mediastinum as well as the heart, nerves and lungs [5]. A VATS resection procedure for a mediastinal mature teratoma is sometimes converted to an open procedure, usually because the tumour shows dense adhesions to surrounding structures. However, large tumours present problems during thoracoscopic procedures, because their size prevents adequate exposure and manipulation. Based on our experience, an open approach may be more appropriate for mature teratomas larger than 6 cm. In addition, in our series, patients who required such conversion had severe preoperative symptoms, including chest pain, back pain and fever, indicating pleuritis that induced dense adhesions between the mass and surrounding organs, such as the pleura, pericardium, large vessels and lung parenchyma. An anterior mediastinal tumour often adheres to the left brachiocephalic vein; thus, conversion to open surgery is needed to safely complete dissection of the vessel. As tumour location is considered important for determination of conversion, we compared the positional relationship between the tumour and left brachiocephalic vein. However, there were no significant differences between the VATS and open groups, indicating that tumour size and adhesion to surrounding organs might be more important than tumour location to determine such conversion.

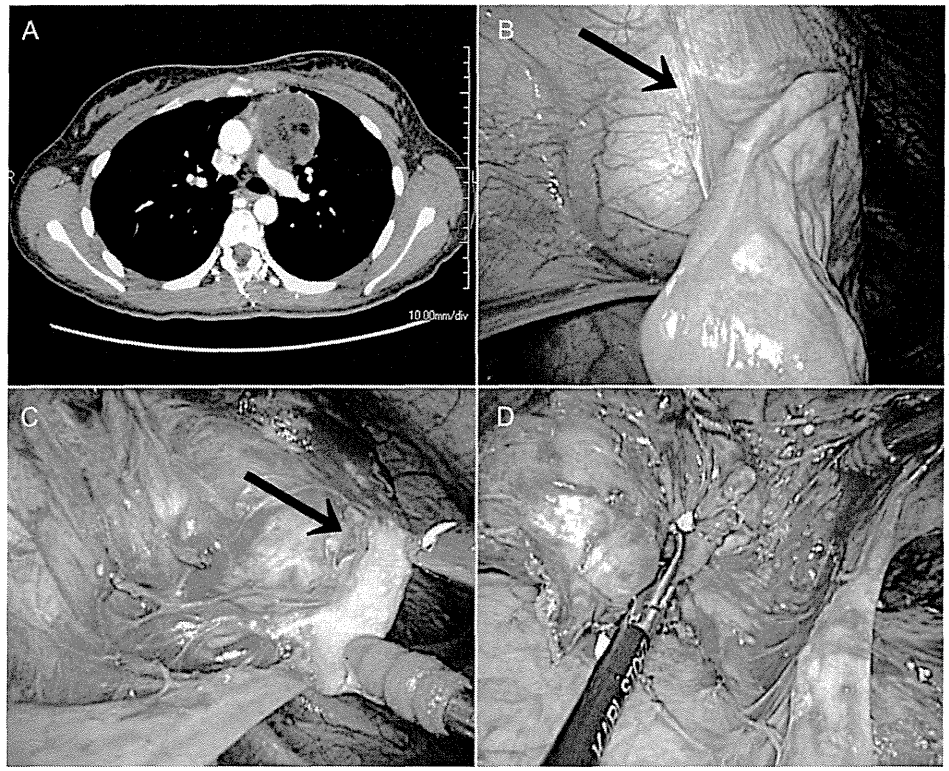


Figure 1: (**A**) Chest computed tomography image showing a well-circumscribed cystic tumour in the anterior mediastinum. (**B**) Thoracoscopic view showing adhesion between tumour and lung (arrow). (**C**) When dissecting the tumour from the phrenic nerve, the contents spilled due to breakage of the tumour capsule (arrow). (**D**) Aspiration of cystic components can provide a wider thoracoscopic view, leading to easier grasping and retraction from the tumour wall.

Table 1: Patient characteristics (VATS vs conversion)

	VATS[a] ($n = 12$)	Conversion[b] ($n = 3$)	*P*
Age (years)	31 ± 7	43 ± 15	0.20
Gender (m/f)	5/7	1/2	0.79
Symptoms (yes/no)	3/9	3/0	0.02
Tumour size (cm)	4.6 ± 1.0	7.0 ± 2.1	0.04
Tumour location[c] (yes/no)	3/0	7/5	0.17
Operation time (min)	158 ± 55	272 ± 107	0.02
Blood loss (ml)	30 ± 23	322 ± 87	0.01

[a]Patients who successfully underwent video-assisted thoracoscopic surgery (VATS).
[b]Patients who required conversion to an open procedure.
[c]Patients with tumour contact to the left brachiocephalic veins on CT findings.

In two of the present patients, the tumour partially ruptured during adhesiotomy from the phrenic nerve and the spilled cystic contents were aspirated. We diagnosed these patients as having benign disease and considered it more important to conserve the phrenic nerve. Furthermore, in cases with a tumour that has dense adhesion to the phrenic nerve, such rupture might not be avoidable even during open surgery. Nakano *et al.* [6] reported that extraction of cystic components with the aid of a tube prior to beginning a thoracoscopic resection can provide a wider thoracoscopic view, leading to easier complete removal. To avoid massive spillage of the tumour contents, an intentional puncture of the tumour wall and prompt aspiration of the spilled cystic contents may be necessary for providing adequate exposure of important organs and manipulation of the mass.

It is important to extirpate the tumour by an open procedure without any exposure when a malignant component is suspected. However, there are few reports of coexistence of malignant components in a mature teratoma [7]. Chang *et al.* [8] noted rapid tumour dissemination in a patient who underwent VATS resection of a teratoma with malignant transformation, in whom intraoperative rupture of the cystic tumour induced dissemination. Although teratomas with malignant transformation are extremely rare, careful and complete resection along with the cystic wall of an intact mediastinal mature teratoma is necessary to avoid a risk of relapse from a potentially malignant component. Preoperative FDG-PET may be useful for determining whether the tumour has malignant potential [9]. In our series, 4 patients had solid-like portions in addition to watery or fatty parts in CT or MRI findings; thus, they also underwent FDG-PET, which revealed a normal or absent uptake of FDG, leading to a clinical diagnosis of a benign mature teratoma.

CONCLUSION

In selected patients, VATS surgery may be considered standard care for resection of a mediastinal teratoma, as most are benign. In contrast, an open approach may be more appropriate for patients with a large tumour or preoperative symptoms.

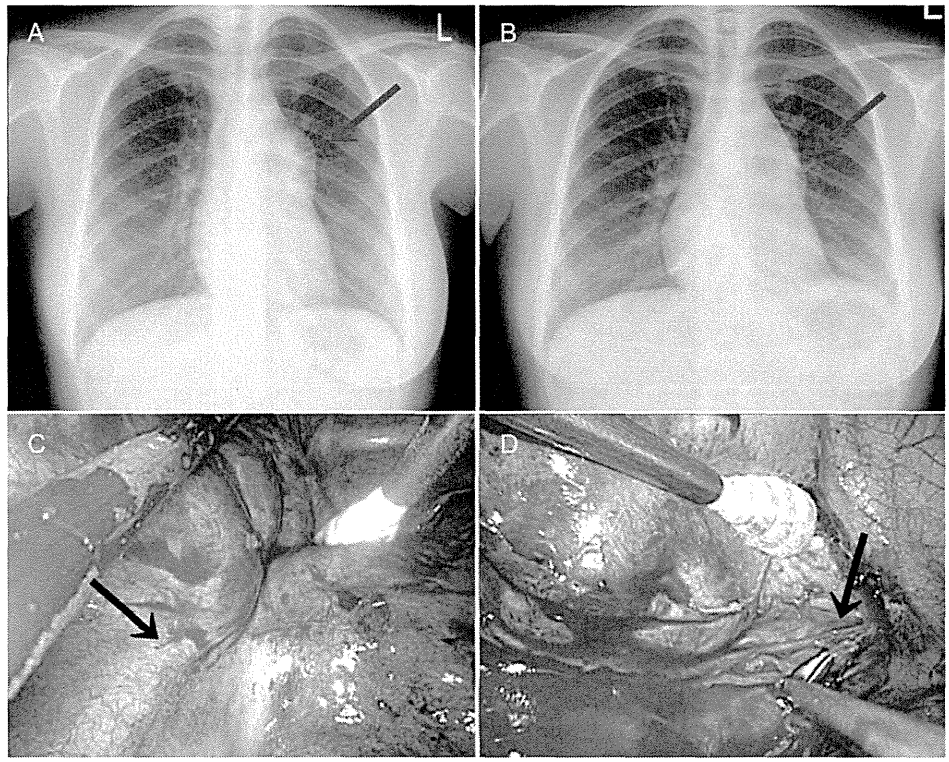
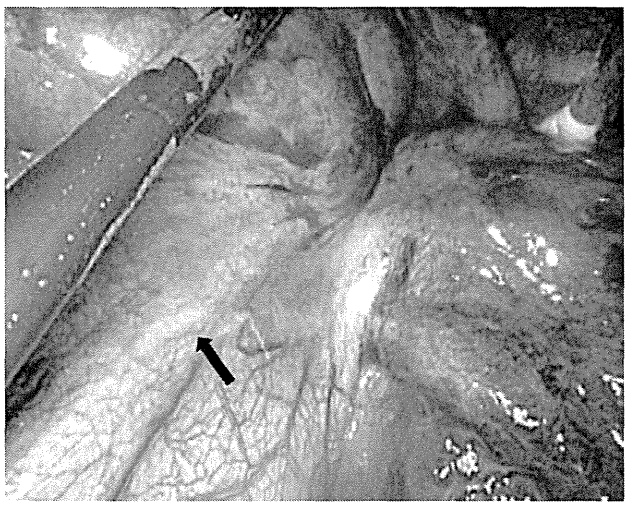


Figure 2: (**A**) Preoperative chest roentgenogram showed abnormal shadow on the left side of the mediastinum (arrow). (**B**) Preoperative chest roentgenogram taken after severe chest pain showing change in shape of an abnormal mass (arrow). (**C**) Thoracoscopic view showing thickened pleura and involvement of the phrenic nerve by the tumour (arrow). (**D**) Thoracoscopic view showing dense adhesions between tumour wall and pericardium (arrow).



Video 2: Thoracoscopic view showing thickened mediastinal pleura, and adhesions between the tumour and lung. The thickened pleura was incised just anterior to the phrenic nerve (arrow). The tumour was densely adhered to the pericardium.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *ICVTS* online.

Conflict of interest: none declared.

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Pulmonary Fibroblasts Induce Epithelial Mesenchymal Transition and Some Characteristics of Stem Cells in Non-Small Cell Lung Cancer

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Background. Fibroblasts are key components of the tumor microenvironment. The purpose of this study was to clarify the role of fibroblasts in tumor progression in non-small cell lung cancer (NSCLC).

Methods. Fibroblasts isolated from surgical exploration were co-cultured with human lung adenocarcinoma cell lines. We defined fibroblasts obtained from tumors as cancer associated fibroblasts (CAFs) and those from normal lung tissue as lung normal fibroblasts (LNFs).

Results. Expression levels of myofibroblast markers were higher in CAFs than LNFs within 5 passages in the absence of continuing interaction with carcinoma cells. Thus, we used at least 2 pairs of these CAFs and LNFs in the following experiments; conditioned medium (CM) from fibroblast-induced epithelial mesenchymal transition and acquisition of cancer stem cell-like qualities in lung cancer cells (A549 and NCI-H358), indicating that CM from fibroblasts was biologically active. Furthermore, the concentration of the transforming growth factor (TGF)-β1 was higher in CM from CAFs as compared with that from LNFs, and phenotypic changes of cancer cells by CM from CAFs were greater than those induced by CM from LNFs. These CAF-induced changes were inhibited by addition of the TGF-β inhibitor SB431542. Subcutaneous co-injection of lung cancer cells and CAFs in mice enhanced tumor growth when compared with cancer cells alone, which was attenuated by administration of SB431542.

Conclusions. Fibroblasts were associated with increased malignant potential and the acquisition of stem cell-like properties in NSCLC tumors. Targeting CAFs as a therapeutic strategy against cancer is an intriguing concept that would benefit from further study.

(Ann Thorac Surg 2013;96:425–33)

Lung cancer is the leading cause of cancer death worldwide [1]. Overall prognosis for affected patients is poor due to metastatic disease and lack of curative systemic therapy, underscoring the need for a better understanding of the biologic changes that promote the aggressive neoplastic phenotype [2].

Epithelial to mesenchymal transition (EMT) is a fundamental biologic process during which epithelial cells lose their polarity and change to a mesenchymal phenotype [3]. When cancer cells invade or metastasize, they use a mechanism similar to EMT [4]. Furthermore, some studies have reported a role for EMT in the development of cancer cell resistance to anti non-small cell lung cancer (NSCLC) agents [5–7]. The cancer stem cells (CSC) theory proposes that cancers are maintained by subpopulations of tumor cells that possess progenitor cell characteristics. These cells can initiate tumor formation, differentiate along multipotent pathways, and are relatively resistant to conventional chemotherapy [8–10]. The EMT generates cells with many of the properties of epithelial stem cells [11]. The induction of EMT in primary lung cancer cell line results in the acquisition of mesenchymal profile and in the expression of stem cell markers [12]. Thus, EMT-driven gain of cancer stem cell properties may be associated with aggressiveness and metastatic spread [13].

The tumor microenvironment is a key component of tumor progression, and the invasion of cancer cells into and through the stroma requires EMT [14]. A specific subset of stromal cells, termed cancer associated fibroblasts (CAFs), show morphologic characteristics of both fibroblasts and smooth muscle cells [15]. The CAFs modulate the behavior of adjacent cancer cells by secreting various growth factors and cytokines, thereby promoting tumorigenesis [16]. Tumor-localized CAFs can comprise up to more than half of the tumor mass, and there is active multi-directional communications

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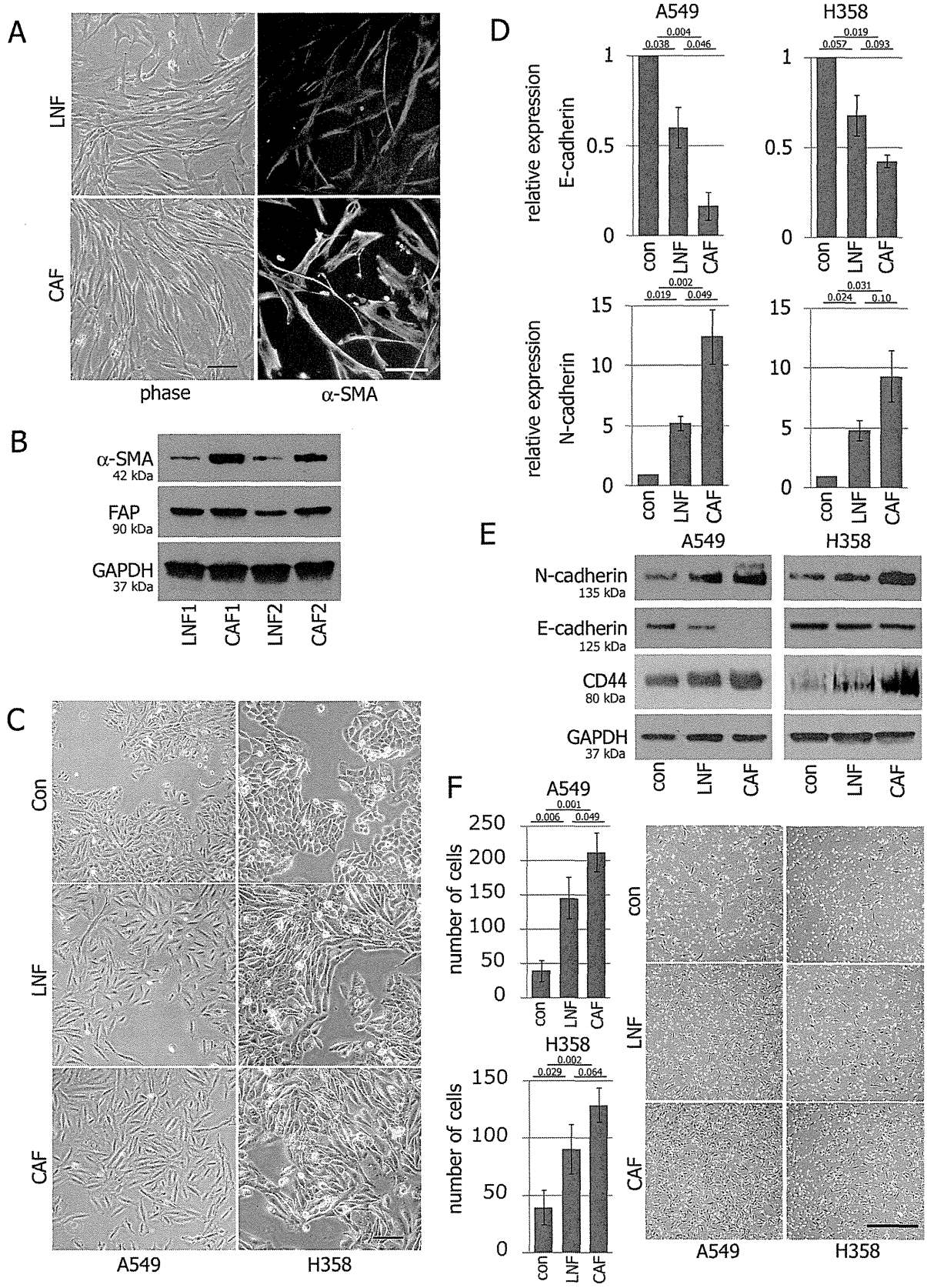
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between these coevolving cell types within cancer tissue [17–19].

In order to identify new targets for prevention of metastasis, it is important to understand the molecular mechanisms that drive EMT. Therefore, the goal of the present study was to clarify the role of CAFs in the induction of EMT and CSC of NSCLC cells.

Material and Methods

Cell Culture and Materials

A549 cells and NCI-H358 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in Roswell Park Memorial Institute 1640 with 10% fetal bovine serum (FBS). Antibodies for western blotting and immunofluorescence were as follows: anti-alpha-smooth muscle actin (α-SMA) pAb (catalog number ab5694; Abcam, Tokyo, Japan), anti-fibroblast activating protein pAb (Abcam, ab53066), anti-glyceraldehyde-3-phosphate dehydrogenase mAb (G9545; Sigma-Aldrich, St. Louis, MO), anti-N-cadherin (sc-59987; Santa Cruz Biotechnology, Inc, Dallas, TX), anti-E-cadherin mAb (M106; Takara, Otsu, Shiga, Japan), and anti-CD44 mAb (156-3C11; Cell Signaling Technology, Beverly, MA). Antibodies for the immunohistochemistry experiments were as follows: anti-E-cadherin mAb (M3612; Dako, Carpinteria, CA), anti-CD44 mAb (156-3C11; Cell Signaling), and Ki67 (M7240; Dako). Cisplatin was purchased from Sigma (catalog# 479306, St. Louis, MO), and transforming growth factor (TGF)-β1 was from R&D Systems (240-B; Minneapolis, MO), and SB431542 was from Tocris Bioscience (1614; Ellisville, MO).

Isolation and Primary Culture of Fibroblasts

Fibroblasts were isolated from surgical explanation. We defined the fibroblasts from the non-necrotic part of the tumors as CAFs and those from normal lung as LNFs. Briefly, cancerous tissue or normal lung far from cancerous tissue were obtained aseptically from 5 patients with NSCLC undergoing pulmonary resection after the Institutional Review Board for Clinical Research at Osaka University Hospital approved our study protocol and written informed consent for surgical intervention was obtained from each patient. Tissues were digested for 6 hours in 1 mg/mL collagenase I (Sigma), and cells were plated in Dulbecco's Modified Eagles medium (DMEM) containing 10% FBS. Two weeks later, proliferated cells were selected using anti-Fibroblast MicroBeads and MACS Columns (Miltenyi Biotec, Auburn, CA), confirmed by positive staining for vimentin and negative staining for pan-cytokeratin, and continued to culture as fibroblasts. Conditioned media (CM) were obtained from 48-hour serum-starved cells (6 mL DMEM for fibroblasts plated semi-confluent to a 10-cm dish) and used freshly. All in vitro experiments were performed in triplicate using 2 pairs of primary cultured CAFs and LNFs.

Real-Time RT-PCR

Total RNA was isolated from cells treated with or without CM from fibroblasts for 24 hours using RNeasy Mini Kit (Qiagen, Tokyo, Japan). Real-time RT-PCR (E-cadherin, Hs00170423_m1; N-cadherin, Hs00169953_m1 [Applied Biosystems, Tokyo, Japan]) was performed using a CFX96 system (BioRad, Tokyo, Japan) and relative expression levels were calculated by the comparative Ct method.

Detergent Extraction, SDS-PAGE, and Immunoblots

Monolayers of cultured cells, treated with or without CM from fibroblasts for 48 hours, were extracted with RIPA buffer (Cell Signaling, catalog# 9806). Cell extracts were resolved by SDS-PAGE [sodium dodecyl sulfate polyacrylamide gel electrophoresis] and immunoblotted as described [20].

Trans-Well Motility Assays

We plated 5×10^5 cells in the upper chamber of polyethylene terephthalate membranes (pore size 8 μm; Becton Dickinson, Franklin Lakes, NJ). The number of cells on the lower side of the filter was counted under microscope after 8 hours incubation.

Spheroids Culture

Cells were cultured in low adherent 35-mm dishes (Corning, Corning, NY) under serum-free condition for 21 days [21]. Briefly, 1×10^3 cells were suspended in 6 mL of DMEM or CM from CAFs or LNFs, supplemented with 10 ng/mL basic fibroblast growth factor. Spheroid bodies were harvested every 5 days and re-cultured with fresh media. To inhibit TGF-β signaling, SB431542 was added to CM. Spheres with a diameter over 50 μm were counted on an inverted microscope.

Cell Viability Assay

For quantitative viability assays, Cell Proliferation Kit I (MTT; 3-[4.5 dimethylthiazol 2-yl]-2,5-diphenyltetrazolium

Fig 1. (A) We defined fibroblasts obtained from tumors as cancer associated fibroblasts (CAFs) and those from normal lung tissue as lung normal fibroblasts (LNFs). Phase contrast pictures of LNFs and CAFs were taken (left panels). Scale bar: 100 μm. The LNFs and CAFs were stained with α- smooth muscle actin (SMA). Scale bar: 50 μm. (B) Two pairs of LNFs and CAFs were analyzed on Western blot for α-SMA, fibroblast activating protein (FAP), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control. (C) A549 and NCI-H358 cells were treated with Dulbecco's Modified Eagles medium (DMEM) as a control, Con) or conditioned medium (CM) from LNFs and CAFs for 2 days. Phase contrast images were taken. Scale bar: 100 μm. (D) A549 and NCI-H358 cells were treated with and without CM from CAFs or LNFs for 24 hours. Cells were analyzed on real-time reverse transcriptase-polymerase chain reaction to detect E-cadherin and N-cadherin. The results are shown as mean ± SD. Significance was tested with the Mann-Whitney test and exact p values are shown. (E) A549 and NCI-H358 cells treated with DMEM (con) or CM from LNFs and CAFs for 2 days were analyzed on Western blot for N-cadherin, E-cadherin, CD44, and GAPDH. (F) Motility assays were performed and images were obtained. Cells transversing the filter were counted. Scale bar: 100 μm. Columns represent mean ± SD and p values are shown.

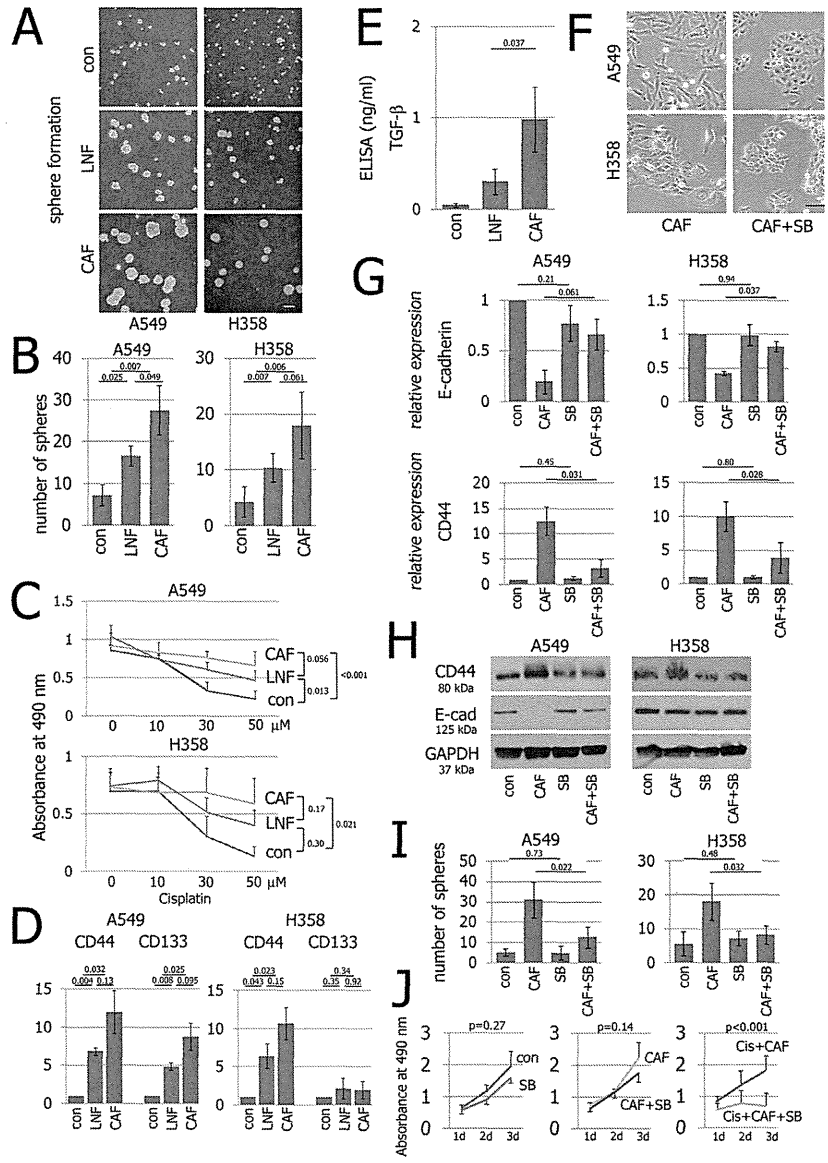


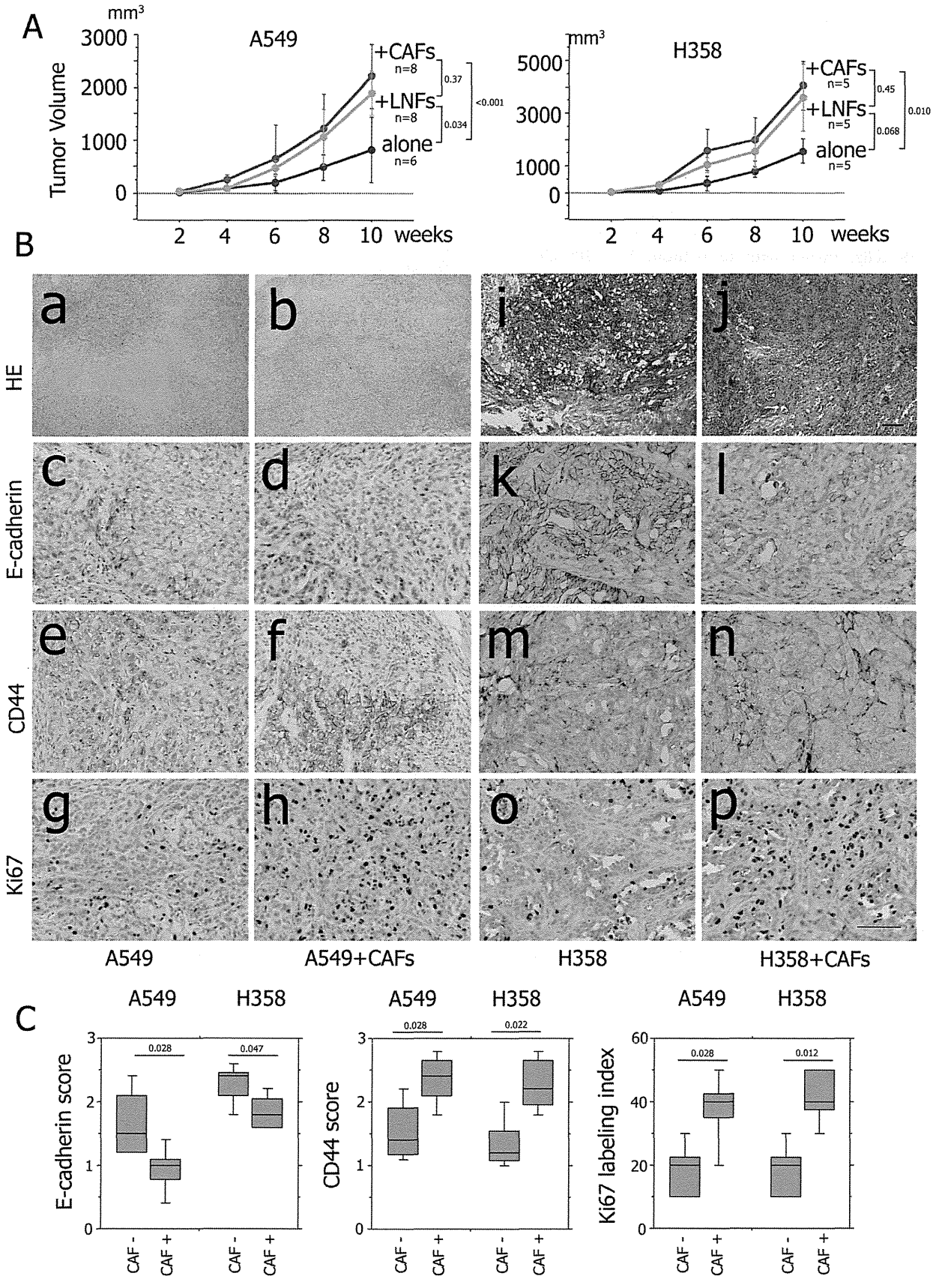
Fig 2. (A) A549 and NCI-H358 cells were grown under spheroid culture condition with or without conditioned medium (CM) from cancer associated fibroblasts (CAFs) or lung normal fibroblasts (LNFs). Floating spheroid bodies were photographed. Scale bar: 50 μm. (B) Floating spheroid bodies were counted and the results are shown as mean ± SD with exact p *values. (C) A549 and NCI-H358 cells were treated with cisplatin with or without CM from CAFs or LNFs. Proliferation of cells 2 days after seeding was quantified and significance was tested with the repeated measures analysis of variance. (D) A549 and NCI-H358 cells were treated with and without CM from CAFs or LNFs for 24 hours. The real-time reverse transcriptase-polymerase chain reaction (RT-PCR) performed to detect CD44 and CD133. (E) CM was collected from 2 pairs of LNFs and CAFs and quantification of transforming growth factor (TGF)-β1 was assessed by enzyme-linked immunosorbent assay; Con, control (culture media for fibroblasts). (F) A549 and NCI-H358 cells were treated with CM from CAFs for 2 days with or without the TGF-β inhibitor; SB431542. Phase contrast images were taken; scale bar: 100 μm. (G) A549 and NCI-H358 cells were cultured in CM from CAFs with or without SB431542 for 24 hours. The real-time RT-PCR performed to detect CD44 and CD133. (H) A549 and NCI-H358 cells cultured in CM from CAFs with or without SB431542 for 48 hours were analyzed on Western blot for CD44, E-cadherin, and glyceraldehyde-3-phosphate dehydrogenase. (I) A549 and NCI-H358 cells were grown under spheroid culture condition with CM from CAFs including SB431542. Floating spheroid bodies were counted and the results are shown as mean ± SD. (J) Proliferation of A549 cells was quantified by MTT assay. A549 cells were cultured in Dulbecco's Modified Eagles medium (DMEM) (as a control, con), DMEM with SB431542 (10 μM, SB), CM from CAF, CM with SB431542 (10 μM, CAF + SB) for 3 days (1d = 1 day; 2d, 2 days; 3d = 3 days after treatment). Cells were also treated with CM including cisplatin (30 μM, Cis + CAF) and CM including both cisplatin (30 μM) and SB431542 (10 μM, Cis + CAF + SB).*

bromide) was obtained from Roche Applied Science (Mannheim, Germany). Cells were plated in 96-well plates (1×10^4 cells/50 μL serum free media for each well). Fifty μL of serum free media, CM from CAFs, or CM from LNFs were added to each well. The cells were treated according to the manufacture's protocol and absorbance was measured at 490 nm using a microplate reader (BioRad) after indicated days. Cisplatin or SB431542 was added at various concentrations.

Enzyme-Linked Immunosorbent Assay (ELISA) for TGF-β1

Around 1×10^6 cells were plated in 10-cm dishes, and cells were fed with serum-free DMEM (6 mL) next day.

Fig 3. (A) Tumor volumes (mean ± SD) after subcutaneous injection of A549 or NCI-H358 cells with or without cancer associated fibroblasts (CAFs) or lung normal fibroblasts (LNFs) into nude mice were plotted and significance was tested with the repeated measures analysis of variance. Exact p *values are shown. (B). Panels a to p show representative findings from primary tumor obtained from mice. Scale bar: 100 μm. (C) Expression of E-cadherin and CD44 were scored in a semiquantitative manner and Ki-67 labeling index was calculated. Bars for the box extend from the 25th to 75th percentile of the data and the line in the middle represents the median. The upper and lower bars represent the distance from the 10th to 90th percentile from the median. (HE = hematoxylin and eosin.)*


A
mm³
A549
Tumor Volume
3000
2000
1000
0
+CAFs
n=8
+LNFs
n=8
alone
n=6
0.37
0.034
<0.001
2 4 6 8 10 weeks
mm³
H358
5000
3000
1000
0
+CAFs
n=5
+LNFs
n=5
alone
n=5
0.45
0.068
0.010
2 4 6 8 10 weeks
B
HE
E-cadherin
CD44
Ki67
a
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e
f
m
n
g
h
o
p
A549
A549+CAFs
H358
H358+CAFs
C
A549
H358
E-cadherin score
0.028
0.047
CAF -
CAF +
CD44 score
0.028
0.022
Ki67 labeling index
0.028
0.012

The CM was collected after 48 hours and quantification of TGF-β1 was assessed by ELISA according to the manufacture's protocol (human Quantikine; R&D System, Minneapolis, MN).

Animal Studies

All manipulations were done in accordance with protocols approved by the Osaka University Institute Animal Care Committee. The 1×10^6 tumor cells (A549 or NCI-H358 cells) mixed with or without 1×10^5 CAFs or LNFs (2 pairs of primary cultured CAFs and LNFs for A549 cells and 1 pair of those for H358 cells [A549 alone, n = 6; A549 + CAFs, n = 8; A549 + LNFs, n = 8; H358 alone, n = 4; H358 + CAFs, n = 4; H358 + LNFs, n = 4]) were injected subcutaneously into the back of 4-week-old nude mice. Tumor volume was calculated according to the formula: tumor volume (mm^3) = $d^2 \times D/2$, where d and D were the shortest and the longest diameters, respectively. The SB431542 was injected intraperitoneally at 10 mg/kg (once per day, 5 times a week for 2 weeks) 1 week after co-injection (1×10^6 A549 cells with or without 1×10^5 CAFs, n = 5 for each group) according to protocols described in previous reports [22, 23].

Immunohistochemistry

Immunohistochemistry was performed as previously described [7]. All sections stained with E-cadherin and CD44 were scored in a semiquantitative manner classified as 0 (no staining), +1 (weak staining), +2 (moderate staining), or +3 (strong staining) in intracellular compartments of carcinoma cells [24]. The Ki-67 labeling index (labeling frequency %) was calculated by the following formula: [number of positive nuclei/total number of represented cells] ×100. All immunohistochemistry results were quantified by counting at least more than 200 tumor cells in randomly selected 5 areas in each specimen.

Statistical Design and Data Analysis

A χ^2 test, Mann-Whitney *U* test, or repeated measures analysis of variance was used to compare the results. All statistical analyses were performed using StatView version 5.0 for Windows (Abacus Concepts, Berkeley, CA).

Results

NSCLC Cell Lines Undergo EMT in Response to Conditioned Medium From Fibroblasts

Primary cultured CAFs and LNFs each expressed α-SMA and fibroblast activating protein, with the levels greater in CAFs than LNFs even after 5 passages (Fig 1A, 1B). Conditioned medium from both also induced EMT in A549 and NCI-H358 cells, which became more spindle shaped (Fig 1C). Furthermore, the expression of E-cadherin decreased and that of N-cadherin increased in cancer cells (Fig 1D, 1E). These changes in EMT markers were significantly greater in A549 cells treated with CM from CAFs as compared with those treated with CM from LNFs, while migration induced by CAFs was significantly greater than that induced by LNFs in A549 cells (Fig 1F). These findings suggest that paracrine interplay between fibroblasts and cancer cells leads to EMT in cancer cells. In addition, they indicate that CAFs can maintain the phenotypic properties of myofibroblasts even in the absence of continuing interaction with carcinoma cells.

Spheroid Formation Ability and Resistance to Treatment by NSCLC Cell Lines in Response to CM From Fibroblasts

When cultured with CM from fibroblasts, both A549 and NCI-H358 cells gave rise to significantly greater spheroid body formation as compared with cells cultured without CM (Fig 2A, 2B). Furthermore, CM-treated cells showed a significant increase in cell viability in response to cisplatin when compared with untreated control cells (Fig 2C).

CAF-Mediated EMT Promoted Generation of Cancer Stem Cells

We evaluated changes in the CSC markers CD44 and CD133. The CM from CAFs increased the expression of CD44 to a greater degree than that seen after treatment with CM from LNFs (Fig 2D). The SDS-PAGE results also showed that the expression of CD44 was increased to a greater degree in cancer cells treated with CM from CAFs than in those treated with CM from LNFs (Fig 1E).

Blocking TGF-β Signaling Inhibited Phenotypic Changes Associated With EMT and Stem Cell-Like Qualities

Transforming growth factor-β is a strong inducer of EMT in epithelial cells. The ELISA findings showed that the concentration of TGF-β1 was greater in CM from CAFs as compared with that from LNFs (Fig 2E). Thus, we blocked TGF-β signaling to clarify the role of TGF-β in CAF-induced EMT. The CAF CM-driven phenotypic changes were inhibited by addition of the TGF-β inhibitor SB431542 to CM (Fig 2F), while down-regulation of E-cadherin and increased CD44 expression (Fig 2G, 2H), and spheroid formation ability induced by CM from CAFs (Fig 2I) were significantly inhibited by SB431542. Although TGF-β inhibition did not change the growth of cancer cells after 2 days, it attenuated the resistance to chemotherapy in vitro (Fig 2J). Taken together, these results suggest that TGF-β signaling may mediate EMT and maintenance of stem cell-like qualities induced by CAFs.

CAF-Mediated Tumor Formation of Human Lung Adenocarcinoma Cell Lines

Although cancer cells co-cultured with fibroblasts did not show an increase in cell proliferation (Fig 2J, compare left panel with middle panel, $p = 0.74$), subcutaneous co-injection of each lung cancer cell line with human CAFs or LNFs into mice resulted in a high rate of tumor formation, as compared with injection of cancer cells alone (Fig 3A). There was no difference between co-injection with cancer cells and CAFs and that with LNFs. We also compared specimens obtained from mice injected with cancer cells alone with those co-injected with cancer cells and CAFs. Hematoxylin and eosin

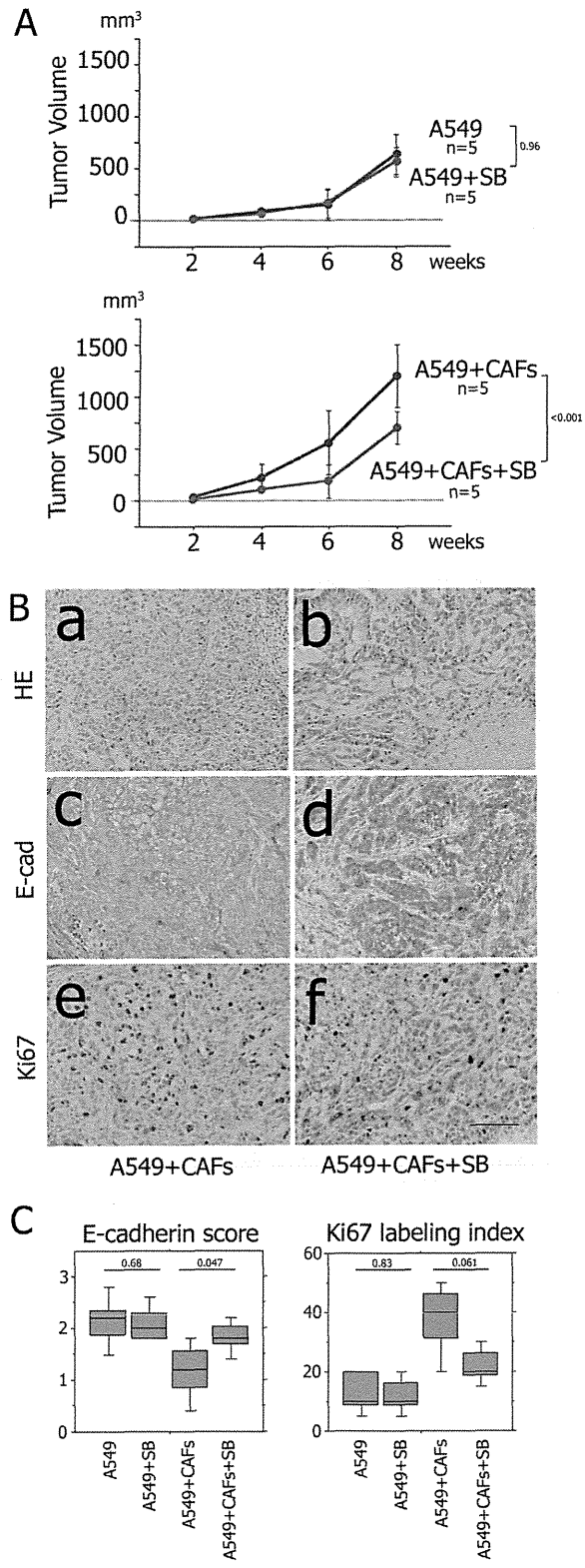

Fig 4. (A) The transforming growth factor (TGF)-β inhibitor, SB431542, was injected intraperitoneally after subcutaneous injection of A549 cells, with or without cancer associated fibroblasts (CAFs), into nude mice. Tumor volumes (mean ± SD) were plotted and significance was tested with the repeated measures analysis of variance. Exact p values are shown. (B) Panels a through f show representative findings from primary tumor obtained from mice treated with or without the TGF-β inhibitor, SB431542. Panels a, c, e: mice with A549 in combination with CAFs; panels b, d, f: mice treated with the TGF-β inhibitor SB431542 (SB) after injection with A549 in combination with CAFs. (C) Expression of E-cadherin was scored and Ki-67 labeling index was calculated, and the results are shown in the same way as Figure 3C.

staining revealed both necrosis and apoptotic cell death in the middle of tumors from mice injected with A549 or H358 cells alone (Fig 3B). On the other hand, E-cadherin and CD44 expressions were changed in tumors from mice that underwent co-injection with cancer cells and CAFs as compared with those with cancer cells alone. We compared these findings using a semiquantification technique, which showed that the expression of E-cadherin was lower and that of CD44 higher in cancer cells injected with CAFs as compared with cancer cells alone (Fig 3C). Furthermore, the Ki-67 labeling index was also higher in tumors from mice injected cancer cells and CAFs as compared with cancer cells alone. These results suggest that co-injection with A549 and CAFs induced EMT and stem cell-like qualities in cancer cells, resulting in rapid tumor growth in vivo.

When compound SB431542 was injected in an intraperitoneal manner, tumor formation enhanced by co-injection with CAFs was attenuated, whereas that compound did not affect tumor growth in mice injected with A549 alone (Fig 4A). Furthermore, the expression of E-cadherin was increased and Ki-67 labeling index was decreased in tumors obtained from mice treated with SB431542 (Fig 4B, 4C).

Comment

In the present study we found that CM from fibroblasts induced EMT in NSCLC cells, while those cells also acquired characteristics of stem cell-like qualities. The EMT changes induced by CAFs in A549 cells were greater than those induced by LNFs, indicating that CAFs may have been more activated in our experimental system as compared with LNFs. Navab and colleagues [25] reported that CAFs were activated to a greater degree than LNFs by showing α-SMA expression and collagen gel contraction. Together, these findings suggest that CAFs play a more crucial role in tumor homeostasis, progression, and maintenance of stem cell-like qualities in cancer cells as compared with normal fibroblasts. However, it is difficult to compare CAFs with LNFs in a rigorous manner. One reason is that the present fibroblasts were isolated from heterogeneous tissues obtained from patients with different backgrounds. Furthermore, while fibroblasts are in an inactive and quiescent state in normal tissues, isolated fibroblasts may become activated and release mediators when cultured with FBS or co-cultured with cancer cells. We found that primary cultured CAFs and LNFs each expressed α-SMA and fibroblast activating protein,

though those levels were greater in CAFs than LNFs after 5 passages. Therefore, the cells used in the present in vitro experiments were collected within the limit of 5 passages. We found that co-injection of each lung cancer cell line with human fibroblasts into mice resulted in a high rate of tumor formation. A previous report [25] noted that CAFs demonstrated a greater ability to enhance the tumorigenicity of lung cancer cell lines in vivo, while co-injection with CAFs increased tumor formation in a manner similar to that seen when co-injected with LNFs in the present experimental system. It is possible that co-injection of LNFs with cancer cells may stimulate LNFs in vivo and change their characteristics in a manner similar to CAFs. Although the marker profile of lung cancer stem cells remains to be fully elucidated, recent findings suggest that CD44 or CD133 may be an effective lung cancer stem cell marker [26, 27]. In addition, CD44 has been associated with a number of signaling cascades that mediate tumor progression and resistance to chemotherapy [28, 29]. Cancer cells in the present study showed increased levels of these CSC markers, supporting the notion that fibroblasts in the tumor stroma maintain cancer stem cell-like properties.

Phenotypic changes associated with EMT and stem cell-like qualities were inhibited by addition of the TGF-β inhibitor SB431542, indicating that TGF-β signaling plays a major role in fibroblast-induced changes. The TGF-β is expressed by both cancer and stromal cells, and establishes interactive pathways in the cross-talk between them [2]. In the efferent pathway cancer cells trigger a reactive response in the stroma, while in the afferent pathway cells responding to modified stromal cells in the surrounding microenvironment have effects on cancer cell response [30, 31]. These interactive pathways, which become established during cross-talk between cancer and stromal cells, may form a malignant signaling cycle for cancer progression. Because CAFs are genetically stable and less likely to develop drug resistance [15], targeting them as a therapeutic strategy against cancer is an intriguing concept that would benefit from further study.

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