**Table 1** Results of the questionnaire survey

| | |
|---|---|
| Institutes which responded to the questionnaire | 17 |
| Doctors who responded to the questionnaire | 26 |
| Standardized follow-up program within the institute? | |
| Yes | 6 |
| No | 11 |
| Continued follow-up for more than 5 years? | |
| Yes | 15 |
| No | 11 |
| Arranging the follow-up schedule by disease stage? | |
| Yes | 15 |
| 1A/1B,2,3 | 6 |
| 1A/1B,2/3 | 4 |
| 1/2/3 | 2 |
| 1A/1B/2,3 | 1 |
| 1/2,3 | 1 |
| 1,2/3 | 1 |
| No | 11 |
| Physical examination? | |
| Yes | 26 |
| No | 0 |
| Blood examination? | |
| Yes | 26 |
| No | 0 |
| Sputum cytology? | |
| Yes | 0 |
| No | 26 |
| Chest radiography? | |
| Yes | 26 |
| No | 0 |
| Abdominal ultrasonography? | |
| Yes | 0 |
| No | 26 |
| CT? | |
| Yes | 26 |
| No | 0 |
| Bone scintigraphy? | |
| Yes | 5 |
| No | 21 |
| Brain MRI or CT? | |
| Yes | 10 |
| No | 16 |
| PET/CT? | |
| Yes | 7 |
| No | 19 |

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CT, Computed tomography; MRI, Magnetic resonance imaging; PET, Positron emission tomography

(CT) routinely. Six doctors performed PET (positron emission tomography) or PET/CT, and nine doctors performed brain MRI or brain CT routinely. None of the doctors performed sputum cytology or abdominal ultrasonography in the setting of postoperative follow-up.

**Table 2** First recurrence site

| | | | |
|---|---|---|---|
| Lung | 95 | Vertebra | 5 |
| Mediastinal lymph node | 64 | Bronchial stump | 5 |
| Brain | 45 | Staple line in lung | 3 |
| Pleural dissemination | 30 | Malignant pericarditis | 3 |
| Liver | 17 | Kidney | 2 |
| Chest wall | 14 | Pulmonary hilar lymph node | 1 |
| Cervical lymph node | 13 | Carcinomatous meningitis | 1 |
| Adrenal | 11 | Spleen | 1 |
| Rib | 9 | Others | 25 |

All recurrence sites were listed in patients with multiple recurrences at the time of the detection of the first recurrence

**Table 3** Examinations and symptoms based on which recurrence was diagnosed or suspected

| | | | |
|---|---|---|---|
| Asymptomatic recurrence | 154 | Symptomatic recurrence | 76 |
| CT | 93 | Respiratory symptoms | 13 |
| PET | 20 | Cranial nerve abnormalities | 24 |
| Chest X-ray | 14 | Pain | 21 |
| Brain MRI | 4 | Others | 18 |
| Elevation of serum CEA | 20 | | |
| Elevation of serum CYFRA | 1 | | |
| Bone MRI | 1 | | |
| Hepatic dysfunction | 1 | | |

*CT* computed tomography, *PET* positron emission tomography, *MRI* magnetic resonance imaging, *CEA* carcinoembryonic antigen, *CYFRA* cytokeratin 19 fragment

Results of our retrospective analysis of the clinical data are summarized in Fig. 1 and Tables 2, 3. The median age was 67 years and 162 patients were male. Histological examination revealed adenocarcinoma in 150 patients, squamous cell carcinoma in 59 patients and other histological types in 21 patients; the p stage was p-Stage IA in

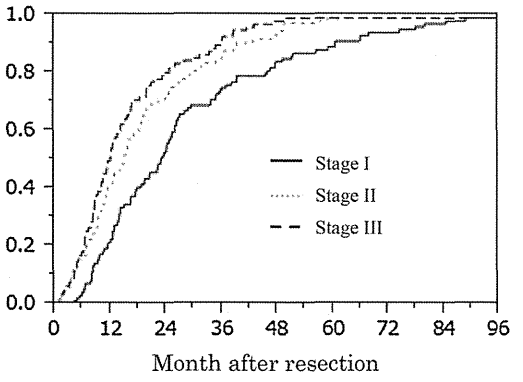


**Fig. 1** Cumulative recurrence curves of the retrospective analysis according to disease stage

36 patients, IB in 63, IIA in 13, IIB in 43, IIIA or more advanced stage in 75. 62.7 % of recurrence developed within 24 months, 81.5 % of recurrence developed within 36 months and 94.7 % of recurrence developed within 5 years. The cumulative recurrence curve according to disease stage is shown in Fig. 1. Recurrences in patients with advanced disease developed significantly earlier than those in patients with early disease. The first recurrence sites are listed in Table 2. The most common site of recurrence was the lung, followed in frequency by the mediastinal lymph node and the brain, in that order. The recurrence was symptomatic in 76 patients (33.0 %) and asymptomatic in 154 patients (67.0 %) (Table 3). In the patients with asymptomatic recurrence, the recurrence was most frequently detected by CT (93 patients, 40.4 %), followed by PET-CT (20 patients, 8.7 %) and chest radiography (14 patients, 6.1 %); and in 21 cases (9.1 %), the recurrence was detected by elevated levels serum tumor markers, including carcinoembryonic antigen (CEA) and cytokeratin 19 fragment (CYFRA).

## Discussed issues

### *Follow-up period*

Several investigators reported that incidence of recurrence decrease 5 years after resection [14, 15]. Similarly, our retrospective analysis of the clinical data showed that approximately 94.8 % of recurrences developed within 5 years after resection (Fig. 1). Lung cancer is generally accepted to be cured if no recurrences develop within 5 years after resection. The follow-up period was, therefore, set as 5 years. Extension of the follow-up period to 7–8 years in total may be acceptable, but extension to over 10 years is not recommended from the standpoint of cost-effectiveness [16].

### *Individual examinations*

*CT* CT is the most efficient examination modality to detect pulmonary lesions and is recommended as the main modality for follow-up surveillance [17–19]. Our retrospective analysis showed that approximately 63 % of recurrences developed during the first 2 years after resection (Fig. 1). Intensive CT follow-up is, therefore, performed every 6 months for the first 2 years after resection and repeated annually thereafter in the Moderate-risk pathway, High-risk pathway and Comprehensive pathway. Since the supraclavicular lymph nodes, liver and adrenal glands are well known common recurrence sites [8], the scan area is set from the lower poles of the thyroid gland to the upper abdomen including the adrenal glands. Enhanced CT is superior to plain CT for detecting small abnormalities, however, owing to concerns about the safety and complexity, the choice between plain and enhanced CT is left to the discretion of the attending doctor.

*Chest radiography* Chest radiography is known to have some limitation in terms of its sensitivity, but it is commonly performed due to the quickness of the test, low radiation exposure and low cost. Our retrospective analysis of the clinical data showed chest radiography detected recurrence in 14 patients out of 154 patients with asymptomatic recurrence. Chest radiography is considered to be effective to detect recurrence and therefore scheduled in all pathways except the Low-risk pathway and is performed between the CT examinations.

*Brain CT or MRI* There is no evidence of periodic brain screening being beneficial to the patients' survival. The results of the questionnaire survey also showed that doctors who performed brain screening in the setting of follow-up were in the minority (Table 1). Brain screening is therefore not recommended in these pathways.

*Blood examinations, including tumor marker measurements* The efficacy of measurement of tumor markers in the setting of postoperative follow-up has not been proven, but our retrospective analysis showed that in approximately 13 % of patients with asymptomatic recurrences, the recurrences were suspected based on elevation of the serum levels of CEA and confirmed by imaging examinations. Similarly, in 0.6 % of patients, recurrence was suspected based on elevation of the serum level of CYFRA (Table 3). Therefore, measurement of tumor markers was considered to be of some benefit for the diagnosis of recurrence. The Japanese national health insurance covers measurement of two kinds of tumor markers. Measurement of the serum CEA is recommended in these pathways, since it is considered to be the most sensitive marker and is also a known prognostic factor [20]. Measurement of any other tumor markers is left to the discretion of the attending medical doctor.

*FDG-PET/CT, bone scintigraphy and abdominal ultrasonography* FDG-PET/CT enables examination of the whole body, excluding the brain, in a non-invasive manner and also differentiation between malignant and benign lesions. The literature provides much evidence of its superior diagnostic performance over CT for the diagnosis of cancer recurrence [21, 22]. Antoniou et al. [23] also reported the usefulness of periodical examination by PET/CT in the setting of postoperative follow-up. The benefits of PET/CT over conventional examinations in the setting of postoperative follow-up had, however, not yet been demonstrated clearly. In addition, FDG-PET/CT is only available at selected institutes and its high cost is another problematic issue. Therefore, it cannot but be said that it is still premature to recommend PET/CT in the setting of postoperative follow-up so far. It might be a good idea to

use PET not in all patients but in selected patient, for example patients with advanced disease. Applying PET/CT in the setting of postoperative follow-up might be re-evaluated and re-considered in the future.

Aokage et al. [24] reported that periodic abdominal ultrasonography is not beneficial as a periodic postoperative follow-up examination. There is no paper addressing the efficacy of bone scintigraphy. The questionnaire survey also showed that only a few doctors performed these examinations in the setting of postoperative follow-up (Table 1). Therefore, abdominal ultrasonography and bone scintigraphy are not recommended in these pathways.

*Frequency and interval of the examinations*

There were two opinions about the degree of aggressiveness to be adopted for follow-up taking into consideration the risk of recurrence. One was that the follow-up intensity should be modified, according to the risk of recurrence. The other was to use a single universal pathway for all patients, for reasons of simplicity and ease of adoption. We held discussions to determine which approach might be superior and should be recommended, but could not arrive at any definitive conclusion. The results of the questionnaire survey also showed that approximately half of the doctors devised the follow-up program according to the risk of recurrence, while the other half used a common follow-up program for all patients (Table 1). Two types of pathways (Risk of recurrence-based pathway and Comprehensive pathway), were, therefore retained in this pathway, and selection between the two pathways for individual patients is left to the discretion of the attending doctor.

*Risk of recurrence-based pathway* The 5-year disease-free survival in patients with MIA or AIS is reported to be almost 100 % [25, 26]. Considering the excellent outcomes after resection for MIA or AIS, while follow-up is necessary in this population, minimal follow-up interventions are considered to be adequate. In contrast, it is estimated that more than 50 % of patients with Stage II or more advanced disease develop recurrence [27]. This patient group should therefore be followed up more intensively; however, a short-term intensive follow-up schedule might be adequate, according to the results of Fig. 1. In this pathway, the surveillance modalities are, therefore classified into three subgroups and modified according to the risk of recurrence (Table 4).

(a) Pathway for the low risk of recurrence group (Low-risk pathway): This pathway is intended for patients with minimal invasive adenocarcinoma (MIA) and adenocarcinoma in situ (AIS).
(b) Pathway for the moderate risk of recurrence group (Moderate-risk pathway): This pathway is intended for patients with stage I cancer other than those with MIA or AIS.
(c) Pathway for the high risk of recurrence group (High-risk pathway): This pathway is intended for patients with stage II or more advanced stage cancer.

*Comprehensive pathway* This is a universal pathway that covers all patients. The examination schedule in the Comprehensive pathway is exactly the same as that for the moderate-risk group in the Risk of recurrence-based pathway.

*Medical cost of the follow-up examinations* The medical cost was calculated, based on the current health insurance system. The 5-year medical cost of the Low-recurrence risk pathway was estimated to be ¥160370. Similarly, the costs for the Moderate recurrence risk pathway and High recurrence risk pathway were estimated to be ¥259220 and ¥328670, respectively.

## Discussion

The distinguishing points of our follow-up pathways are the consistency of the two types of pathways taking into consideration the risk of recurrence and measurement of serum tumor markers. Several medical societies have drawn up their own follow-up programs, however, all are common follow-up programs that cover all categories of patients irrespective of the individual risk of recurrence, and do not include measurement of the serum tumor markers. In our pathways, the follow-up intensity is modified according to the risk of recurrence in the Risk of recurrence-based pathway, and three pathways are proposed according to the risk of recurrence. Considering that patients with AIS or MIA rarely develop recurrence and more than 50 % of patients with stage II or more advanced disease develop recurrence, it might be reasonable to modify the follow-up intensity according to the risk of recurrence. On the other hand, the simplicity and ease of use of the comprehensive pathway may make it more advantageous and acceptable, especially under the circumstance in which the efficacy of postoperative follow-up is still unclear. As Table 1 shows, serum tumor marker measurements are already performed in all institutes in Japan and the test is considered to be already accepted as a standard modality in the setting of postoperative follow-up in lung cancer patients.

Medical cost is a critical issue to discuss the efficiency of follow-up surveillance. If the cost efficiency needs to be evaluated strictly, the concept of cost per life-year gained

**Table 4** Follow-up pathways

| Time after resection (Month) | 1 | 3 | 6 | 9 | 12 | 15 | 18 | 21 | 24 | 27 | 30 | 33 | 36 | 39 | 42 | 45 | 48 | 51 | 54 | 57 | 60 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| I. Risk of recurrence-based pathway | | | | | | | | | | | | | | | | | | | | | |
| I-A: Pathway for the group with a low risk of recurrence | | | | | | | | | | | | | | | | | | | | | |
| Physical examination | ● | ● | | | ● | | | | ● | | | | ● | | | | ● | | | | ● |
| Blood examination | ● | | | | ● | | | | ● | | | | ● | | | | ● | | | | ● |
| Chest radiography | ● | ● | | | | | | | | | | | | | | | | | | | |
| CT | | | | | ● | | | | ● | | | | ● | | | | ● | | | | ● |
| I-B: Pathway for the group with a moderate risk of recurrence | | | | | | | | | | | | | | | | | | | | | |
| Physical examination | ● | ● | ● | | ● | | ● | | ● | | ● | | ● | | ● | | ● | | ● | | ● |
| Blood examination | ● | | ● | | ● | | ● | | ● | | ● | | ● | | ● | | ● | | ● | | ● |
| Chest radiography | ● | ● | | | | | | | | | ● | | | | ● | | | | ● | | |
| CT | | | ● | | ● | | ● | | ● | | | | ● | | | | ● | | | | ● |
| I-C: Pathway for the group with a high risk of recurrence | | | | | | | | | | | | | | | | | | | | | |
| Physical examination | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | | ● | | ● | | ● | | ● |
| Blood examination | ● | | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | | ● | | ● | | ● | | ● |
| Chest radiography | ● | ● | | ● | | ● | | ● | | ● | ● | ● | | | ● | | | | ● | | |
| CT | | | ● | | ● | | ● | | ● | | | | ● | | | | ● | | | | ● |
| II. Comprehensive pathway | | | | | | | | | | | | | | | | | | | | | |
| Physical examination | ● | ● | ● | | ● | | ● | | ● | | ● | | ● | | ● | | ● | | ● | ● | |
| Blood examination | ● | | ● | | ● | | ● | | ● | | ● | | ● | | ● | | ● | | ● | ● | |
| Chest radiography | ● | ● | | | | | | | | | ● | | | | ● | | | | ● | | |
| CT | | | ● | | ● | | ● | | ● | | | | ● | | | | ● | | | ● | |

(LYG) needs to be discussed. This estimation is, however, difficult to perform, and the medical economic burdens described above are considered to be acceptable under the current medical environment in Japan.

Another important concern of the postoperative follow-up would be a risk of radiation exposure by chest radiography and CT performed during the follow-up. Several investigators suggested that excessive radiation exposure by medical examinations might lead the second malignancy and might be harmful [28–30]. Chest CT is repeated 7 times and chest radiograph repeated 5 times and patients receive in total approximately 49 mSv during the 5-year follow-up period in the Comprehensive pathway and the pathway for the moderate-risk group in the Risk of recurrence-based pathway. Considering benefits and potential risks of the postoperative follow-up, this amount of radiation exposure during the postoperative follow-up might be justified and acceptable. Chiu et al. reported of usefulness of low dose CT in the postoperative follow-up. Low dose CT might minimize the risk of radiation exposure in the course of the post-follow-up [5].

In patients with Stage II or more advanced disease, adjuvant chemotherapy with platinum doublet is recommended [31–34]. In these patients, the postoperative follow-up pathway would be adopted after finishing adjuvant chemotherapy.

Brain is one of the most frequent sites of recurrence, and the quality of life is known to deteriorate rapidly once brain metastasis develops and becomes symptomatic [35–37]. Treating brain metastasis in the asymptomatic stage might therefore contribute to a better quality of life of the patients. However, there is little evidence to justify screening for brain metastasis until today and brain screening is not recommended in the pathways.

The Ministry of Health, Labour and Welfare has promoted cooperation between the cancer institute and a family doctor. The concept of the cooperation is that the cancer institute plays a main role of treatment of cancer and a family doctor takes care of daily care. Considering a participation of this cooperation in the postoperative follow-up pathways presented in this paper, it is considered be adequate that the cancer institute performs main examinations such as CT examination, while a family doctor performs blood examination, chest radiography, daily care and refers a patient to the cancer institute, if abnormality is detected. The way of the cooperation between cancer institute and family doctor is in the middle of improvement and needs further discussion.

The limitations of our follow-up pathways are that they were designed based on the retrospective analysis and clinical experience and the feasibility and efficacy, such as an optimal frequency, intervals of the examinations, the

5-year successful execution rate, recurrence detection rate and survival benefits, etc., are unknown. A prospective study is now ongoing to attempt to answer these questions and is not designed to be a randomized control study between the Risk of recurrence-based pathway and Comprehensive pathway. Patients are assigned to either the Risk of recurrence-based pathway or the Comprehensive pathway according to attending doctors' choice and followed up according to each schedule. The primary endpoint is the recurrence detection rate during the first 3 years after resection of each postoperative pathway. The secondary endpoints are the 5-year successful execution rate, 5-year survival rate. This study is now open and it is planned to recruit approximately 400 patients. The examination schedule such as the frequency and interval proposed in the pathways will be revised according to results of this prospective study.

In conclusion, we proposed established pathways for postoperative follow-up of lung cancer patients after complete resection. Two pathways, the Risk of recurrence-based pathway and the Comprehensive pathway, are proposed. A prospective study is now ongoing to determine the feasibility and efficacy of these follow-up pathways.

**Conflict of interest** None of the authors has any financial or other potential conflicts of interests to declare.

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◎コラム

# LVRS

## 肺気腫治療の1つとして考慮される肺容量減少手術

山下 素弘* YAMASHITA, Motohiro

重症肺気腫に対するLVRS (lung volume reduction surgery) は，GOLD*1 Ⅳ期の治療法の1つに，肺移植とともに挙げられており，日本の「COPD診断と治療のためのガイドライン」[1]でも外科治療法の1つとされている。Cooperら[2]がその臨床成績を発表し，それまでの包括的呼吸リハビリテーションと，気管支拡張薬を中心とする保存的治療法での限界を大きく変える可能性のある治療として，急速に脚光を浴びた。その治療概念は，肺の気腫性変化の不均一性heterogeneityに着目し，気腫性変化の比較的強い領域を外科的に切除し，肺の容積を減少させることで，残存肺および過膨張になった胸郭の機能や運動効率を改善することにある。

本稿では，外科的切除によるLVRSの治療成績について，米国で大規模に行われた臨床試験NETT*2の結果[3~6]を解説し，その後の状況と問題点，さらにLVRSに代わる治療法となる可能性のある内視鏡的治療についても触れる。

## 背景

肺気腫は，肺の呼吸細気管支より末梢の気腔拡大により気流閉塞を生じる病態で，肺弾性収縮力の低下による肺の過膨張と空気とらえこみair trappingがみられる。気腔の拡大は，程度の差はあるものの全肺野にみられる。びまん性に気腫性変化が生じていても，気腫性変化（空気とらえこみ）の特に強い領域が局在するheterogeneousなタイプが存在する。この気腫性変化により機能しなくなっている領域を切除し，胸郭の過膨張を減少することで換気メカニズムを改善し，残存する気腫肺のガス交換効率をより改善させるという概念は，1957年にBrantiganによって報告された。しかし，当時の手術関連死亡率の高さから，広く認識される治療法とはならなかった[2]。1995年にCooperら[2]は，気腫性変化に局在性がある重症肺気腫（heterogeneousタイプ）に対し，両側一期的LVRSで，肺の約20~30%の容積を減少させ，肺気腫患者の呼吸機能，呼吸困難感，運動能，クオリティオブライフ（QOL）の改善効果を報告すると，LVRSは急速に広まった。

## 手技

硬膜外麻酔併用の全身麻酔下に，気腫化した肺のなかでも特に気腫性変化の強く肺血流の乏しい領域（target area）を自動縫合器を用いて切除する。target areaは上葉に存在するのが理想的であり，肺容積の20~30%程度を目安に切除する。重症気腫肺は非常に脆弱であるため，切除に際して特に愛護的な操作が必要で，また切除ラインにかかる機械的ストレスの軽減のため，自動縫合器にバットレスbuttressとよばれる補強材料を用いる。補強材料として，現在では，自動縫合器のサイズに応じた生体吸収性の布製の製品（ネオベールシート）が用いられることが多い[2,7]。

手術アプローチは，胸骨正中切開で体位変換なしで両側肺切除する方法と，左右片側ずつ体位変換して胸腔鏡下に肺切除する方法があり，どちらの方法でも効果には差がない。当初，この具体的適応や手術法および周術期管理が十分に把握されないままLVRSが行われため，その合併症の多さ，死亡率の高さが問題となった。これらの問題を解明すべく，特に曖昧であったLVRSの患者選択基準と予後を明らかにし，保存的治療と比較した耐運動能やQOLにおける効果を検討する目的で，全米の17施設による多施設共同第Ⅲ相試験，NETTが1998年から開始された[3~6]。

*四国がんセンター 胸部外科

*1 GOLD：Global Initiative for Chronic Obstructive Lung Disease
*2 NETT：National Emphysema Treatment Trial


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＊3　1秒当たりの努力呼気量（$FEV_1$）の予測値に対する%
＊4　一酸化炭素肺拡散能の予測値に対する%

表1　NETTの対象患者（抜粋）

| |
|---|
| **inclusion criteria** |
| 4か月以上禁煙 |
| 呼吸機能および画像上肺気腫に合致 |
| ランダム化時にプレドニゾロン内服20 mg以下 |
| BMI：男性≦31.1 kg/$m^2$，女性≦32.3 kg/$m^2$ |
| 呼吸機能：%$FEV_1$≦45%（70歳以上では%$FEV_1$：15～45%），%TLC≧100%，%RV≧150% |
| 血液ガス所見：$PaCO_2$≦60 mmHg，$PaO_2$≧45 mmHg (room air) |
| 心機能：循環器科医による耐術可能の判断 |
| 運動能：無負荷から3分ごとにサイクルエルゴメーター参加可能で，リハビリテーション後の6分間歩行テスト≧140 m |
| 呼吸器内科・麻酔科・胸部外科医による，リハビリテーション前後とも耐術可能との判断 |
| **exclusion criteria** |
| 開胸術の既往 |
| 循環器疾患：心エコー上LVEF＜45%や重篤な不整脈症例，6か月以内の心筋梗塞と不安定な循環器疾患，コントロールされていない高血圧。心エコー上，収縮期PAP≧45 mmHgが予想される場合は，右心カテーテル検査で収縮期PAP≧45 mmHgを確認。 |
| 呼吸器疾患：繰り返す呼吸器感染，間質性肺炎，臨床上問題となる喀痰，気管支拡張症，巨大肺嚢胞，運動時の6Lを超える酸素吸入 |
| %DLco≦20%* |
| 全身状態：90日以内の10%以上の体重減少，呼吸リハビリテーション後の6分間歩行テスト≦140 m |
| %$FEV_1$：予測1秒量に対する%，%TLC：予測全肺気量に対する%，%RV：予測残気量に対する%，PAP：肺動脈圧，%DLco：予測一酸化炭素肺拡散能に対する% |
| ＊%DLco≦20%は2001年の報告後追加された。 |
| National Emphysema Treatment Trial Research Group. Patients at high risk of death after lung-volume-reduction surgery. N Engl J Med 2001；345：1075-83. |
| Rationale and design of the National Emphysema Treatment Trial (NETT)：A prospective randomized trial of lung volume reduction surgery. J Thorac Cardiovasc Surg 1999；118：518-28より作成 |

## NETT

### 研究デザイン

NETTは，①LVRSによって恩恵の得られる肺気腫患者を明らかにし，②その効果の期間と生存率を評価する目的で開始された。プライマリエンドポイントは，LVRS群と非手術群の生命予後と最大運動能を評価することで，2500人の症例集積と4年半の経過観察の予定で開始された[3～5]。1998年から開始され，LVRSの理想的適応に合致しない可能性のある肺気腫も対象に含めた患者設定（homogeneousタイプも含まれる）であった。

途中の効果安全性の評価により，2001年に，LVRSのリスクの高い%$FEV_1$[＊3]＜20%かつ，homogeneousタイプもしくは%DLco[＊4]＜20%のグループ（以降：高リスク群）が明らかになった。2001年5月以降はこれら高リスク群を除外し，2002年7月までの症例が集積された[6]。

### 対象（表1）

LVRSの患者選択基準と予後・運動能に対する効果を明らかにする目的で，対象となり得る肺気腫患者3777人を評価し，6～10週の包括的呼吸リハビリテーションと服薬治療ののち，1218人がランダム化された。当初，手術対象となる可能性のある肺気腫患者を広く対象とし，CT画像で「heterogeneous/homogeneous」，気腫の偏在性で「上葉型/下葉型/びまん性型（後に上葉型とそれ以外の2種類に分類）」に層別化してランダム化した。

安全性評価（2001年の1033人登録時）により，%$FEV_1$＜20%かつ，homogeneousタイプもしくは%DLco＜20%の肺気腫患者に対するLVRSの死亡率が高いことが明らかになった[6]。この2001年の報告以後，これらの高リスク群を対象から除外し，最終的にLVRSに608人，保存的治療に610人が割り付けられた。言い換えれば，患者集積の途中まで，LVRSの治療概念に合致しない可能性のある肺気腫も対象に含めた患者設定（homogeneousタイプも含まれる）であったことになる。

リハビリテーション後のランダム化時の最大運動能評価で，男性では40 W，女性では25 Wを閾値として「低運動能/高運動能」に分類し，まず2年間の予後，呼吸機能，活動性，耐運動能，QOLを評価し[3,8,9]，最終評価は登録終了後4年余りの調査期間を経て報告された[10]。

## 結果

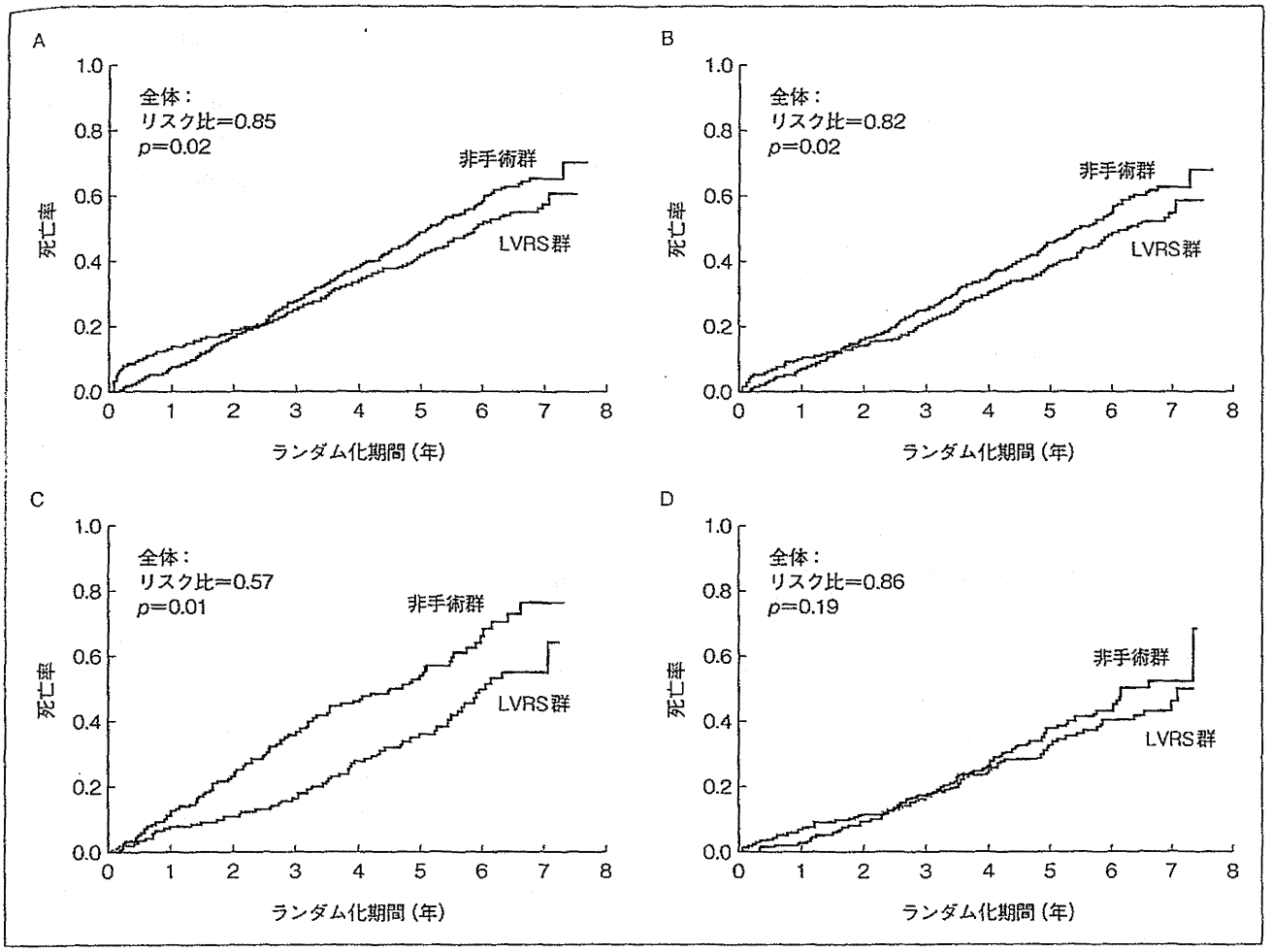
2001年の中間解析で，高リスク群のLVRS術後30日以内の死亡が16%〔95%信頼区間（CI）8.2～26.7〕，非手術群0%（$p$＜0.001），90日以内の死亡もLVRS群28%（95%CI 18.4～40.8），非手術群0%（95%CI 0～5.1）とLVRS群で高く，死亡原因は両群ともに呼吸器関連が約90%を占め，次いで循環器合併症が10%台であった[6]。平均観察期間29.2か月での報告[3]が2003年になされ，高リ

図1　ランダム化後のKaplan-Meier死亡曲線

A：全症例（$n$=1218），B：非高リスク患者（$n$=1078），C：上葉優位肺気腫で低運動能患者（$n$=290），D：上葉優位肺気腫で高運動能患者（$n$=419）

LVRS群全体（A）の生存率は5年の時点で有意に良好で（5年生存率LVRS群58%，非手術群51%，$p$=0.02），LVRSが有益とされた上葉型で低運動能の肺気腫患者群（C）では効果が際立っていた（生存率$p$=0.003，最大運動能$p$<0.001，健康関連QOL $p$<0.001）。上葉型で高運動能の肺気腫患者群（D）ではLVRSは生存率の改善はなかった（$p$値はFisher検定で算出し，平均観察期間は4.3年のもの）。

（Naunheim KS, et al. Long-term follow-up of patients receiving lung-volume-reduction surgery versus medical therapy for severe emphysema by the National Emphysema Treatment Trial Research Group. Ann Thorac Surg 2006；82：431-43より作成）




スク群も含む全体での死亡者数は，LVRS群157人，非手術群160人であった。90日以内の死亡者数はLVRS群7.9%（95%CI 5.9～10.3），非手術群1.3%（95%CI 0.6～2.6）（$p$<0.001）で有意にLVRS群が高かったが，観察期間全体での死亡割合は両群ともに0.11/person-yearであった。

運動能力はリハビリテーション終了時をベースラインとして，10W以上改善した患者数で示すと，2年目の時点で全LVRS群15%，非手術群3%（$p$<0.001）とLVRS群が上回っており，高リスク群を除くLVRS群では16%，非手術群3%（$p$<0.001）であった。QOL評価でも同様に全LVRS群の33%，非手術群の9%（$p$<0.001）と有意にLVRS群が良好であった。LVRSが有益とされた上葉型で低運動能の肺気腫患者群では，90日以内の死亡2.9%（95%CI 0.8～7.2），非手術群3.3%（95%CI 1.1～7.6）と差がなく，平均29.2か月の観察期間の死亡リスクは，LVRS群が有意に低く（リスク比0.47，$p$=0.005），経過での運動機能もQOLも有意に良好であった。高リスク患者群と，非上葉型で高運動能の肺気腫患者群では呼吸機能面での改善はわずかで，LVRSには適さないとの結果であった[3]。

さらにその後約2年の追跡調査（合計

表2 最大運動能の改善者割合経過

ベースラインから10W以上の最大運動能の改善した者の割合（母数は各群のベースライン時の数で算出）

| | | 1年 | 2年 | 3年 |
|---|---|---|---|---|
| 全症例 | LVRS群 ($n$=608) | 23% | 15% | 9% |
| ($n$=1218) | 非手術群 ($n$=610) | 5% | 3% | 1% |
| | オッズ比 | 5.79 | 5.06 | 7.43 |
| | $p$値 | <0.001 | <0.001 | <0.001 |
| 非高リスク群 | LVRS群 ($n$=538) | 24% | 17% | 10% |
| ($n$=1078) | 非手術群 ($n$=540) | 5% | 4% | 1% |
| | オッズ比 | 5.72 | 5.41 | 9.46 |
| | $p$値 | <0.001 | <0.001 | <0.001 |
| 上葉型で低運動能群 | LVRS群 ($n$=139) | 42% | 30% | 21% |
| ($n$=290) | 非手術群 ($n$=151) | 6% | 2% | 0% |
| | オッズ比 | 12.5 | 26.1 | — |
| | $p$値 | <0.001 | <0.001 | <0.001 |

Naunheim KS, et al. Long-term follow-up of patients receiving lung-volume-reduction surgery versus medical therapy for severe emphysema by the National Emphysema Treatment Trial Research Group. Ann Thorac Surg 2006；82：431-43より作成

表3 非高リスク群のLVRS術後90日以内死亡リスク

| | | 相対リスク | $p$値 |
|---|---|---|---|
| 手術関連死亡（90日以内） | 非上葉型肺気腫 | 2.99 | 0.009 |
| 呼吸器合併症（30日以内） | 年齢 | 1.05 | 0.02 |
| | %$FEV_1$ | 0.97 | 0.05 |
| | %DLco | 0.97 | 0.01 |
| 循環器合併症（30日以内） | 年齢 | 1.07 | 0.004 |
| | ステロイド内服 | 1.72 | 0.04 |
| | 非上葉型肺気腫 | 2.67 | <0.001 |

%$FEV_1$：予測1秒量に対する%，%DLco：予測一酸化炭素肺拡散能に対する%

Naunheim KS, et al. Predictors of operative mortality and cardiopulmonary morbidity in the National Emphysema Treatment Trial. J Thorac Cardiovasc Surg 2006；131：43-53より

平均4.3年）の結果，全症例の解析[9]で，死亡者はLVRS群で283人，非手術群で324人と有意にLVRS群の死亡が少なかった（図1）。留意すべきは周術期の死亡を除けば，いずれの曲線もLVRS群の傾きが緩やかで死亡が少ないことである。LVRS群全体の生存率は5年の時点で有意に良好で（図1-A），最大運動能は3年間，健康関連QOLは4年間良好であった（表2）。LVRSが有益とされた上葉型で低運動能の肺気腫患者群では効果が際立っていた（図1-C）。上葉型で高運動能の肺気腫患者群ではLVRSは生存率の改善はなかったものの（図1-D），最大運動能で3年間，健康関連QOLで4年間，有意に良好であった[9]。

## 手術関連死亡や合併症の予測因子

LVRSで大きな問題となる手術関連死亡や合併症の予測因子の検討は，実際にLVRSを受けた患者のうち上記の高リスク群に該当した69人を除いて検討された[10,11]（表3）。30日死亡率はLVRS群で2.2%，保存的治療群で0.2%（$p$<0.001）で，90日死亡率はLVRS群で5.2%，保存的治療群で1.2%（$p$=0.001）で，呼吸器合併症を29.9%，循環器合併症を20%で認めた。死亡原因は呼吸器関連（肺炎，ARDS，誤嚥，呼吸不全）で12例（43%），循環器関連（心筋梗塞，不整脈，肺動脈血栓塞栓症）で5例（18%），多臓器不全2例（7%），脳血管障害と敗血症が各1例，詳細不明が7例であった。頻度の高い合併症は，再挿管呼吸管理22%，不整脈19%，肺炎18%，人工呼吸器管理14%であった[10]。多変量解析の結果，手術関連死亡の予測因子は非上葉型肺気腫で，呼吸器合併症の予測因子は年齢，%$FEV_1$，%DLcoで，循環器合併症の予測因子は非上葉型肺気腫のみが独立した危険予測因子であった。

…

これらから，LVRSは上葉型で低運動能の肺気腫患者に推奨でき，上葉型で高運動能の肺気腫患者にも症状の緩和の意味で考慮され得ると結論された。さらに，NETTにおいて切除された標本の病理学的検討から，肺気腫にさまざまなタイプが存在し，とりわけ病理学的に慢性気管支炎所見があるものでは効果が得られにくい[11]ことも示唆されている。

## 費用効率

費用効率に関しては医療面だけでなく介護関連費用も含めて検討され，LVRS群では手術および周術期管理費用などで6万ドルと高額となること，術後7～36か月の総医療費は約36,200ドル vs. 49,600ドルで，保存的治療群より少なくなるが，最終的に5年までの治療経過にかかる費用の総額は，手術費用のためLVRS群が高額になる[12,13]ことも明らかにされた。

一方，LVRSの効果を最初に提唱した

*5 VENT：Endobronchial Valve for Emphysema Palliation Trial

Cooperら Washington大学のグループは，NETTのデータを活用した検討[14]から，上葉のheterogeneousタイプの肺気腫では，術前運動能のいかんにかかわらずLVRS群の生存率が優位に良好で（5年生存率LVRS群70%，保存的治療群60%，$p=0.02$），呼吸機能でもQOLでもLVRS群が良好な成績を維持できたと報告している。さらにWashington大学の連続250例との比較[15]で，周術期死亡率や合併症の頻度も類似し，5年に及ぶ生存曲線はほぼ一致し，heterogeneousタイプの肺気腫を選択することが大切であるとしている。

実際には，2001年に発表された，高リスク群はLVRSの治療に適さないとの結果報告が，すべてのタイプの肺気腫に対しLVRSの効果がないものとして誤解を生じた可能性がある[11, 16]。この発表以降，NETTへの患者登録は著しく減少し，予定集積期間に目標とした2500人の登録人数に達せず，1218人での解析となった。また，集積終了後4年半の追跡調査と合わせて7年の試験期間は，LVRSに対する関心をさらに低下させる結果となった。実際，米国で2004年に行われたLVRS 254件が2006年には105件と減少していると報告され，日本の2012年の集計[17]では年間35件であった。

## 肺気腫に対するLVRSの可能性

では，肺気腫に対するLVRSは行われなくなるのであろうか。筆者らの岡山大学の経験[7]では，30日以内の手術関連死亡は0%で，治療後の呼吸機能，6分間歩行距離などの指標は3年以上にわたり有意に改善していた。しかしながら，いったん改善した呼吸機能は経年的に低下する。つまりLVRSは肺気腫を根治させる治療法ではなく，呼吸機能を改善させる手術として位置づけられ，ほかの非手術療法と比べて手術関連リスクを上回るベネフィットが求められる。前述のとおり，NETT試験から明らかになったような，切除の対象となる気種性変化が上葉に存在した低運動能患者が，比較的安全に最大の効果が期待できると考える。ただし，周術期死亡率は通常の肺がん手術より高く，綿密な周術期の継続的で包括的な呼吸循環管理が必要と考える。そのためには，単に手術件数の多い呼吸器外科だけでなく，よく洗練された呼吸器・循環器・麻酔科・理学療法科のチーム医療が実践できる施設でのLVRS治療が，よりよい成果をもたらすと考えられる。

## 新たな手法でのLVRSの試み

LVRSの周術期の危険性や合併症の問題点を補うべく，新しく考えられた治療法が，経気管支的に処置を行い，容積減少治療を行う方法である。LVRSに比べ，侵襲が少なく，より合併のある患者にも対象とできる可能性などから，LVRSに代わって注目を集め始めている。気管支内処置法の違いによりいくつかの方法があるが，一方向弁を有するendobronchial valve（EBV）で最も大規模な臨床試験，VENT*5が行われており，その結果[18, 19]も報告されているので紹介する。

### 研究デザイン

その基本となる治療概念はLVRSと同様であるが，気胸などに使われる一方向弁をシリコンで作成し，小型の脱着可能なデバイスとして，過膨張をきたした区域あるいは葉気管支に挿入し，過膨張になった領域を脱気し，無気肺にする治療法である。

2004年11月～2006年4月までに米国で肺気腫患者977人を対象にスクリーニングし，321人が登録され，EBVと保存的治療に2対1の比率でランダム化された。高分解能（HR）CTを用い，治療の目標となる領域を肺気腫評価専用のソフトウェアで半自動的に評価し決定した。

プライマリエンドポイントは治療後6か月間の安全性として，6項目（死亡，膿胸，大量喀血，処置部以遠の肺炎，気胸，7日以上の肺漏もしくは24時間以上の人工呼吸器管理を要する呼吸不全）を評価し，効果評価では$FEV_1$と6分間歩行テストを比較した。セカンダリエンドポイントの効果判定にはQOL，呼吸困難度，エルゴメーターでの運動機能，酸素投与量とし，heterogeneityとHRCTでのfissure completenessを効果予測因子として評価した。

### 対象

対象のinclusion criteriaはNETTとほぼ同じで，具体的手技は原著に譲るが，経気管支鏡的に片側の目標とする肺葉が無気肺になるように葉気管支，区域気管支，あるいは亜区域気管支にEBVを挿入した[18, 19]。

### 結果

平均留置EBV数は3.8個（1～9個）で，装着に要した時間は平均約30分，目標肺葉は右上葉52.3%，左上葉24.3%，左下葉14%，右下葉9.3%であった。

$FEV_1$はEBV群で4.3%増加し，保存的治療群では2.5%減少していた（$p=0.005$）。6分間歩行テストも同様の傾向であった〔EBV群2.5%で9.3 m増加と，保存的治療群3.2%で10.3 m減少（$p=0.02$）〕。90日までの評価で入院を要するCOPD増悪は，EBV群で7.9%に対し，保存的治療群では1.1%（$p=0.03$），喀血はEBV群で6.1%，保存的治療群では0%（$p=0.01$）であった。12か月までの目標領域の肺炎はEBV群で4.2%にみられた。

EBV群の死亡はEBV挿入に関係しない呼吸不全3例，悪性腫瘍，虚血性腸炎，大量喀血が各1例でみられ，1年死亡率はEBV群で3.7%，保存的治療群では3.5%で，治療効果予測に関してはheterogeneityとfissure completenessが予

測因子となる。同様の多施設共同の臨床試験はヨーロッパでも行われ，呼吸機能，QOL，運動能力への効果は同様の結果であった。1年での死亡率はEBV群5%，保存的治療群7%で有意差なく，入院を要する呼吸不全増悪，喀血，気胸，肺炎などの合併症には有意差を認めなかったとしているが，症例数不足で有意差を検出するだけのパワーが足りない可能性を示唆している[19]。

結論として，heterogeneousな重症肺気腫に対するEBV治療は，軽度の呼吸機能，運動能，症状の改善効果を認めるが，COPD急性増悪，肺炎，喀血の頻度を増加させた。よりheterogeneityの際立ったfissure completenessの明確な肺気腫症例でEBV治療の有効性が期待できるが，その立証にはさらに洗練された臨床研究で確認する必要がある，としている。

…

そのほか，経気管支的に薬物を注入する方法，気管支内コイル塞栓法，気道内バイパス作成法に関して小規模な臨床試験も行われているが，明らかな有効性を示す所見は現在のところ得られていない。

この20年余りの間に，NETTをはじめとする各種の臨床試験や研究により，肺気腫に対する理解と治療法の開発が急速に進んできている。本稿によって，LVRSの適応と効果が正しく理解され，今後も増加する肺気腫治療の一手段として認識されるための一助となることを期待する。

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## Summary


- 肺気腫の特定の患者群では，LVRSが呼吸機能，運動能とクオリティオブライフ（QOL）の臨床的に意義のある改善効果を示し，特に上葉優位で低運動能の肺気腫群ではその生存率も改善する。
- 周術期合併症の多さと費用対効果の問題点などから，現実的にはあまり行われていない実情があるが，NETTの報告で最初に出されたLVRS高リスク患者群の報告の誤った解釈により，LVRSがすべてのタイプの肺気腫で効果に乏しく危険であると考えられている懸念がある。
- 長期成績も含めたNETTの最終結論は，対象とした患者群に対して，LVRSは生命予後，運動機能，QOLを改善する。
- %$FEV_1$<20%かつ，homogeneousタイプもしくは%DLco<20%の肺気腫に対するLVRSは，死亡率が高く推奨できない。
- LVRSは上葉型で低運動能の肺気腫患者に最も推奨でき，上葉型で高運動能の肺気腫患者にも症状の緩和の意味で考慮され得る。

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ORIGINAL ARTICLE - CANCER RESEARCH

# Ezrin-expressing lung adenocarcinoma cells and podoplanin-positive fibroblasts form a malignant microenvironment

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**Abstract**
*Purpose* Cancer cells and cancer-associated fibroblasts (CAFs) together create the tumor microenvironment, which affects malignant behavior. Lung adenocarcinomas with CAFs expressing podoplanin (PDPN) are clinically aggressive, but the molecular mechanism underlying this phenomenon has not been established. So we identified the characteristic immunophenotype of lung adenocarcinoma cells coexisting with PDPN-expressing CAFs (PDPN-CAFs) and examined how it relates to an aggressive clinicopathological outcome.
*Methods* We analyzed the clinicopathological characteristics of 119 adenocarcinomas with a uniform size (2–3 cm). The expression levels of ten invasiveness-related proteins which related to cell adhesion and invasiveness, such as Ezrin, were examined in cancer cells from PDPN-CAFs (+) cases and from PDPN-CAFs (−) cases ($n = 20$ each). To examine the functional importance of the identified protein on the invasion phenotype, we performed wound healing and a Matrigel invasion assay using shRNA-knockdown lung adenocarcinoma cells (PC-9).
*Results* The PDPN-CAFs (+) cases had significantly higher rates of node metastasis ($p < 0.01$) and vascular invasion ($p < 0.01$). The cancer cells from the PDPN-CAFs (+) cases also had a significantly higher staining score for Ezrin ($p < 0.01$) than those from the PDPN-CAFs (−) cases. The migration and invasion activities of the shEzrin-induced PC-9 cells were significantly lower than those of the control cells.
*Conclusions* Our results indicated that within a tumor microenvironment composed of PDPN-CAFs, increased Ezrin expression in cancer cells might play a key role in the invasiveness of lung adenocarcinoma.

**Keywords** Cancer microenvironment · Ezrin · Podoplanin · CAFs · Lung cancer

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## Introduction

Primary adenocarcinoma of the lung is known to have a poor prognosis, even if surgery is successful, and approximately 15 % of patients develop recurrences (Asamura et al. 2008; Devesa et al. 2005). The 5-year disease-free survival rate for patients with stage I disease is about 75 % (Yoshizawa et al. 2011). Thus, improved knowledge of the molecular mechanisms underlying the development and progression of lung adenocarcinoma is essential to the development and establishment of effective therapeutic modalities.

Cancer tissue is composed of not only cancer cells, but also stromal cells, such as fibroblasts, endothelial cells,

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and inflammatory cells; together, these cells create the tumor microenvironment. Previous studies have demonstrated that the biological behavior of cancers is influenced by the tumor microenvironment (Ishii et al. 2005; Ito et al. 2007). Extensive clinical evidence and experimental models have shown that certain types of cancer-associated fibroblasts (CAFs) may have a tumor-promoting phenotype (Gottschling et al. 2013; Ito et al. 2007; Korc 2007; Okusa et al. 1999; Xing et al. 2010).

We previously reported that CAFs expressing podoplanin (PDPN), a mucin-like transmembrane glycoprotein that is known as a lymphatic endothelial marker, was correlated with a poor prognosis in patients with all-stage lung adenocarcinoma,(Kawase et al. 2008) and a high rate of recurrence in patients with stage I lung adenocarcinoma (Ito et al. 2012a). Moreover, the co-transplantation of A549 (a human lung adenocarcinoma cell line) and PDPN-positive fibroblasts significantly increased the efficiency of tumor implantation, and the short hairpin RNA (shRNA) knockdown of PDPN in fibroblasts decreased the augmenting effect on A549 tumor formation in a mouse xenograft model (Hoshino et al. 2011). These results suggested that both cancer cells and CAFs expressing PDPN might create a more malignant microenvironment for tumor tissue. The biological characteristics of PDPN-CAFs are gradually being elucidated (Ito et al. 2012b), but the characteristics of cancer cells in a microenvironment containing PDPN-CAFs have never been analyzed.

Accordingly, we attempted to identify the immunohistochemical characteristics of lung adenocarcinoma cells coexisting with PDPN-expressing CAFs (PDPN-CAFs) and to examine how these immunohistochemical characteristics relate to aggressive outcomes.

## Materials and methods

### Patient selection

A total of 695 consecutive patients underwent surgical resection for primary lung cancer between January 2011 and June 2013 at the National Cancer Center Hospital East, Chiba, Japan. We reviewed the patients' clinicopathological information in their medical records and selected 119 cases of invasive lung adenocarcinoma with a tumor size of 2–3 cm in diameter. Patients who received preoperative treatment and cases with multiple primary lung cancer were excluded. All the surgical specimens were collected and analyzed after receiving the approval of the Institutional Review Board of the National Cancer Center Hospital East.

### Histological studies

The surgical specimens were fixed in 10 % formalin and were serially sectioned at 5-mm intervals; all the sections were then embedded in paraffin. The sections were stained using the hematoxylin and eosin (HE) method, the Alcian blue-periodic acid-Schiff (AB-PAS) method for the detection of cytoplasmic mucin production, or the Victoria-blue van Gieson (VVG) method for the detection of elastic fibers. All the histological materials included in this series were reviewed by two pathologists (S.S. and G.I.). The pathological stage was determined based on the TNM classification of the International Union Against Cancer (UICC), seventh edition. Histological typing of the primary tumors was performed based on the World Health Organization classification of cell types, third edition. Invasive lung adenocarcinomas were classified into the following subtypes: lepidic predominant, papillary predominant, acinar predominant, micropapillary predominant, and solid predominant. The predominant component was defined as the histological component that comprised the largest percentage among the components.

### Antibodies and immunohistochemical staining

The antibodies used in this study are summarized in Supplementary Table 1. The slides were deparaffinized in xylene and dehydrated in a graded ethanol series, and endogenous peroxidase was blocked with 3 % hydrogen peroxide in absolute methyl alcohol. After epitope retrieval, the slides were washed with phosphate-buffered saline and were incubated overnight at 4 °C using mouse or rabbit antihuman antibodies at their final dilution in the blocking buffer. The slides were washed again and incubated with EnVision (Dako, Glostrup, Denmark) for 1 h at room temperature. The reaction products were stained with diaminobenzidine; lastly, the slides were counterstained with Meyer hematoxylin.

### Calculation of immunohistochemical scores

All the stained tissue sections were semiquantitatively scored and evaluated independently under a light microscope by two pathologists (S.S. and G.I.); when the evaluation results differed, the final report was determined based on a consensus reached between the two pathologists who evaluated the slides together under a conference microscope. We graded the podoplanin expression by the CAFs as follows: grade 0, podoplanin-positive CAF area/stromal area $\times$ 100 = <10 %; grade 1, podoplanin-positive CAF area/stromal area $\times$ 100 = 10–50 %; and grade 2, podoplanin-positive CAF area/stromal area $\times$ 100 = >50 %.

The grades were based on a previously reported protocol (Kawase et al. 2008). The immunostaining score was evaluated based on the staining intensity and the percentage of cancer cells that showed positive staining. The following scoring system was used: 0 (negative staining, defined as no immunoreactivity); 1+ (weak staining intensity); and 2+ (strong staining intensity). We also evaluated the extent of staining in a lesion as a percentage (0–100 %). The staining scores were calculated by multiplying the percentage values by the staining intensity, with the scores ranging from 0 to 200. We confirmed that the positive control tissues were stained by each antibody, and we also performed negative control studies without the primary antigen for all the antibodies.

Immunohistochemical scores of cancer cells within PDPN-CAFs (+) areas and PDPN-CAFs (−) areas

We selected 20 adenocarcinomas with a PDPN-CAF expression grade of 1, which had sparsely PDPN-CAF (+) areas. One or two PDPN-CAFs (+) and PDPN-CAFs (−) areas (objective lens 20×; 0.24 $mm^2$) were selected, and the immunoreactivity of the cancer cells in each area was examined. The average staining score was determined, and the results were recorded as the score for that case.

Cell culture and reagents

The human lung adenocarcinoma cell line PC-9 was purchased from the European Collection of Cell Culture. The PC-9 cell lines were maintained in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10 % heat-inactivated fetal bovine serum (FBS, Nichirei Bioscience, Tokyo, Japan), 1 % glutamine (Sigma-Aldrich), and antibiotics (1 % penicillin and streptomycin [Sigma-Aldrich]). The cultures were incubated at 37 °C in an atmosphere containing 5 % $CO_2$.

Lentiviral vectors

For the Ezrin shRNA experiments, oligonucleotides were chemically synthesized. Oligonucleotides encoding both strands of the targeting sequence (Supplementary Table 2) were annealed and inserted into the *BglII*/*Xba*I sites of pENTR4-H1 (RIKEN BioResource Center, Japan). A cassette containing the H1 promoter plus the shRNA was transferred to a self-inactivating (SIN) LV construct using Gateway® LR ClonaseII™ Enzyme Mix (Invitrogen), generating CS-H1-shRNA-CG. Then, 293T cells were transfected with 3 plasmids: PCAG-HIV, pCMV-VSV-G-RSV-Rev (RIKEN BioResource Center), and CS-H1-shRNA-CG. The transfection was achieved using Lipofect AMINE 2000 reagent (Invitrogen) according to the manufacturer's instructions. Virus-containing medium was filtered through a 0.45-mm filter, and 8 μg/mL (final concentration) of polybrene (Sigma) was added for target cell transduction. The transduction efficiency was evaluated using a flow cytometry analysis to detect the positivity of enhanced green fluorescent protein (EGFP), the expression of which was under the control of the CMV gene promoter.

Western blot

The Western blot analysis was performed as previously reported (Hoshino et al. 2011). The blots were incubated overnight at 4 °C with antihuman mouse monoclonal Ezrin antibody (Cell signaling Technology, Inc.). After washing in TBS-T, the membranes were incubated with HRP-rabbit anti-mouse IgG (Zymed). ECL Western Blotting Detection Reagents (GE Healthcare) were used to develop the high-performance chemiluminescence film (GE Healthcare).

Wound healing assay

A single scratch was made in the monolayer using a micropipette tip. Subsequently, the cells were washed and then incubated with growth medium. After 7 h of observation, we measured the area occupied by the cells healing the wound and calculated the invasion rate.

Matrigel invasion assay

A Matrigel invasion assay was performed using 24-well culture chambers and a growth factor-reduced, Matrigel-coated filter with a pore size of 8 μm (Becton–Dickinson Labware). The lower chamber contained 0.6 mL of RPMI-1640 + 10 % FBS. In the upper compartment, $2 \times 10^4$ of shLuc or shEzin-induced PC-9 cells were placed in triplicate wells and were incubated for 24 h. After incubation, the cells that had passed through the filter were stained with hematoxylin and were counted under a microscope in 9 predetermined fields (950 × 650 μm/field).

Statistical analysis

For the univariate analysis, the Pearson chi-square test was used to determine the statistical significance of the differences between two groups. For the staining scores, the Mann–Whitney *U* test was used because it did not have a normal distribution. For pathological factors such as vascular invasion, lymphatic permeation, and pleural invasion, the differences in variables were evaluated using the log-rank test. All of the reported *p* values were two-sided, and the significance level was set at $p < 0.05$. All the analyses were performed using SPSS Statistics version 21.0 for Windows (SPSS, Chicago, IL, USA).

**Table 1** Relationship between grade of podoplanin expression in CAFs and clinicopathological characteristics

| Variables | The number of cases | | | *p* value | | |
|---|---|---|---|---|---|---|
| | PDPN-CAFs grade 0 ($n = 65$) | PDPN-CAFs grade 1 ($n = 34$) | PDPN-CAFs grade 2 ($n = 20$) | Grade 0 vs 1 | Grade 1 vs 2 | Grade 0 vs 2 |
| Gender | | | | | | |
| Male | 36 | 21 | 13 | 0.67 | 0.81 | 0.61 |
| Female | 29 | 13 | 7 | | | |
| Age | | | | | | |
| ≥70 | 32 | 15 | 10 | 0.68 | 0.78 | 0.95 |
| <70 | 33 | 19 | 10 | | | |
| Smoking status (B.I.) | | | | | | |
| ≥400 | 29 | 19 | 9 | 0.30 | 0.57 | 0.97 |
| <400 | 36 | 15 | 11 | | | |
| Node metastasis | | | | | | |
| *n* (+) | 2 | 6 | 6 | 0.02 | 0.32 | <0.01 |
| *n* (−) | 63 | 28 | 14 | | | |

*PDPN-CAFs* podoplanin-expressing cancer-associated fibroblast, *B.I.* Brinkmann index

## Results

### Relationship between grade of podoplanin expression in CAFs and clinicopathological characteristics in adenocarcinoma patients with pathological lesions of 2–3 cm in diameter

Table 1 shows the relationship between the grade of PDPN expression in CAFs and the clinicopathological characteristics of the 119 adenocarcinoma patients with pathological lesions of 2–3 cm in diameter. Sixty-five patients (54.6 %) were PDPN-CAF-negative (grade 0) (Fig. 1a), 34 (28.6 %) were PDPN-CAF-positive with a grade of 1, and 20 (16.8 %) were PDPN-CAF-positive with a grade of 2 (Fig. 1b).

In a univariate analysis, the PDPN-CAF expression grade (podoplanin-positive CAF area/stromal area × 100) was significantly associated with the rate of node metastasis (grade 0 vs. grade 1: $p = 0.02$, grade 0 vs. grade 2: $p < 0.01$) (Table 1). We also evaluated the local invasiveness of cancer cells, including vascular invasion, lymphatic permeation, and pleural invasion. Vascular invasion (grade 0 vs. grade 2: $p < 0.01$) significantly increased with an increased grade of PDPN expression in CAFs. For pleural invasion, there was a borderline significance (grade 0 vs. grade 2: $p = 0.05$) (Fig. 1c). These results were partly consistent with our previous reports of stage I adenocarcinoma cases (Ito et al. 2012a).

### Phenotypical differences of cancer cells in PDPN-CAFs (+) adenocarcinoma and PDPN-CAFs (−) adenocarcinoma

Since a correlation between PDPN-CAFs (+) and the increased invasive and metastasis abilities of cancer cells was confirmed, we examined the expression of invasion-related molecules, such as adhesion molecule and the epithelial-mesenchymal transition (EMT) markers, of cancer cells with grade 2 PDPN-CAFs (+) surrounding areas and grade 0 PDPN-CAFs (−) surrounding areas ($n = 20$, each). The clinicopathological characteristics of the extracted cases are shown in Supplementary Table 3. The results of the staining scores for the cancer cells in the grade 0 and grade 2 PDPN-CAF cases and the significance of these scores in univariate analyses are shown in Table 2. The Ezrin and E-cadherin scores of the cancer cells were significantly higher in the PDPN-CAF grade 2 cases (Fig. 2b, d) than in the grade 0 cases (Fig. 2a, b) (Ezrin: 32.5 vs. 73, E-cadherin: 57 vs. 93). The median staining scores of the other molecules did not show any significant differences.

### Phenotypical differences in cancer cells between PDPN-CAFs (+) areas and PDPN-CAFs (−) areas within the same tumor

To validate the anatomical correlation between Ezrin and E-cadherin-overexpressed cancer cells and PDPN-CAF, we examined the Ezrin and E-cadherin expressions in cancer cells within PDPN-CAFs (+) areas (Fig. 3c) and PDPN-CAFs (−) areas (Fig. 3d) within the same tumor using 20 PDPN-CAF grade 1 cases (Fig. 3a, b). Of the 2 antibodies, only the Ezrin staining score for cancer cells within PDPN-CAFs (+) areas (Fig. 3e) was significantly higher than that for cancer cells within PDPN-CAFs (−) areas (Fig. 3f) (score of 70 vs. 42.5, $p < 0.01$) (Table 3). No significant differences in the staining scores for E-cadherin were observed.

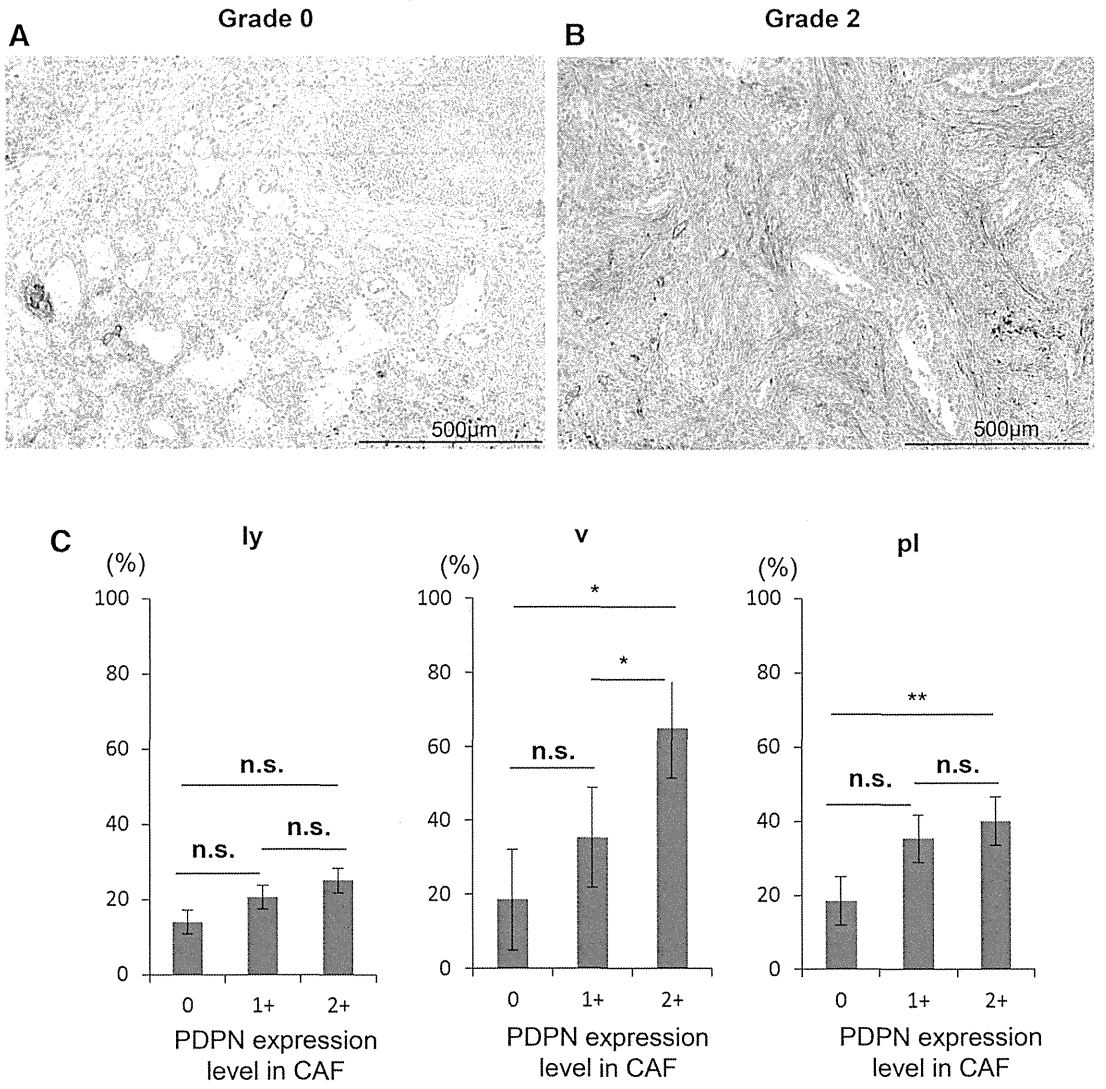


**Fig. 1** **a**, **b** Grading for PDPN-CAFs. **a** Grade 0: no PDPN-CAFs are present in the invasive area of the adenocarcinoma. **b** Grade 2: PDPN-CAFs are found in 50–100 % of the invasive area of the adenocarcinoma. **c** Frequency of lymphatic permeation, vascular invasion, and pleural invasion according to the PDPN-CAF grade in 119 adenocarcinoma patients. Vascular invasion significantly increased with an increased grade of PDPN expression in CAFs (*$p < 0.05$). For pleural invasion, there was a borderline significance (**grade 0 vs. grade 2: $p = 0.05$)

Ezrin-knockdown lung adenocarcinoma cells exhibited lower migration and invasive activities

To examine whether Ezrin expression in lung adenocarcinoma cells is involved in cell migration and invasiveness, we generated Ezrin-knockdown PC-9 cells. Short hairpin RNA for Ezrin or luciferase was transduced into PC-9 cells. The expression levels of Ezrin in the transduced cells were confirmed using a Western blot analysis (Supplementary Figure 1) and RT-PCR (data not shown). In the wound healing assay, the migration rate of each shEzrin (shEzrin 1 to shEzrin 3)-induced PC-9 cell line was significantly lower than that of a control. Moreover, in a Matrigel invasion assay, the invasive activities of the shEzrin-induced PC-9 cell lines were also significantly lower than those of the control cells (Fig. 4).

## Discussion

We previously reported that PDPN-CAFs were associated with a tumor-promoting phenotype in both in vitro and in vivo studies (Hoshino et al. 2011). The current study using adenocarcinomas of relatively uniform size showed that

**Table 2** Staining score of cancer cells in PDPN-CAFs (−) and PDPN-CAFs (+) cases

| Category | Antibody | Staining score of cancer cells[a] | | *p* value |
|---|---|---|---|---|
| | | PDPN-CAFs grade 0 | PDPN-CAFs grade 2 | |
| Cell adhesion and invasion | Integrin β1 | 10 (0–82) | 34 (0–132) | 0.16 |
| | Laminin 5 | 26 (4–92) | 41 (4–100) | 0.41 |
| | CD44 | 20 (0–88) | 40 (0–180) | 0.14 |
| | Ezrin | 32.5 (0–70) | 73 (20–100) | <0.01 |
| Growth factor receptor | EGFR | 3 (0–56) | 5 (0–78) | 0.43 |
| | cMET | 75 (14–134) | 52 (10–128) | 0.19 |
| EMT | E-Cadherin | 57 (20–92) | 93 (14–170) | <0.01 |
| | Fibronectin | 0 (0–24) | 0 (0–76) | 0.28 |
| | Clusterin | 4 (0–52) | 12 (0–136) | 0.46 |
| | Caveolin | 0 (0) | 0 (0–56) | 0.29 |

*PDPN-CAFs* podoplanin-expressing cancer-associated fibroblast, *EMT* epithelial-mesenchymal transition

[a] Median (range)

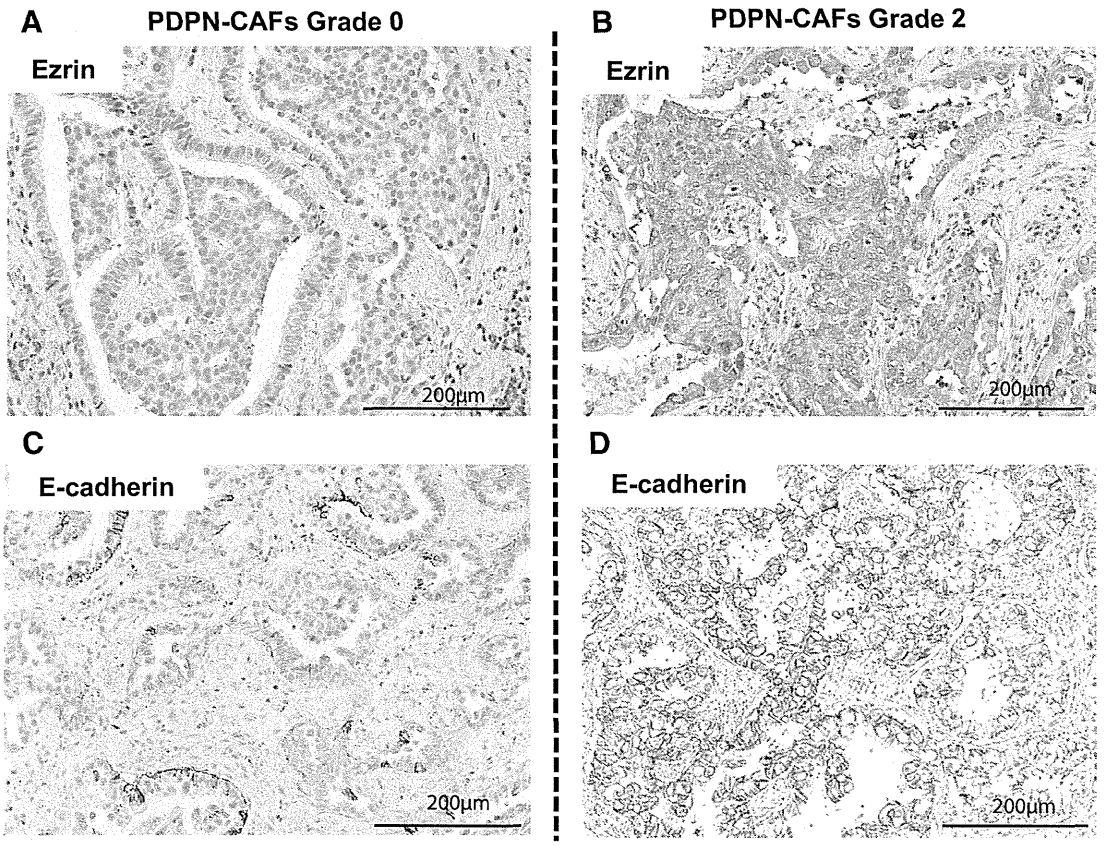


**Fig. 2** Phenotypical differences in cancer cells in PDPN-CAFs (+) adenocarcinoma and PDPN-CAFs (−) adenocarcinoma. **a** Ezrin expression in cancer cells in PDPN-CAFs (−) adenocarcinoma. **b** Ezrin expression in cancer cells in PDPN-CAFs (+, grade 2) adenocarcinoma. **c** E-cadherin expression in cancer cells in PDPN-CAFs (−) adenocarcinoma. **d** E-cadherin expression in cancer cells in PDPN-CAFs (+, grade 2) adenocarcinoma

vascular invasion, pleural invasion, and node metastasis were associated with an increased grade of PDPN expression in CAFs, which was partly consistent with our previous clinicopathological reports (Kawase et al. 2008; Ito et al. 2012a). These results suggested that adenocarcinoma cells coexisting with PDPN-CAFs have a high malignant potential, such as invasiveness. In light of these results, we examined invasiveness-related immunohistochemical

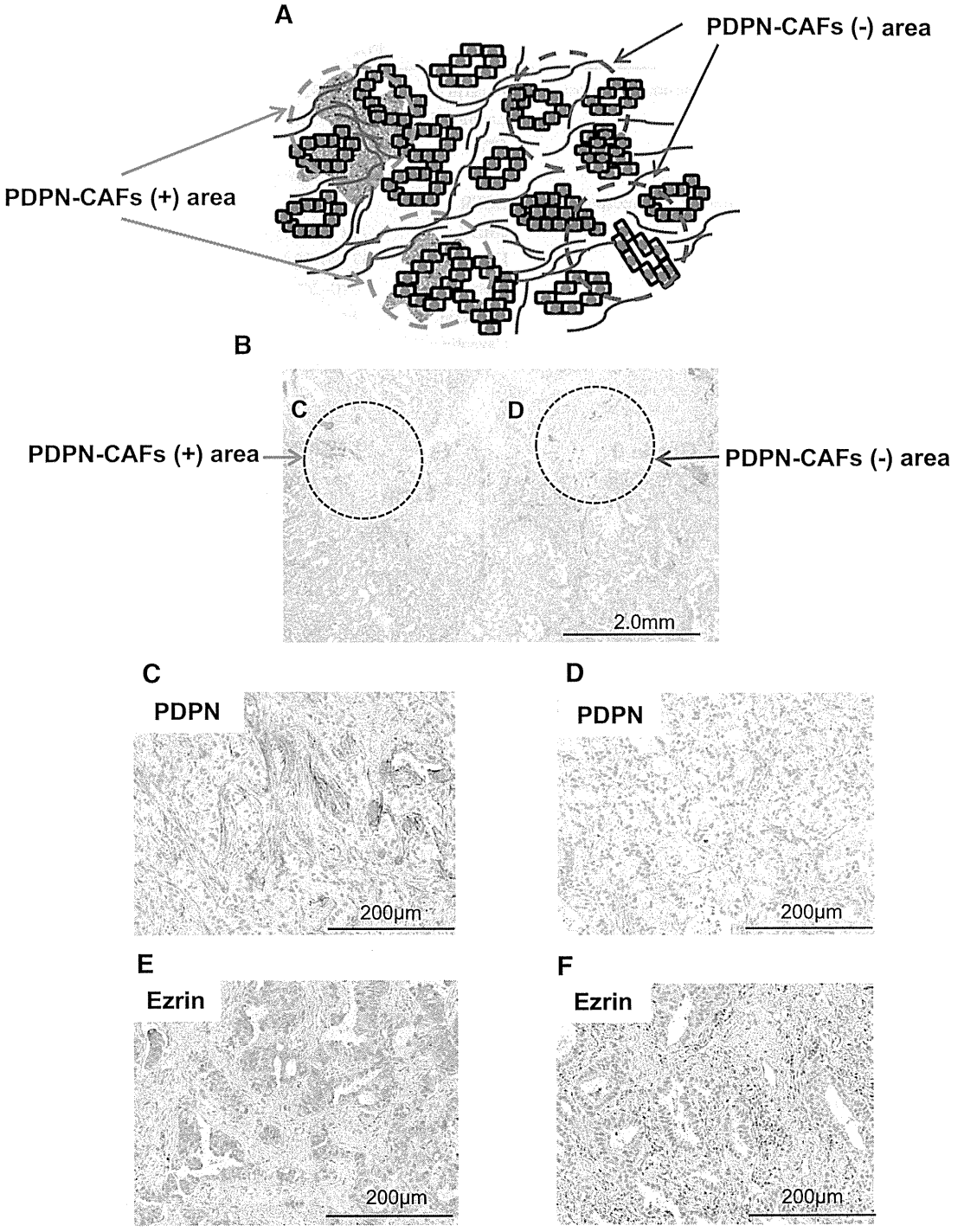


**Fig. 3** Phenotypical differences between cancer cells in PDPN-CAFs (+) area and PDPN-CAFs (−) area within the same tumor. **a** Schematic representation of PDPN-CAFs (+) area. **b** PDPN-CAFs grade 1 case which has PDPN-CAFs (+) area and PDPN-CAFs (−) area within the same tumor. **c** Area in which CAFs expressed PDPN. **d** Area in which CAFs did not express PDPN. **e** Ezrin expression in cancer cells within area (**c**). **f** Ezrin expression in cancer cells within area (**d**)