Differences of tumor microenvironment between stage I lepidic-positive and lepidic-negative lung adenocarcinomas

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Glossary of abbreviations:

Lep+ adenocarcinoma: lepidic-growth positive adenocarcinoma

Lep- adenocarcinoma: lepidic-growth negative adenocarcinoma

PDPN: podoplanin

CAFs: cancer-associated fibroblasts

TAMs: tumor-associated macrophages

CT: Computed tomography

TSCT: thin-section computed tomography

GGO: ground-glass opacity

HE: hematoxylin-eosin

VVG: Victoria blue-van Gieson

IHC: immunohistochemical

PBS: phosphate-buffered saline

DAB: diaminobenzidine

CEA: Carcinoembryonic antigen

EGFR: Epidermal growth factor receptor

AIS: adenocarcinoma in situ

MIA: minimally invasive adenocarcinoma
53 LPA: lepidic predominant adenocarcinoma

54 PET: positron emission tomography

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Central message:

Lower cancer cell-specific expression levels of hypoxia markers and a smaller number of tumor-promoting stromal cells in invasive component were characteristic features of Lep+ lung adenocarcinomas.
Perspective statement:

We characterized the clinicopathological differences of the invasive components between Lep+ and Lep- invasive size-matched tumors belonging in pathological stage I. Given that Lep+ adenocarcinoma is less invasive with less metastatic potential, early stage small Lep+ adenocarcinomas may be treated with limited resection techniques such as wide-wedge resection and segmentectomy.
Lep+ adenocarcinomas are less malignant than invasive size-matched Lep- ones.
Abstract

Objective:
Lepidic growth is a non-invasive component of lung adenocarcinoma. Many adenocarcinoma cases contain coexistent lepidic and non-lepidic (invasive) components (lepidic-growth positive; Lep+ adenocarcinoma); however, some cases comprise only non-lepidic components (lepidic-growth negative; Lep- adenocarcinoma). The aim of this study was to investigate the biological differences between the invasive components of Lep+ and Lep- adenocarcinoma.

Methods:
We investigated the clinicopathological characteristics of 232 adenocarcinomas (116 size-matched tumor pairs from Lep+ and Lep- adenocarcinomas). We then evaluated the cancer cell-specific expression levels of cancer stem-cell, hypoxia, and invasion molecules in these lesions. The number of tumor-promoting stromal cells, including podoplanin-positive cancer-associated fibroblasts (PDPN+ CAFs) and CD204-positive tumor-associated macrophages (CD204+ TAMs) was also analyzed.

Results:
Among cases with size-matched invasive components, significant differences were shown in total tumor size and predominant subtype in invasive component between
Lep+ and Lep- adenocarcinomas. The expression levels of hypoxia related molecules were significantly lower in Lep+ adenocarcinomas (GLUT1: 0 vs. 10, p<0.01; CA IX: 0 vs. 0 (mean: 4.7 vs. 14.1), p=0.01). The number of PDPN+ CAFs and CD204+ TAMs was significantly lower in Lep+ adenocarcinomas (PDPN+ CAFs: 0 vs 0 (mean: 1.6 vs. 11.6), p<0.01; CD204+ TAMs: 8.7 vs 24.7, p<0.01).

Conclusion:

Our results indicated that lower cancer cell-specific expression levels of hypoxia markers and a smaller number of tumor-promoting stromal cells in invasive component were characteristic features of Lep+ adenocarcinomas.
Lung cancer is one of the leading global causes of mortality. Adenocarcinoma, the most common histological subtype of this disease,\(^1\) is heterogeneous with respect to its molecular biology; clinical, pathological, and radiological features; and surgical approach. Adenocarcinoma is primarily classified as one of five histopathological subtypes, including lepidic, papillary, acinar, solid, and micropapillary. It is widely accepted that adenocarcinoma cells exhibiting a lepidic growth pattern are non-invasive tumor components.\(^2\)\(^3\) Therefore, the prognostic importance of invasive component size, excluding the lepidic component, has gained gradual recognition.\(^4\) Indeed, the TNM classification was drastically revised in 2016, especially in T category, and the size of the invasive component is now predominantly used to determine the T category.\(^2\)

Although the 8th TNM classification reflects the prognosis of lung cancer patients well, many patients with stage I disease still have unfavorable prognoses.\(^5\) This discrepancy indicates that factors other than invasive component size may influence prognosis.

The biological and morphological characteristics of the invasive component are important determinants of cancer cell malignant potential.\(^6\)\(^7\) In addition to cancer cells, the invasive component comprises several stromal cell types, including macrophages, inflammatory cells, and fibroblasts. These stromal cells can interact with cancer cells
and create a specific tumor microenvironment, influence cancer cell proliferation, invasion, and metastasis. Given the influence of stromal cells on clinical tumor features, characterization of stromal cell phenotypes may improve our understanding of patient prognosis.  

Typical adenocarcinoma cases contain both lepidic and non-lepidic (invasive) components (Lep+ adenocarcinomas). Computed tomography (CT) findings of these cases often include both ground-glass opacity (GGO) in the periphery and a solid component in the center. Conversely, some purely invasive cases, such as papillary, acinar and solid adenocarcinoma (Lep- adenocarcinoma), typically only exhibit a solid component on CT. In clinical investigations, the presence of a GGO component may indicate a good prognosis in early clinical stage patients. Interestingly, Moon et al. reported that the lack of a lepidic component was associated with an unfavorable prognosis, even in stage I patients. As a result, the presence of a lepidic GGO pattern in lung adenocarcinoma may confer a favorable prognosis.  

Herein, we hypothesized that the tumor microenvironment of the invasive component of Lep+ adenocarcinoma would differ from that of Lep- adenocarcinoma, and that this relationship would persist in lesions with size-matched invasive (non-lepidic) components. To this end, we characterized the clinicopathological differences between
the invasive components of Lep+ and Lep- invasive size-matched tumors.

Materials and Methods

Patients

A retrospective review of data from accumulated Thoracic Surgical Database in National Cancer Center Hospital East was performed. Patients who underwent R0 resection by lobectomy or greater for lung adenocarcinoma at our institution from January 2003 to December 2011 were enrolled in this study. We excluded patients who underwent preoperative treatment and those with insufficient data. We classified them according to the pathological status defined by the 2004 World Health Organization classification and the 8th edition of the TNM classification of the Union for International Cancer Control. Stage IA adenocarcinomas measuring 3 cm or less (n=575) were then divided into two groups: Lep+ adenocarcinomas (lepidic component ratio in their tumor of 10% or greater) and Lep- adenocarcinomas (lepidic component ratio of less than 10%) (Table1, Figure 1).

All studies involving human participants were performed in accordance with the ethical standards of the institutional and national research committees, and with the 1964 Declaration of Helsinki, its later amendments, or comparable ethical standards.
Comprehensive informed consent was obtained in the study.

**Histopathological evaluation**

Resected specimens were fixed with 10% formalin and embedded in paraffin, and 4-μm sections were stained with hematoxylin-eosin (HE) stain. Vascular and lymphovascular invasion were identified by HE and the Victoria blue-van Gieson (VVG), visualizing elastic fibers, staining. Histologic subtype, as well as total and invasive size, were reviewed by S.K. and G.I, and tumors were classified according to the 2015 World Health Organization Classification of Tumours of the Lung, Pleura, Thymus and Heart. Tumor staging was based on the 8th edition of the TNM classification of the International Association for the Study of Lung Cancer.

**Matching of invasive size**

We matched the invasive component sizes of selected tumors (optimal matching with no caliper) statistically using EZR (Saitama Medical Center, Jichi Medical University; see Statistical Methods). Patients were allocated in a 1:1 ratio, and two groups with the same number of patients (n=116 per group) were selected for further analysis (Table 2). Standardized differences of both pre- and post-matched cohort were shown in Table1.
Immunohistochemical staining

As we predicted that the biological differences between Lep(+) and Lep(-) adenocarcinoma would be found even in early stage cancer, we selected 62 pathological T1b tumors from each group of matched cases for immunohistochemical (IHC) staining. One case with variant type (showing only intrabronchial growth) in Lep-adenocarcinomas was excluded (n=62 Lep+ adenocarcinomas; n=61 Lep-adenocarcinomas). Slides were deparaffinized with xylene and dehydrated with a graded ethanol series. Endogenous peroxidase was blocked with 3% hydrogen peroxide in absolute methyl alcohol. After epitope retrieval, slides were washed with phosphate-buffered saline (PBS) and incubated overnight at 4°C with primary antibodies at their final dilution in the blocking buffer. The primary antibodies we used were shown in Supplemental Table 1. We chose cancer stem-cell related molecule (ALDH-1, CD44), because higher expression level of cancer stem-cell related molecules was reported to be a prognostic marker and associate with subtype of adenocarcinoma. To investigate the potential of invasiveness, proliferation or hypoxic condition in cancer cells, we selected invasion related molecule (laminin-5), and
hypoxia related molecules (GLUT1, CA IX). And to research the tumor promoting  
stromal cells, we investigated PDPN+ CAFs and CD204+ TAMs. Then after  
washing them with PBS again, they were incubated with EnVision (Agilent  
Technologies, Santa Clara, CA) for 1 hour at room temperature. After washing them  
with PBS, the color reaction was performed with diaminobenzidine (DAB). Finally,  
these slides were counterstained with Meyer’s hematoxylin, dehydrated and mounted.

Immunohistochemical scoring method

Two pathologists (S.K. and G.I.) independently evaluated all tissue sections using light  
microscopy. Non-invasive and invasive component areas were determined using HE or  
VVG-stained sections. The IHC staining scores of ALDH1, CD44, CA IX, GLUT-1,  
and laminin-5 were evaluated on the basis of the staining intensity and the percentage,  
corresponding to every ten percentages (0-100%), of stained cancer cells. We used the  
following scoring system: 0 (negative staining, defined as no immunoreactivity); 1+  
(weak staining intensity); 2+ (strong staining intensity). We calculated staining scores  
by multiplying the stained percentages by the staining intensity, ranging from 0 to 200,  
in each slide. We excluded three laminin-5-stained slides (2 Lep+ cases; 1 Lep- case)  
owing to unsatisfactory staining. Evaluation of scoring for podoplanin (PDPN) + CAFs
was calculated based on the staining intensity and the stained percentage of CAFs with the method mentioned ahead. CD204+ tumor associate macrophages (TAMs) in the stroma of the tumor invasive component were enumerated across three high-power fields and averaged.

Survival analysis

All patients were basically followed-up for 5 years after surgery at least and up to 10 years. Recurrence or survival information was obtained as possible by following letter in the case of patients who can’t make regular clinics visit up to 10 years. The length of overall survival was defined as the period between the date of surgery and the last follow-up date or death due to any cause. In the same way, the period of relapse free survival was calculated in months from the date of resection to the date of first recurrence or last follow-up and death from any cause. Observations were censored at the last follow-up when the patient was alive or lost to follow-up. The data cut-off date was May 2016 at our institution.

Statistical methods

We performed matching for patients based on the invasive component size using the
following algorithm: 1:1 optimal match with no caliper and no replacement. Associations between clinicopathological factors and the presence of lepidic growth were analyzed using the multivariate logistic regression analysis (for categorical variables) or the Mann-Whitney U-test (for continuous variables). Histological subtypes and IHC results were assessed using the Mann-Whitney U-test. Overall survival (in months) was calculated from the date of resection to the date of death from any cause, and relapse-free survival was calculated to the date of death from any cause and first recurrence or last follow-up. Survival probabilities were calculated by a Kaplan-Meier method, and assessed by a Cox regression analysis adjusted for some factors (age, sex, smoking status, total tumor size). Two-sided P-values of less than 0.05 were considered statistically significant. Observations were censored at the date of last follow-up when the patient was alive, or when the patient was lost to follow-up. The date of data cut off is May in 2016 at our institution. All data were analyzed with EZR version 1.32, a graphical user interface for R (The R Foundation for Statistical Computing). More precisely, EZR is a modified version of R commander that incorporates frequently used biostatistic functions.

Results
Clinicopathological differences between Lep+ and Lep- adenocarcinomas

We identified 459 Lep+ and 116 Lep- adenocarcinomas, the clinicopathologic differences between which are shown in Table 1. No significant differences in patient age, sex distribution, preoperative serum CEA (carcinoembryonic antigen) levels or total tumor size were detected, but we did observe significant differences in the smoking history of the two groups. With respect to pathological characteristics, Lep+ adenocarcinomas exhibited a smaller invasive component size (10.5 mm. vs. 19.5 mm.; p<0.01), and were more likely to be early pathological T stage (p<0.01). The frequencies of both vascular and lymphovascular invasion were not significantly different between two groups, whereas the frequency of EGFR (epidermal growth factor receptor) mutations was significantly higher in Lep+ adenocarcinomas (p<0.01).

Clinicopathological differences of tumors matched for invasive component size

Table 2 shows the clinicopathological features of Lep+ and Lep- tumors matched for invasive component size. There were no significant differences in invasive component size or pathological T status between the two groups. Most of their invasive components were composed of mixed subtypes, but the dominant subtypes in the invasive component were significantly different between two groups (p<0.01). Almost all of the
differences of characteristics were not significant, but total tumor size was significantly
greater in Lep+ adenocarcinomas (25 mm. vs. 20 mm, $p<0.01$).

Prognostic impact of the presence of lepidic growth

Supplemental Figure 1 shows the Kaplan-Meier curves for the overall and relapse-free
survival of patients with Lep+ and Lep- adenocarcinoma. We did not observe significant
differences for both overall survival and relapse-free survival between the two groups.

Immunohistochemical staining score

IHC staining results are summarized in Table 3.

1. Evaluation of cancer cells

(1) Cancer stem-cell related molecule

Cancer cell-specific ALDH1, which exhibited a cytoplasmic staining pattern, did not
differ between the two groups. As for the staining of CD44, expressed in the membrane
and cytoplasm of cancer cell, the score was significantly higher in Lep+
adeno-carcinomas (50 vs. 20, $p<0.01$) (Figure 2).

(2) Hypoxia related molecules

Lep+ adenocarcinomas exhibited significantly lower GLUT1 and CA IX staining
(3) Invasion related molecule

There were no significant differences in the expression levels of laminin-5 between the two groups.

2. Evaluation of stromal cells

(1) Podoplanin+ CAFs

The staining score for PDPN+ CAFs was significantly lower in Lep+ adenocarcinomas (0 vs. 0 (mean: 1.6 vs. 11.6), p<0.01) (Figure 2).

(2) M2 macrophages

The number of CD204+ TAMs was significantly lower in Lep+ adenocarcinomas (8.7 vs. 24.7, p<0.01) (Figure 2).

Discussion

In this study, we compared the clinicopathological features of Lep+ and Lep- adenocarcinomas with size-matched invasive components. In survival analysis, there were no significant differences between Lep+ and Lep- adenocarcinomas. On IHC staining (Figure 3), both hypoxia marker scores (GLUT-1 and CA IX), as well as scores
of the tumor-promoting stromal cells (CD204+ TAMs and PDPN+ CAFs), were significantly lower in Lep+ adenocarcinomas. These results indicate that the invasive component of Lep+, but not Lep-, adenocarcinoma is associated with a less malignant microenvironment. To our knowledge, this is the first study to clarify the clinicopathological differences between the invasive components of Lep+ and Lep- adenocarcinomas.

GLUT-1 and CA IX are established hypoxia markers. Hypoxia is an important factor that influences tumor proliferation and malignant progression. Elevated expression of GLUT-1 and CA IX in solid cancers, including lung cancer, is known as a poor prognostic factor, and is associated with a greater risk of vascular invasion and metastasis. In our study, the expression of GLUT-1 and CA IX was significantly attenuated in Lep+ adenocarcinomas. This result indicates that the invasive component of Lep+ adenocarcinoma exhibit a reduced invasive potential. And Lep(+) adenocarcinomas had significantly lower percentage of solid component than Lep(-) adenocarcinomas (Supplementary Table2). Chiu et al. reported the strong correlation of solid growth pattern and GLUT1 expression, and this result was consistent with ours.

Compared to Lep- adenocarcinomas, Lep+ tumors contained a lower number of PDPN+ CAFs and CD204+ TAMs (M2 macrophages). Given that these stromal cells
promote cancer cell invasion and tumor metastasis, we conclude that Lep+ adenocarcinomas are associated with a less invasive microenvironment.

Using an animal hypoxia model, Zhang et al. reported that hypoxia significantly promotes lung cancer metastasis accompanied by an increased infiltration of M2 macrophages. In addition, Kolenda et al. reported that with human glioma cell lines LN308 and U87MG cells, a robust increase in podoplanin mRNA was observed with those in the condition of 72h hypoxia compared with those in the normoxic condition. Therefore, lower numbers of tumor-promoting stromal cells in Lep+ adenocarcinomas may be associated with less hypoxic condition.

In the present study, expression levels of CD44 were significantly higher in Lep+ adenocarcinomas. CD44 expression is reportedly correlated with long survival and predominantly lepidic subtypes in lung adenocarcinoma, consistent, in part, with our results. In fact, one of the supportive explanations is that the interaction between CD44 and its ligand of hyaluronic acid may inhibit angiogenesis and progression of cancers.

The current WHO classification scheme proposes that the new AIS and MIA categories represent a stepwise progression of Lep+ adenocarcinoma. Interestingly, Naito et al. reported that this morphological progression is associated with elevated cancer cell
specific-expression of invasion-related molecules, as well as increased recruitment of tumor-promoting stromal cells.\textsuperscript{11} Thus, Lep\textsuperscript{+} adenocarcinomas may slowly acquire malignant potential as their invasive components undergo stepwise changes (i.e., “multistage carcinogenesis”) (Figure 3, upper).

On the contrary, the mechanism of Lep\textsuperscript{−} adenocarcinoma tumorigenesis is poorly understood. In a process known as vessel (or vascular) co-option, tumor cells may grow adjacent to pre-existing blood vessels, thereby facilitating tumor cell-mediated acquisition of oxygen and essential nutrients without obligate neovascularization.\textsuperscript{30-32} Progression of Lep\textsuperscript{+} adenocarcinoma can be supplied with co-opted vessels, because they grow up slowly with preservation of the alveolar structure. However, Lep\textsuperscript{−} adenocarcinoma often grows rapidly and destructively, leading to widespread disruption of co-opted vessels and the development of a hypoxic core (Figure 3, lower).\textsuperscript{32 33}

A previous study reported a strong correlation between the sizes of solid and invasive tumor components as determined by thin-section computed tomography (TSCT).\textsuperscript{34} So, given that tumors lacking GGO on TSCT are likely Lep-adenocarcinomas, treatment strategies for those tumors, especially performing limited resection, must be carefully selected and additional preoperative examination like positron emission tomography (PET)-CT may give us more information.\textsuperscript{35}
We investigated patients who underwent lobectomy and the survival differences between two groups were slight but not significant. However, the opportunity of performing limited resection for the patients with small-size tumors has been increasing and our IHC results suggest that performing limited resection for Lep(-) adenocarcinomas may have the possibility of under treatment. Therefore, carefully patient selection will be required when we plan to perform limited resection. And to clarify this hypothesis, further investigation for survival data from the patient with Lep(-) adenocarcinoma who underwent sublobar resection should be absolutely necessary.

Study limitation

First, this study was a retrospective and conducted in a single institution. Second, in the IHC staining scoring, we couldn’t be blinded to the presence or absence of lepidic component. Third, the associations we observed may be due to chance so that further verification in other studies will be required.

Conclusion

Our current study indicated that lower levels of cancer cell-specific hypoxia marker
expression, as well as a smaller number of tumor-promoting stromal cells in the
invasive component, were characteristic features of Lep+ adenocarcinoma. These
results suggest that the invasive component of Lep+ adenocarcinoma is associated with
a less malignant tumor microenvironment. Given that Lep+ adenocarcinoma is less
invasive with less metastatic potential, early stage small Lep+ adenocarcinomas may be
treated with limited resection techniques such as wide-wedge resection and
segmentectomy. Further investigation based on survival data from the patient with
Lep(-) adenocarcinoma who underwent sublobar resection will be required.
References


cancer-associated stromal cells in patients with stage I lung adenocarcinoma.

*Chest.* 2012;142:151-158.


17. Pardridge WM, Boado RJ, Farrell CR. Brain-type glucose transporter (GLUT-1) is selectively localized to the blood-brain barrier. Studies with quantitative western blotting and in situ hybridization. The Journal of biological chemistry. 1990;265:18035-18040.


Figure 1: Microscopic features of Lep+ and Lep- adenocarcinomas

A: Low-power view of Lep+ adenocarcinomas

B: Low-power view of Lep- adenocarcinomas

a: High-power view of Lep+ adenocarcinomas; 1: peripheral region, 2: central region

b: High-power view of Lep- adenocarcinomas; 1: peripheral region, 2: central region

Figure 2: Distinct immunohistochemical staining patterns of Lep+ and Lep-
adencarcinomas

Left: Lep+ adenocarcinomas; the staining score of CD44 is higher

Right: Lep- adenocarcinomas; the staining score of hypoxic marker (GLUT-1, CA IX) and tumor-promoting stromal cells (PDPN+CAFs and CD204+TAMs) are higher

There were no significant differences in the expression levels of laminin-5 between the two groups.

Figure 3: Schematic of a model for Lep+ and Lep- adenocarcinoma development

AIS: adenocarcinoma in situ, MIA: minimally invasive adenocarcinoma, LPA lepidic predominant adenocarcinoma
Upper: Lep+ adenocarcinomas progress slowly with preservation of the alveolar structure as stepwise progression, which can be maintained with co-opted vessels.

Lower: Lep- adenocarcinomas often grow rapidly and destructively and widespread vascular disruption and regression of co-opted vessels may occur in the invasive area, resulting in a hypoxic core.

Supplemental Figure 1: Comparison of overall and relapse-free survival between patients with Lep+ and Lep- adenocarcinomas (adjusted for age, sex, smoking status, and total tumor size)

A: Overall survival

B: Relapse free survival

(+) Lep+ adenocarcinomas; (−) Lep- adenocarcinomas

Supplemental Figure 2: Comparison of overall and relapse-free survival between patients with Lep+ and Lep- adenocarcinomas performed IHC staining (adjusted for age, sex, smoking status, and total tumor size)

A: Overall survival

B: Relapse free survival
(+): Lep+ adenocarcinomas; (−): Lep- adenocarcinomas