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Prognostic impact of microscopic vessel invasion and visceral pleural invasion and their correlations with epithelial–mesenchymal transition, cancer stemness, and treatment failure in lung adenocarcinoma

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ABSTRACT

Objectives: Microscopic vessel invasion (MVI) and visceral pleural invasion (VPI) have been recently reported as poor prognostic factors of non-small cell lung cancer. Epithelial–mesenchymal transition (EMT) and cancer stemness (CS) are known malignant phenotypes that induce resistance to cancer therapy. We aimed to assess the prognostic significance of MVI and the correlations among VPI/MVI, EMT, CS, and treatment failure for recurrent tumor.

Materials and methods: From 2002 to 2013, 1034 consecutive patients with pathological T1-4N0-2M0 lung adenocarcinoma underwent complete resection. Moreover, we established 206 tissue microarray (TMA) samples from 2002 to 2007. We then evaluated the prognostic impact of MVI, including conventional clinicopathological factors, and analyzed the VPI/MVI, EMT, CS, and treatment failure by TMA immunohistochemical staining.

Results: Among the 1034 cases, the proportion of patients with a 5-year overall survival (OS) period was 63.9% and 88.2% (MVI: +/−; $p < .001$). Multivariate analysis revealed that both MVI and VPI were independent predictors of OS (HR 1.57 and 1.47, respectively). Significant separation of the OS rate curves was observed among the 3 groups [VPI/MVI: both positive (2), either positive (1), and both negative (0)]. Among the 206 TMA cases, these 3 groups of VPI/MVI were significantly correlated with EMT and CS. The median time to progression after recurrence were 3.8, 8.9, and 15.9 months, respectively (VPI/MVI: 2/1/0; $p = 0.016$).

Conclusion: MVI and VPI are significant prognostic factors of lung cancer, and they are correlated with EMT, CS, and treatment failure for recurrent tumor.

1. Introduction

Visceral pleural invasion (VPI), a pathological invasive finding, has been reported to be a poor prognostic factor in patients with non-small cell lung cancer (NSCLC) [1–3]. Accordingly, VPI has been incorporated in the current 8th edition of the TNM classification [4,5]. Recent studies have demonstrated that microscopic vessel invasion (MVI), including blood vessel invasion (BVI) and lymphatic vessel invasion (LVI), is a strong independent factor of poor prognosis in patients with NSCLC [6–11].

Epithelial–mesenchymal transition (EMT) and cancer stemness (CS) of cancer cells are known to play important roles in invasion, metastasis, recurrence, and resistance to treatment [12–15]. EMT follows 2 processes: i) loss of epithelial characters such as E-cadherin and EpCAM and ii) gain of mesenchymal characters such as N-cadherin, SNAIL, TWIST, and vimentin. Cancer stem cells (or cancer-initiating cells) possess stem cell-like properties such as self-renewal, multi-lineage differentiation, and resistance to therapy. Various studies have reported outcomes of high level of malignancy by EMT and CS, with a poor prognosis [16–20]. The concepts of EMT and CS in cancer cells have been established based on *in vitro* and *in vivo* experimental models. However, they remain to be fully examined among cancer patients in

Abbreviations: MVI, microscopic vessel invasion; VPI, visceral pleural invasion; EMT, epithelial-mesenchymal transition; CS, cancer stemness

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clinical practice, because the mechanisms of EMT and CS within tumor tissues have not been fully observed and thus remain unclear. Therefore, the associations among EMT, CS, and histopathological findings need to be investigated.

In this study, we assessed the prognostic significance of MVI in lung adenocarcinoma patients and investigated the correlations among VPI/MVI, EMT, CS, and treatment failure for recurrent tumor.

2. Materials and methods

2.1. Patients

From December 2002 to December 2013, 1034 consecutive patients with pathological T1-4N0-2M0 lung adenocarcinoma underwent complete resection at the Kyoto University Hospital. We defined complete resection as undergoing segmentectomy/lobectomy/pneumonectomy with nodal dissection up to 2a group. Patients who received induction chemotherapy or radiotherapy and those with evidence of residual tumor at the surgical margin were excluded from the study. The tumors staged according to the 8th edition of the TNM classification [4,5] were histologically subtyped and graded according to the World Health Organization guidelines [21]. Informed consent for the use and analyses of clinical data was preoperatively obtained from each patient. The collection and evaluation of the clinical data were approved by the Institutional Review Board, and the study followed the methods of our previous studies [9,22–25].

2.2. Histopathological studies

All surgical specimens were fixed in 10% formalin and then embedded in paraffin. Serial 4-μm sections were stained with hematoxylin and eosin (H&E). BVI and VPI were evaluated by H&E and, whenever necessary, by elastic van Gieson staining. LVI was evaluated by H&E and, whenever necessary, by lymphatic endothelial (D2-40) staining. The blood vessels were identified by the presence of erythrocytes in the lumen or by that of elastic tissue around the larger vessels. The presence of BVI or LVI was determined by identifying conspicuous clusters of intravascular cancer cells surrounded by the blood or lymphatic vessels, as reported elsewhere [9]. MVI was defined as the presence of BVI and/or LVI. When tumor cells could be seen in the thin-wall vessels and these findings of MVI could not be distinguished, elastic or lymphatic endothelial staining was performed. Pathological evaluation was reviewed for consistency by a single pathologist (A.Y.).

2.3. Tissue microarray

In the establishment of tissue microarrays (TMAs), we used the samples of lung cancer surgically resected which had a 2-mm tissue core of the most morphologically representative region with the patient's consent. TMAs of lung adenocarcinoma were performed using 206 of the resected tumor samples until December 2007 by pathologists at the Department of Diagnostic Pathology in Kyoto University Hospital [23,26] using an approach similar to that described previously [27]. After the case-selection process described above, paraffin-embedded tumor blocks with sufficient tissue samples were selected for TMA. The most representative regions of the tumors were based on the morphology of the H&E-stained slide. Tissue cores measuring 2 mm in diameter were punched out from each donor tumor block using thin-walled stainless steel needles (Azumaya Medical Instruments Inc., Tokyo, Japan) and were arrayed in a recipient paraffin block. We used these TMA samples for the evaluation of EMT/CS.

2.4. Evaluation of clinicopathological factors

The clinical characteristics were retrieved from the clinical records and our division database: age (dichotomized at the mean age of 66 years), gender, and smoking habits (non-, former, or current-smoker). The following factors were reviewed and confirmed by the pathologists: the diameter of the tumor in the resected specimens (≤ 3 cm or > 3 cm), differentiation (well, moderate, or poor), VPI (the presence or absence defined using the 8th edition of TNM classification), MVI (presence or absence), and lymph node metastasis (N0, N1, or N2, as defined using the 8th edition of the TNM classification).

2.5. Immunohistochemical analyses

Immunohistochemical staining was performed according to the procedure described in our previous reports [22,23,25]. Immunostaining against E-cadherin, vimentin, and CD133 was performed with mouse anti-human E-cadherin monoclonal antibody (36B5; dilution 1:300, Leica Biosystems, Newcastle, UK), mouse anti-human vimentin monoclonal antibody (SRL33; dilution 1:300, Leica Biosystems), and mouse anti-human CD133 monoclonal antibody (W6B3C1; dilution 1:10, Miltenyi Biotec, Auburn, CA). After antigen retrieval, individual slides were incubated overnight at 4 °C using primary antibodies. The slides were washed 3 times with PBS and then incubated with EnVisionTM (Dako, Denmark) for 1 h at the room temperature. After washing with PBS, the color reaction was developed for 3 min in 2% 3,3′-diaminobenzidine in 50 mM Tris-buffer (pH 7.6) containing 0.3% hydrogen peroxidase. Finally, the sections were counterstained with Meyer's hematoxylin, dehydrated, and then mounted. The immunostained sections were examined by 2 authors (T.M. and T.S.) blinded to the patient characteristics based on our previously published paper [23]. Cases with discrepancies were jointly reevaluated until a consensus was reached. After immunostaining, the expressions of these proteins were examined in 4 distinct fields, each with a minimum of 500 cells. The proportion of positive cells was measured and classified as 0 (no staining), +1 (weak), +2 (moderate), and +3 (strong), and each specimen was categorized as negative (0, +1) or positive (+2, +3). EMT was defined as the presence of vimentin expression and/or the absence of E-cadherin expression of cancer cells. CS was defined as the presence of CD133 expression of cancer cells.

2.6. Genetic mutation analyses

As previously reported, we analyzed mutations in the epidermal growth factor receptor (EGFR) (exons 18–21), human epidermal growth factor receptor 2 (HER2) (exons 19–20), p53 (exons 5–8), and BRAF (exon 15) genes using the polymerase chain reaction-single-strand conformational polymorphism (PCR-SSCP) technique [24,28,29]. We used a modified mutagenic PCR-restriction fragment-length polymorphism (PCR-RFLP) method to detect KRAS codon 12 mutations [29]. In addition, we performed ALK FISH assays to screen for ALK translocation [30].

2.7. Statistical analyses

Correlations among categorical outcomes were evaluated using Fisher's exact test. Treatment failure was defined as discontinuation of treatment for any reason, including disease progression, treatment toxicity, and death. The overall survival (OS) time was measured from the date of surgery until the date of death from any cause or the last follow-up. The length of the disease-free survival (DFS) time was measured from the date of surgery until the date of the first event of recurrence/death or last follow-up. The time to progression (TTP) was measured from the date of the first recurrence to the date of the first appearance of progression/relapse, cancer-related death, or last follow-up. For the analysis of TTP, patients who died without progression or who were known to possess no progression at the date of last contact were censored. The cumulative survival, disease-free, or progression rates were calculated by the Kaplan–Meier method, and the differences in the survival curves were compared using the log-rank test. The Cox

Table 1
Clinicopathological characteristics of 1034 patients.

Variable	n	(%)
Age, yrs	Mean 66 y.o. (66 ± 9.9)	
> 65	581	(56.2)
≤ 65	453	(43.8)
Sex		
Female	524	(50.7)
Male	510	(49.3)
Smoking history		
Current	201	(19.4)
Former	339	(32.8)
Never	494	(47.8)
Surgery		
Segmentectomy	262	(25.3)
Lobectomy	766	(74.1)
Pneumonectomy	6	(0.6)
Pathological stage		
IA	634	(61.3)
IB	184	(17.8)
IIA	70	(6.8)
IIB	48	(4.6)
IIIA	95	(9.2)
IIIB	3	(0.3)
Differentiation		
Well	255	(24.7)
Moderate	618	(59.8)
Poor	158	(15.3)
NA	3	(0.3)
Tumor size, cm		
≤ 3	768	(74.3)
> 3	266	(25.7)
pN status		
pN0	881	(85.2)
pN1	72	(7.0)
pN2	81	(7.8)
VPI		
Absent	830	(80.3)
Present	204	(19.7)
MVI		
Absent	854	(82.6)
Present	180	(17.4)

NA indicates not available.
MVI, microscopic vessel invasion; VPI, visceral pleural invasion.

proportional hazard model was used in the univariate and multivariate analyses of OS and DFS. The significance level was set at $P < 0.05$. All statistical analyses were performed using the statistical software SPSS 11.0 (SPSS Inc., Chicago, IL, USA) and JMP 9 (SAS Institute, Cary, NC, USA).

3. Results

Table 1 summarizes the patient characteristics. A total of 1034 patients [510 males (56.2%) and 524 females (43.8%)] aged 23–86 years (mean age, 66 years) were enrolled in the study. The study patients underwent lobectomy (74.1%), segmentectomy (25.3%), and pneumonectomy (0.6%). The median follow-up period was 52 months. The 5-year OS rate for patients with MVI was 63.9%, which was significantly lower than that for patients without it (88.2%; $P < 0.001$) (Fig. 1A). The 5-year DFS rate for patients with MVI was 43.9%, which was significantly lower than that for patients without it (81.2%; $p < 0.001$) (Fig. 1B). Supplemental Table 1 shows the association between MVI and clinicopathological factors. MVI was significantly correlated with smoking history, surgery, pathological stage, differentiation, tumor size, pathological nodal (pN) status, and VPI (Supplemental Table 1).

Univariate analysis of OS using the Cox regression model revealed that age, sex, smoking status, differentiation, tumor size, pN status, VPI, and MVI were significantly associated with OS (Table 2A). Multivariate analysis showed that age, pN status, VPI, and MVI were significant independent predictors of OS (Table 2A). Univariate analysis of DFS using the Cox regression model showed that age, sex, differentiation, tumor size, pN status, VPI, and MVI were significantly associated with recurrence (Table 2B). Multivariate analysis demonstrated that age, tumor size, pN status, VPI, and MVI were significant independent factors of recurrence (Table 2B). The strongest factors for OS and DFS, following the pN status and age, were VPI and MVI. These findings were previously reported in another cohort [7,9], which suggested that VPI and MVI were strong predictors of poor outcomes.

We divided the subjects into 4 groups according to the status of VPI and MVI. In the 4 groups with or without VPI/MVI (−/−, +/−, −/+, and +/+), the proportions of patients with a 5-year OS period were 90.5% (n = 737), 74.9% (n = 117), 71.7% (n = 93), and 55.7% (n = 87), respectively (Supplemental Fig. 1A). Because the difference in the OS curves between the VPI+/MVI− and VPI−/MVI + groups was not statistically significant ($p = 0.99$), we divided the cases into 3 groups according to the number of the risk factors for VPI/MVI (Fig. 2A). This finding showed a significant separation in the OS curves between these 3 groups: both VPI and MVI absent (0), either VPI or MVI present (1), and both VPI and MVI present (2). A significant correlation between the number of risk factors for VPI/MVI and recurrence was noted (Supplemental Table 2). These definite survival separations were also noted among the DFS curves (Fig. 2B; Supplemental Fig. 1B). These results were consistent with our previously documented results obtained by analyzing a different cohort [9].

Next, we analyzed the association among EMT, CS, and the status of VPI/MVI using 206 TMA samples. Supplemental Table 3 depicts the characteristics of 206 patients. In IHC staining of the tumors, we noted the presence of vimentin expression and/or the absence of E-cadherin expression of cancer cells, which was defined as EMT (Fig. 3A, B), and the presence of CD133 expression of cancer cells, which was defined as

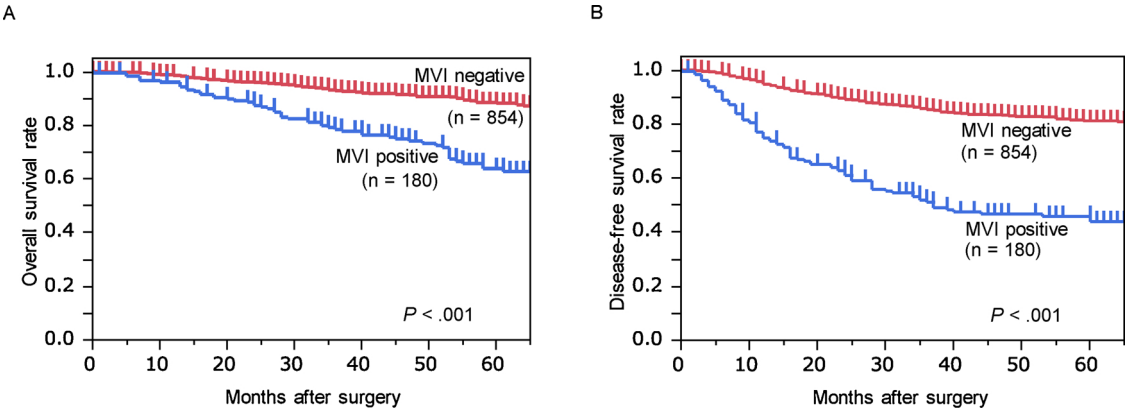

Fig. 1. (A) Overall survival curves of patients with or without MVI. (B) Disease-free survival curves of patients with or without MVI.

Table 2A
Univariate and Multivariate Cox Regression Analysis of Overall Survival.

Factors	Risk Factors	Univariate Analysis			Multivariate Analysis		
		HR	95% CI	*p value*	HR	95% CI	*p value*
Age	> 65	1.96	1.42–2.74	< .001	2.08	1.50–2.91	< .001
Sex	Male	1.78	1.30–2.46	< .001	1.52	1.00–2.35	0.052
Smoking status	Ever smoker	1.68	1.23–2.32	0.001	1.09	0.71–1.69	0.697
Differentiation	Poorly differentiated	2.55	1.80–3.56	< .001	1.31	0.90–1.90	0.161
Tumor size, cm	> 3	2.20	1.61–3.00	< .001	1.13	0.79–1.59	0.495
pN status	pN1-2	5.29	3.88–7.21	< .001	3.77	2.64–5.37	< .001
VPI	Present	3.18	2.32–4.33	< .001	1.47	1.03–2.09	0.035
MVI	Present	3.24	2.35–4.43	< .001	1.57	1.08–2.28	0.019

CI, confidence interval; HR, hazard ratio; MVI, microscopic vessel invasion; VPI, visceral pleural invasion.

Table 2B
Univariate and Multivariate Cox Regression Analysis of Disease-free Survival.

Factors	Risk Factors	Univariate Analysis			Multivariate Analysis		
		HR	95% CI	*p value*	HR	95% CI	*p value*
Age	> 65	1.72	1.33–2.24	< .001	1.89	1.45–2.47	< .001
Sex	Male	1.41	1.10–1.82	0.006	1.35	0.97–1.88	0.08
Smoking status	Ever smoker	1.40	1.09–1.80	0.009	0.97	0.70–1.37	0.878
Differentiation	Poorly differentiated	2.40	1.80–3.15	< .001	1.18	0.86–1.60	0.302
Tumor size, cm	> 3	2.77	2.16–3.55	< .001	1.46	1.11–1.92	0.007
pN status	pN1-2	6.01	4.66–7.73	< .001	3.82	2.86–5.10	< .001
VPI	Present	3.41	2.65–4.39	< .001	1.58	1.19–2.09	0.002
MVI	Present	3.90	3.01–5.02	< .001	1.86	1.38–2.52	< .001

CI, confidence interval; HR, hazard ratio; MVI, microscopic vessel invasion; VPI, visceral pleural invasion.

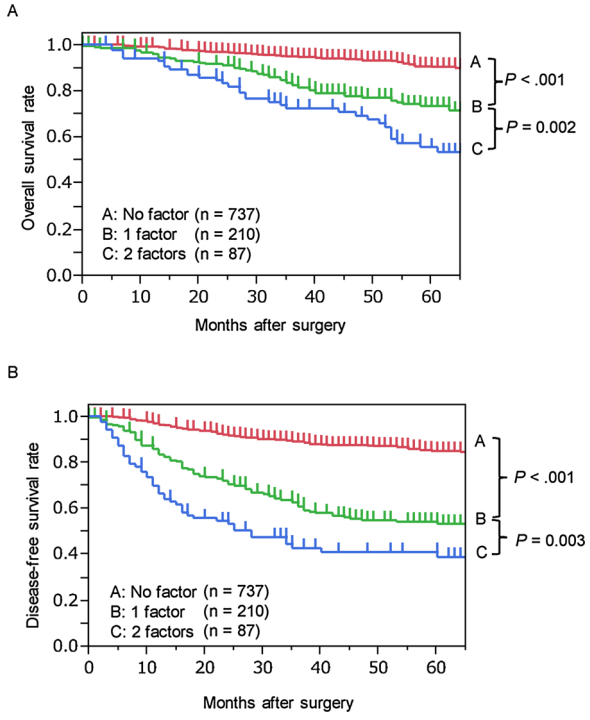

Fig. 2. (A) Overall survival curves of patients with or without MVI and VPI. (B) Disease-free survival curves of patients according to the number of the risk factors.

CS (Fig. 3C) [23]. Significantly positive mutual correlations were noted among the numbers of risk factors for VPI/MVI, EMT, and CS (Table 3; Supplemental Table 4). Moreover, we investigated the correlation between them and oncogenic mutations such as EGFR, HER2, KRAS, p53, BRAF mutations, and ALK translocation. [31–33] Based on the results of our previously reported studies [24,28–30], no significant differences were noted between the risk factors for VPI/MVI and these oncogenic mutations. In fact, we only observed a significantly positive correlation between KRAS mutation and CS as well as significantly negative correlations between EGFR mutation and EMT or EGFR mutation and CS (Supplemental Table 5).

Of the 206 patients with TMA, 57 (27.7%) developed recurrence. We analyzed the correlations between recurrence and EMT and CS and the risk factors for VPI/MVI. There were significantly positive correlations between recurrence and EMT or between recurrence and VPI/MVI as well as a marginally positive correlation between recurrence and CS (Supplemental Table 6). Of the patients who developed recurrence, 52 (91.2%) underwent treatment for recurrence. The recurrence sites and the initial treatment for recurrence are shown in Supplemental Tables 7 and 8, respectively. The durations of median TTP for patients with the numbers of risk factors for VPI/MVI [(0), (1), and (2)] were 15.9, 8.9, and 3.8 months, respectively ($p = 0.016$) (Supplemental Fig. 2A). The median TTP for patients with EMT(+) was 7.8 months, which was significantly shorter than that for patients with EMT(−) (15.9 months; $p = 0.022$) (Supplemental Fig. 2B). The median TTP for patients with CS(+) was 5.1 months, which was significantly shorter than that for patients with CS(−) (14.6 months; $p = 0.036$) (Supplemental Fig. 2C). We divided the patients into 4 groups based on their status of EMT and CS. In the 4 groups without or with EMT/CS (−/−, −/+, +/−, and +/+), the durations of median TTP were 15.9 (n = 15), 23.1 (n = 2), 8.3 (n = 27), and 5.1 (n = 8) months, respectively ($p = 0.003$) (Supplemental Fig. 2D).

4. Discussion

The presence of MVI acted as an independent poor prognostic factor for OS and DFS in patients with pT1-4N0-2M0 lung adenocarcinoma. The risk factors for VPI/MVI strongly affected the poor prognosis, as also supported by previous studies. [7,9] Moreover, in this study, the pathological invasive findings of VPI/MVI were found to be significantly associated with biologically malignant phenotypes of EMT and CS.

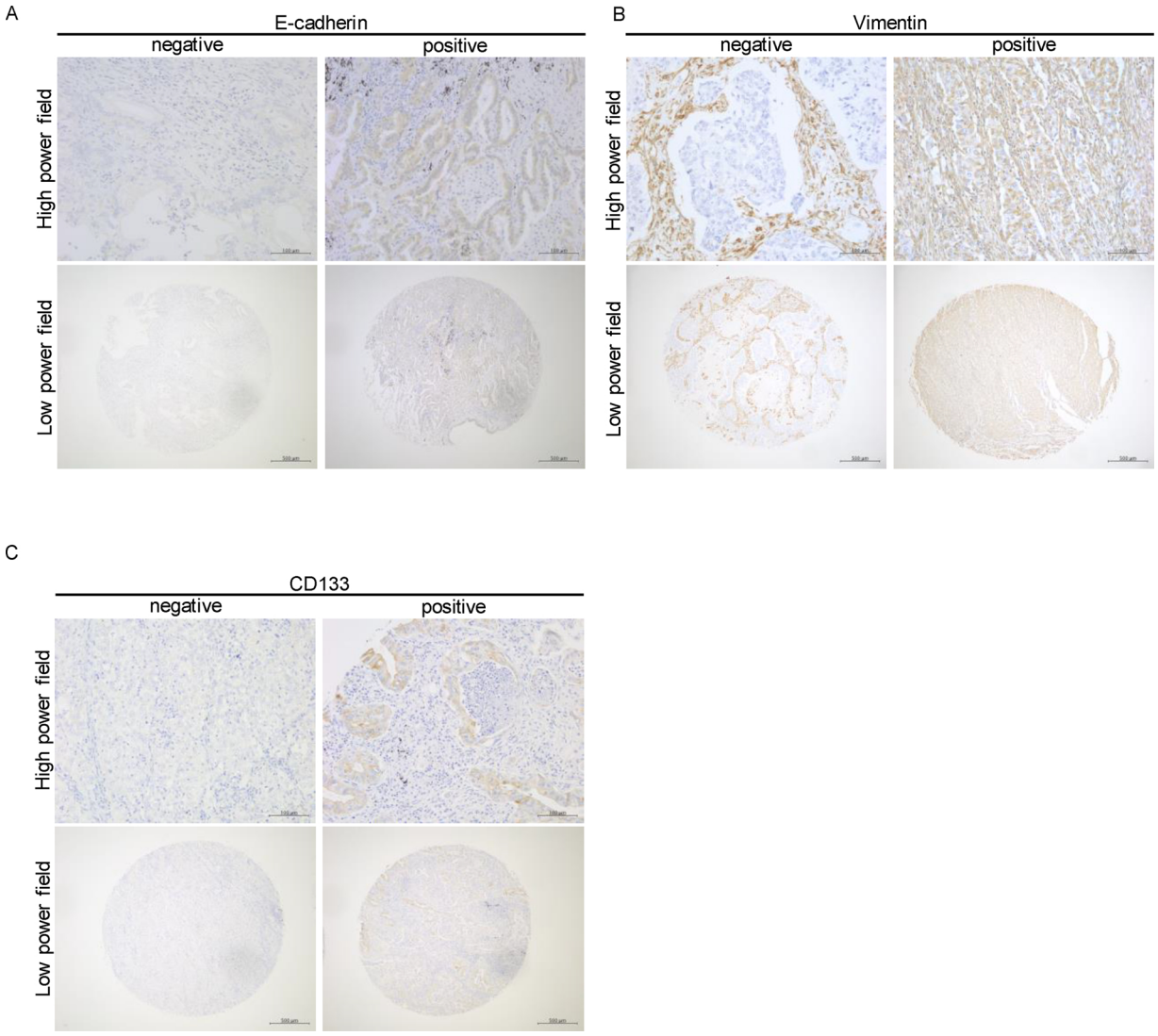

Fig. 3. Immunohistochemical staining of lung adenocarcinoma sections with anti-E-cadherin (A), vimentin (B), and CD133 (C) antibodies.

Table 3
Correlation between EMT, CS and the risk factors of MVI and VPI.

		EMT					CS				
		Negative	(%)	Positive	(%)	*p value*	Negative	(%)	Positive	(%)	*p value*
No. of MVI and VPI	0	63	(52.5)	57	(47.5)	0.038	57	(95.0)	6	(5.0)	0.001
	+1	22	(37.3)	37	(62.7)		46	(78.0)	13	(22.0)	
	+2	8	(29.6)	19	(70.4)		22	(81.5)	5	(18.5)	

CS, cancer stemness; EMT, epithelial-mesenchymal transition; MVI, microscopic vessel invasion; VPI, visceral pleural invasion.

EMT is a malignantly activated phenotype of invasion and metastasis [12,13]. CS is also pivotal to metastasis, recurrence, and drug resistance [13–15]. The concepts of EMT and CS in cancer have been established based on *in vitro* and *in vivo* experimental models. However, the mechanisms of EMT and CS within the tumor tissue of clinical samples remain unclear.

In this study, we defined EMT as the presence of vimentin expression and/or the absence of E-cadherin expression of cancer cells, and CS as the presence of CD133 expression of cancer cells, because we have previously demonstrated that these markers are significantly correlated with poor prognosis [23]. We investigated whether EMT and CS are associated with histopathological findings and oncogenic mutations by analyzing clinical samples. The pathological invasive findings of VPI/MVI were significantly correlated with EMT and CS. As for TTP in the patients who underwent treatment for recurrence, the patients presenting with risk factors for VPI/MVI as well as those with EMT(+) or CS(+) showed poor prognosis. The duration of median TTP for patients with both EMT(+) and CS(+) was found to be 5.1 months (n = 8) and these patients showed the poorest prognosis in TTP, although this result was exploratory because of the fewer patients. The EMT status in cancer cells generates cancer cells with stem cell-like properties [33–37], and EMT maintains CS in cancer cells through the Wnt signaling pathway in breast and lung cancers [38,39]. Moreover, lung cancer-initiating cells expressing $CD133^+/CXCR4^+/EpCAM^-$ or $Twist^+/E\text{-cadherin}^-/Sox15^-/CD133^+$, which have both phenotypes of EMT and CS, possess high abilities of invasion and metastasis [38,40]. Taken together, EMT

in cancer cells possibly not only stimulates other types of cells to promote cancer development in the tumor microenvironment [9,41–43] but also maintains CS in themselves and induces some malignant subtypes of cancer cells with increased invasiveness and resistance to therapy.

As for E-cadherin expression in cancer cells, we observed cytoplasmic expression as well as membranous expression in the 206 TMA samples (5/206, 2.4%). There is a study that E-cadherin could show decreased membranous expression accompanied with increased cytoplasmic expression in some lung adenocarcinomas [44]. However, we observed very few samples with cytoplasmic expression of E-cadherin, and no relationship with membranous E-cadherin expression and prognosis (data not shown). Then, when cancer cells have acquired malignant phenotypes, even cytoplasmic E-cadherin expression has been reported to decrease [45]. Therefore, we assessed and measured the intensities of E-cadherin expression by its total expression.

Regarding oncogenic mutations, KRAS mutation was correlated with CS and EGFR mutation was negatively correlated with EMT and CS. KRAS mutation in cancer cells reportedly preserves cells with a stem-like phenotype in intestinal cancer and lung adenocarcinoma [46,47]. Some of the EGFR-mutated cancer cells have been demonstrated to acquire resistance to EGFR tyrosine kinase inhibitors by up-regulation of CD44 and induction of EMT [48,49]. In our study, we analyzed mutations from tumor samples resected without chemotherapy or radiotherapy. These negative correlations suggested that low-grade cancer cells with EMT-negative and CS-negative statuses account for the major proportion of EGFR-mutated cancer cells with no treatment.

CS has been recognized by several markers of cancer cells in various tumor types [50,51]. CS markers such as CD133, CD44, and aldehyde dehydrogenase 1 (ALDH1) are well known in lung cancer [51–55]. Additionally, we have previously documented that CD133-positive lung cancer is one of the most important CS markers in association with EMT and poor prognosis [23]. We also found that CD133-positve lung cancer was significantly associated with VPI/MVI. However, the CS markers of ALDH1 and CD44 were not associated with prognosis, EMT, and VPI/MVI (data not shown).

In conclusion, we investigated the prognostic significance of VPI/MVI and the correlations among VPI/MVI, EMT, CS, and treatment failure for recurrent tumors. The markers of EMT and CS have not yet been used in clinical practice, warranting further investigation into the mechanisms of EMT and CS in cancer cells with regard to the histopathological invasion findings of VPI/MVI. However, these results suggest that the risk factors of VPI and MVI could be surrogate markers of EMT and CS as well as that for poor prognosis and treatment failure (Supplemental Fig. 3). Moreover, lung adenocarcinoma patients with EMT, CS, or VPI/MVI risk factors are considered good candidates for adjuvant therapy.

Conflict of interest

All authors declare that they have no conflicts of interest associated with this study.

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Author contributions: T.M. is the guarantor of the paper and takes responsibility for the integrity of the work as a whole. S.N. and T.M. performed experiments, collected data, analyzed data, and drafted an initial version of the manuscript. T.S. performed experiments, collected data, and analyzed data. Y.Y., D.N., M.H., A.O., T.C., T.S., M.S., A.Y., H.H., H.D. contributed to data collection and data analysis. All authors reviewed the final version of the manuscript and gave their approval.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.lungcan.2018.12.001.

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