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Clinicopathological analysis of pleomorphic carcinoma of the lung: Diffuse ZEB1 expression predicts poor survival

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ABSTRACT

Objectives: Pleomorphic carcinoma (PC) of the lung is a rare epithelial tumor. The clinicopathological characteristics and prognostic factors of PC are controversial. The information on the ZEB1 gene, which crucially impacts survival of patients with other malignant tumors, is limited for PC.
Materials and methods: Clinicopathological characteristics of 62 patients with PC were investigated in this study. Associations between immunohistochemical expression of ZEB1 and clinical factors, including patient prognosis, were examined. The patient population consisted of 51 (82.2%) men and 11 (17.8%) women, with a mean age of 65.5 years (range, 31–81 years).
Results: The overall survival rate of the 42 patients, for whom follow-up was available, was 68.3% at 5 years. Using TNM criteria, 7 (11.3%), 11 (17.7%), 3 (4.8%), 21 (33.8%), 15 (24.2%), 2 (3.2%), and 3 (4.8%) patients were classified under pathological stage IA, IB, IIA, IIB, IIIA, IIIB and IV carcinomas, respectively. Fifteen (24.1%) patients had tumors consisting entirely of spindle and giant cells (PC component). The other 47 (75.8%) cancers contained additional carcinoma components (i.e., adenocarcinoma (34/62, 54.8%), squamous cell carcinoma (7/62, 11.3%), adenosquamous carcinoma (4/62, 6.5%) and large cell carcinoma (2/62, 3.2%)). Four of 7 (57.1%) stage IA (<20 mm) tumors consisted only of spindle and giant cells. ZEB1 expression was observed only in the PC component. Diffuse expression of ZEB1, was defined as positive nuclear staining in ≥75% of cancer cells, and was found in the PC component in 12 patients. Multivariate analysis revealed that lymph node metastasis, pleural invasion, and diffuse ZEB1 expression in the PC component predicted poorer disease-specific survival ($p = 0.007$, 0.022, and 0.016, respectively).
Conclusion: This is the first report to indicate that ZEB1 may be used as an immunohistochemical prognosticator of PC, which may be useful for histological assessment of PC in biopsy and surgical specimens.

1. Introduction

According to the World Health Organization (WHO) classification of lung tumors, pleomorphic carcinoma (PC) is one of five subgroups of sarcomatoid carcinomas [1]. PC is defined as a group of poorly differentiated non-small cell lung carcinomas (NSCLCs; e.g., adenocarcinoma, squamous cell carcinoma, and large cell carcinoma), which contain spindle and/or giant cells, or carcinomas consisting only of spindle or giant cells. The pleomorphic component (PC component) should comprise at least 10% of the neoplasm. The reported values for incidence of PCs have ranged from 0.8% to 2.1% of all lung carcinomas [2–4]. Patients with PC are usually diagnosed at a more advanced stage of disease progression, and experience a more aggressive clinical course of treatment, compared with other NSCLCs [2]. Patient with PC generally have a poor response to systemic chemotherapy [4]. However, due to its rarity, no consensus exists on the clinical effects of the clinicopathological characteristics on the prognosis for patients with PC.

Epithelial–mesenchymal transition (EMT)-related transcriptional factors are increasingly recognized as important contributors to tumor progression and metastatic spread [5–7]. High expression

Abbreviations: WHO, World Health Organization; PC, pleomorphic carcinoma; NSCLC, non-small cell lung carcinoma; EMT, epithelial–mesenchymal transition; TNM, tumor-node-metastasis; EGFR, epidermal growth factor receptor.

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of these transcriptional factors has a negative effect on patient survival. Expression of ZEB family members correlates with the aggressive phenotype in various other malignancies [8–11]. Expression is associated with more mesenchymal and invasive properties in cancer cell lines, and invasive and metastatic properties and poorer clinical prognosis in primary carcinomas. The ZEB family of transcriptional factors has an important role in developmental processes such as gastrulation, neural crest formation, heart morphogenesis, and formation of the musculoskeletal system and craniofacial structures. In cancer cells, the functions of the ZEB family members are to control cell cycle progression, apoptosis, and senescence, and to induce the epithelial dedifferentiation leading to cancer progression [12,13].

We retrospectively evaluated the clinical and histological features of PC in a patient population from a single institution. We also used immunohistochemical staining to investigate the expression of transcriptional factors (ZEB1), and analyzed associations with clinicopathological parameters. This report is the first to show that high expression of ZEB1 is an independent and significant negative prognostic factor in PC patients.

2. Materials and methods

2.1. Patients

Sixty-two cases of PC were detected from a review of 2328 cases of resected tumors in the lung cancer file of the Department of Pathology, Fukuoka University Hospital (Fukuoka, Japan). The tumors were obtained from surgeries performed between January 1988 and October 2011 at the Department of General Thoracic Surgery, Fukuoka University Hospital. The study protocol was approved by the Ethics Committee of the Fukuoka University School of Medicine. Anonymous use of redundant tissue is part of the standard treatment agreement with patients in our hospitals when no objection has been expressed. The pathological stage was determined using the tumor-node-metastasis (TNM) classification criteria for malignant tumors (International Union Against Cancer) [14]. The stage IIIB cases were upgraded after surgery because of the pathological diagnoses of nodal metastasis. For stage IV cases, partial resections of the primary lung tumor were performed for histological diagnosis to treat brain metastases.

2.2. Pathologic evaluation

The tumors were classified according to the criteria of the current WHO histologic classification scheme [1]. The coexisting carcinoma components of each PC were further classified as adenocarcinoma, squamous cell carcinoma, adenosquamous cell carcinoma, or large cell carcinoma. The PC components were subclassified as predominantly spindle cell carcinoma or predominantly giant cell carcinoma. The clinicopathological parameters that were considered in this study were age, gender, tumor size, lymph node metastasis, pleural invasion, lymphatic permeation, and vascular involvement.

2.3. Immunohistochemistry

The surgically resected specimens were fixed in 10% formalin, and processed in paraffin blocks. Tissue sections (4 μm) were deparaffinized and immersed in 0.3% hydrogen peroxide in methanol for 15 min at room temperature to block endogenous peroxidase activity. They were then heated in 10 mM citrated buffer (pH 6.0) in a microwave oven (700 W) for 10–15 min to retrieve epitopes. The sections were incubated with polyclonal antibodies against ZEB1 (1:500 dilution; Novus, Littleton, CO) overnight at 4 °C. The sections were then washed and incubated with Dako ChemMate EnVision (Dako, Carpinteria, CA) for ZEB1 Immunoreactive proteins were visualized using diaminobenzidine (Dako, Carpinteria, CA), followed by counterstaining with hematoxylin. Immunohistochemical staining of ZEB1 was divided into five groups; group 1: 0%, group 2: 1–24%, group 3: 25–49%, group 4: 50–74%, and group 5: 75–100%, and then classified as a diffuse expression group if ≥75% cancer cells exhibited nuclear staining, or, as a focal expression group if <75% of the cancer cells were stained. Most tumors of the focal group showed ZEB1 positivity in up to 50–60% of cells, and therefore diffuse (≥75%) group were selected easily.

2.4. Statistical analysis

All statistical analyses were performed using StatMate IV for Windows (ATMS, Tokyo, Japan). The relationships between clinicopathological parameters and histopathological subgroups were evaluated using the Chi-square and Fisher's exact tests. Survival was analyzed for the 42 patients, because 20 of the 62 patients were excluded from the analysis. These excuded patients who (1) did not have radical surgery, (2) died of causes other than lung cancer, or (3) died within 30 days after surgery. Patient survival time was calculated from the date of surgery until the date the first recurrence was diagnosed (relapse-free survival) or until death from cancer (disease-specific survival). The Kaplan–Meier method was used for survival curve analysis, and differences between survival curves were analyzed using the log-rank test. A univariate analysis was performed for each clinicopathological parameter. Multivariate regression analysis and the Cox proportional-hazard model were used to determine the independent prognostic factors. A p-value less than 0.05 was considered to be statistically significant.

3. Results

3.1. Clinical and histopathological findings

Table 1 presents the results for the clinicopathological characteristics of the patients. A total of 62 patients (51 male and 11 female; male to female ratio, 4.6:1) were included in the study. Age at the time of diagnosis ranged from 31 to 81 years, with a mean age of 65.5 years. Lobectomy was performed in 55 patients (88.7%), pneumonectomy in 6 patients (9.7%), and a combination of bilobectomy, segmentectomy, and partial resection was performed for 1 patient (1.6%). Regional lymph node dissection was performed in 55 patients, and lymph node metastasis was found in 15 patients (27.3%). Ten of these 15 patients had metastasis with coexisting carcinoma components (9 adenocarcinoma and 1 squamous cell carcinoma). The other five cases included three cases with spindle cells and two cases with giant cells. Nodal status was classified as pN0 in 40 (64.5%) cases, pN1 in 2 (3.2%) cases, pN2 in 12 (19.4%) cases, and pN3 in 1 (1.6%) case. The TMN pathological stages of PC were classified as: 7 (11.3%) cases with stage IA, 11 (17.7%) with stage IB, 3 (4.8%) with stage IIA, 21 (33.8%) with stage IIB, 15 (24.2%) with stage IIIA, 2 (3.2%) with stage IIIB, and 3 (4.8%) with stage IV carcinoma. The coexisting carcinoma component ranged from 0% to 90% of the whole tumor area and consisted of adenocarcinoma in 34 patients (54.8%), squamous cell carcinoma in 7 patients (11.3%), adenosquamous cell carcinoma in 4 patients (6.5%), and large cell carcinoma in 2 patients (3.2%). Fifteen patients (24.2%) had no coexisting carcinoma component. The PC component accounted for 10–100% of tumor volume, and was spindle cell in 18 patients (29.0%), giant cell in 7 patients (11.3%), and was mixed, in the remaining 37 patients (59.7%).

Table 1
Clinicopathological characteristics ($N = 62$).

	Number of patients (%)
Age, years	
Median	65.5
Range	31–81
Gender	
Male	51 (82.3)
Female	11 (17.7)
Tumor size (mm)	
Median	56.1
Range	10–150
p-N	
0	40 (64.5)
1–3	15 (24.2)
x	7 (11.3)
Pleural invasion	
Positive	41 (66.1)
Negative	21 (33.9)
Lymphatic permeation	
Positive	27 (43.5)
Negative	35 (56.5)
Vascular involvement	
Positive	19 (30.6)
Negative	43 (69.4)
Necrosis	
Positive	52 (83.9)
Negative	10 (16.1)
Coexisting carcinoma component	
Adenocarcinoma	34 (54.8)
Squamous cell carcinoma	7 (11.3)
Adenosquamous cell carcinoma	4 (6.5)
Large cell carcinoma	2 (3.2)
None	15 (24.2)
Predominant pleomorphic component	
Spindle cell	42 (67.7)
Giant cell	20 (32.3)

The coexisting carcinoma portions occurred more frequently in larger PCs. The mean percentages of coexisting carcinoma portions in the entire tumor were 8.5% in tumors ≤20 mm, 26.9% in tumors >20 mm and ≤50 mm, and 23.7% in tumors >50 mm. There were seven cases of p-T1a cancer (≤20 mm diameter, mean 16.1 mm, range 10–20 mm) (Table 2). Three (42.9%) of the seven cases had a coexisting carcinoma component, one was an adenocarcinoma component and two squamous cell carcinoma component. They occupied approximately 10%, 10%, and 40%, respectively, of each entire tumor. Spindle cells were detected as a predominant PC component in all but one case. Ipsilateral recurrence occurred in one patient, who died within 1 year after surgery. However, the other six patients were alive after 1 year and did not experience recurrence. These six patients did not receive chemotherapy or radiotherapy. The 5-year disease-specific survival rate of these six stage IA PC patients (pT1a-size) was 83% (one patient was lost during follow-up).

Table 3
Results of univariate analysis of prognostic factors affecting disease-specific and relapse-free survival after surgical resection ($N = 42$).

	p	
	Relapse-free survival	Disease-specific survival
Gender	0.39	0.76
Tumor size	0.25	0.52
p-N positive	0.037*	0.042*
Pleural invasion	0.031*	0.014*
Lymphatic permeation	0.16	0.075
Vascular involvement	0.93	0.97
Necrosis	0.89	0.92
ZEB1, diffuse expression	0.034*	0.031*
Pleomorphic component (≥75%)	0.32	0.135

* Statistically significant.

Twenty three patients with adjuvant chemotherapy (p-stage II or III), those with diffuse ZEB1 expression had significantly shorter overall survival compared with those with focal expression ($p = 0.030$, data not shown).

3.2. Immunohistochemical analysis of ZEB1

ZEB1 expression was detected only in the nuclei of spindle or giant cells of the PC component (Fig. 1). Diffuse expression of ZEB1 was found in 12 patients (19.4%).

3.3. Patient survival

Fig. 2A and B presents the post-surgery disease-specific survival and relapse-free survival curves, respectively, by tumor stage. The 5-year disease-specific survival rate was 84.6% for stage I, 70.5% for stage II, and 45.4% for stage III, PC patients. There was a statistically significant difference between survival of stages I and III PC patients ($p = 0.015$). The 5-year relapse-free survival rate in stages I, II, III was 76.9%, 52.9%, and 41.6%, respectively. The difference between stages I and III ($p = 0.021$) was statistically significant.

Patients with diffuse expression of ZEB1 had shorter disease-specific ($p = 0.031$) and relapse-free ($p = 0.034$) survival times compared with patients with focal expression of ZEB1, and the differences were statistically significant.

Table 3 presents the results for the univariate analysis of clinicopathologic predictors of survival in the PC patients. Lymph node metastasis, pleural invasion, and diffuse expression of ZEB1 predicted poorer relapse-free survival and poorer disease-specific survival. The epithelial component or PC component subtypes did not affect prognosis (data not shown).

A multivariate analysis of patient relapse-free survival revealed that lymph node metastasis and diffuse expression of ZEB1 were independent prognostic factors for poor survival (Table 4, upper). Lymph node metastasis, pleural invasion and diffuse expression of

Table 2
Characteristics of seven patients with a tumor ≤2 cm diameter.

Age/gender	Coexisting carcinoma component	Predominant pleomorphic component	pT (mm)	pN	Survival
74/M	AD (10%)	Spindle cell	2a[a] (16)	0	Dead
66/M	None	Spindle cell	1a (20)	0	Alive
59/M	SQ (40%)	Giant cell	1a (10)	0	Alive
71/M	None	Spindle cell	2a[a] (15)	0	Alive
71/M	SQ (10%)	Spindle cell	1a (17)	0	Alive
46/M	None	Spindle cell	1a (15)	0	Alive
73/F	None	Spindle cell	1a (20)	0	Alive

AD, adenocarcinoma; SQ, squamous cell carcinoma.
[a] By reason of pleural invasion.

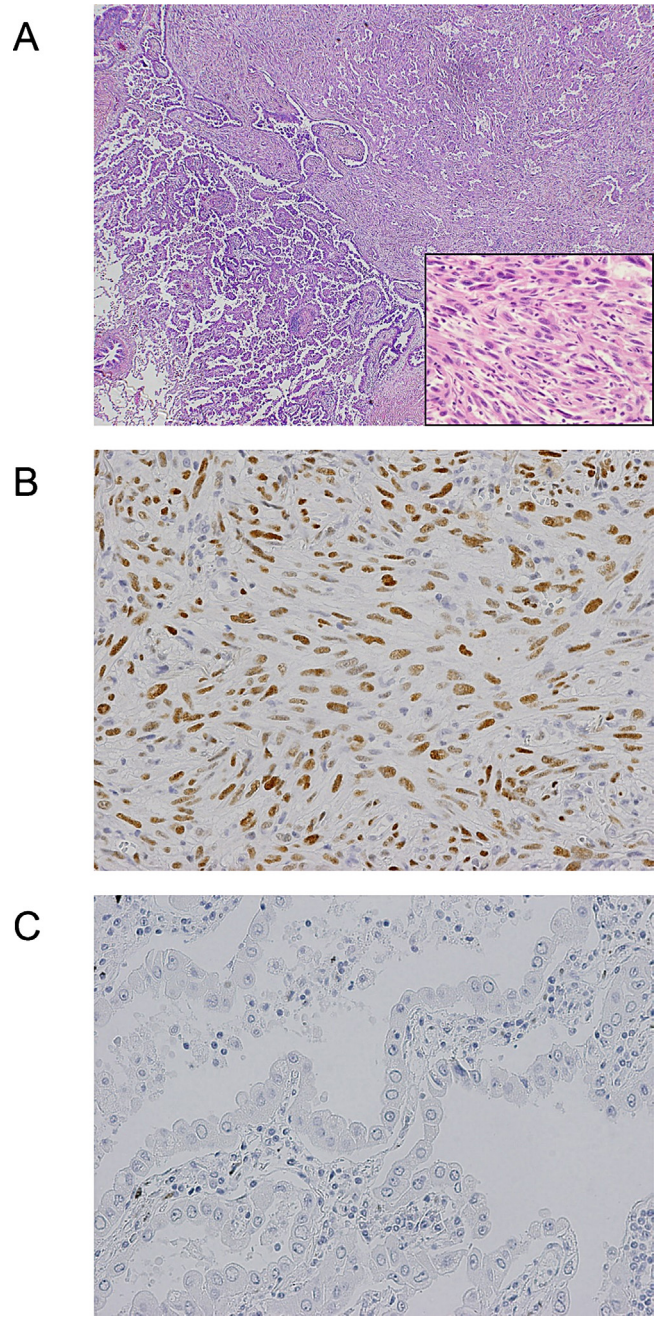

Fig. 1. Expression of ZEB1 in pleomorphic carcinoma (PC). A representative case of PC with spindle cell and adenocarcinoma components is shown in (A). Inset in (A) shows the spindle cell component in a PC. Diffuse ZEB1 expression occurred in the spindle cell nuclei (B), but the adenocarcinoma components were ZEB1-negative (C). (A) HE ×12.5, inset ×400; (B) and (C) ZEB1 immunohistochemistry ×200

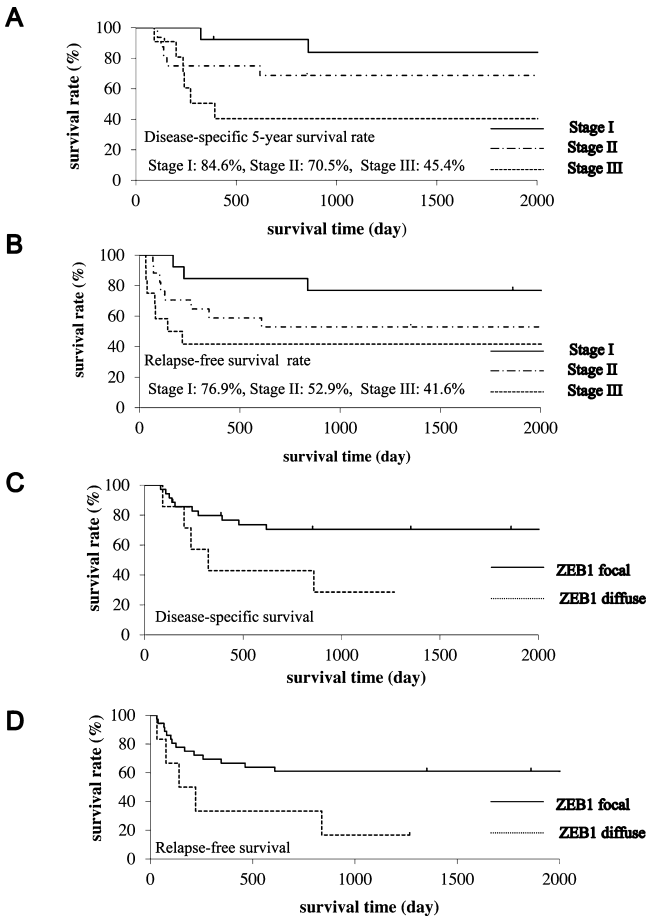

Fig. 2. (A) Kaplan–Meier disease-specific survival curve for pleomorphic carcinoma, stratified by stage at diagnosis ($n = 42$). The solid line indicates stage I; dash-dotted line, stage II; broken line, stage III. There was a statistically significant difference between stages I and III ($p = 0.015$). There were no significant differences between stages I and II, stage II or stage III. (B) Kaplan–Meier relapse-free survival curve for pleomorphic carcinoma, stratified by to stage at diagnosis ($n = 42$). The solid line indicates stage I; dash-dotted line, stage II; broken line, stage III. There was a statistically significant difference between stages I and III ($p = 0.021$). The differences between stages I and II, and between stages II and III, were not statistically significant. (C) Kaplan–Meier disease-specific survival curve for pleomorphic carcinoma, stratified according to ZEB1 expression. The dotted line indicates diffuse expression of ZEB1; solid line, focal expression of ZEB1. There was a statistically significant difference between the two groups ($p = 0.031$). (D) Kaplan–Meier relapse-free survival curve for pleomorphic carcinoma, stratified by ZEB1 expression. The dotted line indicates diffuse expression of ZEB1; solid line, focal expression of ZEB1. There was a significant difference between the two groups ($p = 0.034$).

ZEB1 were independent prognostic factors for poor disease-specific survival (Table 4, lower).

4. Discussion

We are the first to report that diffuse ZEB1 expression in the PC component (spindle or/and giant cells) was significantly associated with shorter relapse-free and disease-specific survival in PC patients. The results of multivariate analysis indicated that diffuse ZEB1 expression was an independent poor prognostic factor. Lymph node metastasis and pleural invasion were also independent poor prognostic factors for relapse-free and disease-specific

Table 4
Results of multivariate analysis of prognostic factors affecting disease-specific and relapse-free survival after surgical resection ($N = 42$).

	Relative risk	95% CI	p
Relapse-free survival			
p-N positive	3.804	1.366–10.594	0.010*
ZEB1, diffuse expression	3.929	1.423–10.843	0.008*
Pleural invasion	2.083	0.949–4.570	0.067
Tumor Size	0.442	0.092–2.118	0.307
Lymphatic permeation	1.374	0.485–3.890	0.549
Gender	0.442	0.092–2.118	0.307
Disease-specific survival			
p-N positive	4.396	1.487–12.99	0.007*
ZEB1, diffuse expression	3.297	1.244–8.738	0.016*
Pleural invasion	3.134	1.172–8.375	0.022*
Tumor Size	0.935	0.406–2.156	0.876
Lymphatic permeation	0.694	0.266–1.808	0.455
Pleomorphic component (≥75%)	0.179	0.023–1.385	0.099

CI indicates confidence interval.
* Statistically significant.

survival or disease-specific survival, respectively. Moreover, an analysis of smaller p-T1a size PCs indicated higher percentages of tumors without a coexisting carcinoma component, and longer survival. The 5-year disease-specific survival rate was 83%.

Several reports have described various relationships between ZEB1 expression and clinicopathological parameters for different cancers. A high expression of ZEB1 is associated with liver metastasis and low survival rates in colorectal cancer patients [15]. In pancreatic cancer patients, a decrease in E-cadherin expression and an increase in ZEB1 or ZEB2 expression are associated with a poorer prognosis and nodal metastasis [8]. Up-regulation of ZEB1 expression is linked to disruption of cell–cell interactions that lead to cancer cell migration and invasion in patients with malignant endometrial cancer [16]. One of the well-known functions of ZEB1 in cancer cells is to cause down-regulation of E-cadherin, which is a hallmark of EMT, the generation of motile mesenchymal cells from epithelial sheets. In this study, we investigated the expression of E-cadherin in PC and in coexisting carcinoma components (data not shown). E-cadherin immunoreactivity was variably observed in normal epithelium and the coexisting carcinoma component, but was completely absent in the PC component in all cases, irrespective of the varying degrees of ZEB1 expression. Thus, there was no inverse correlation between expression levels of E-cadherin and ZEB1 within the PC component.

Recently, ZEB1 functions that are unrelated to EMT have been reported. The non-EMT functions include regulation of cell cycle progression, apoptosis and senescence [12]. ZEB1 directly binds to the promoters of genes encoding cyclin-dependent kinase inhibitors (e.g., p21, p27 and p57) to control the cell cycle [17]. ZEB1 also interferes with a complex regulatory network of p53 family members and their targets to support either pro-survival or proapoptotic responses [12]. Furthermore, ZEB1 appears to be a factor that override drug resistance and oncogene addiction. Silencing ZEB1 using siRNA restores cell sensitivity to DNA damaging agents, such as gemcitabine, 5-fluorouracil (5-FU), and cisplatin [18]. Moreover, ZEB1 depletion sensitizes head and neck squamous cell carcinoma cells to the epidermal growth factor receptor (EGFR) inhibitor, erlotinib [12,19]. In our study, the prognosis did not significantly different between the patients who received adjuvant chemotherapy or not. However, diffuse expression of ZEB1 significantly correlated with poor prognosis compared with focal expression in the 23 patients with adjuvant chemotherapy. This result has the possibility of predictive factor of response against adjuvant chemotherapy. However, further studies are required to investigate the biological functions of ZEB1 in PCs in this point. Diffuse ZEB1 expression was not significantly correlated with clinicopathological parameters such as size, presence of necrosis, lymphovascular involvement, pleural invasion, and predominant pleomorphic or coexisting carcinoma components. The precise mechanisms via which ZEB1 expression affected PC patient survival remain to be elucidated, but the previously mentioned EMT and non-EMT-related mechanisms may both be involved.

The contributions of unfavorable clinicopathological factors, such as nodal involvement [20,21], no surgery [3,4], higher pathologic stages [22,23], and pleural invasion [24] have been reported. Massive coagulation necrosis and lymphatic permeation are also independent prognostic factors that predict a poor outcome [2]. The results of our study indicated that lymph node metastasis and pleural invasion were independent prognostic factors for disease-specific survival of PC patients. However, the type of coexisting carcinoma and the PC component did not affect prognosis (data not shown). The percentage of PC component in the entire tumor did not affect prognosis in early-stage or in advanced-stage tumors. As favorable prognostic factors, it is reported that lymphoplasmacytic infiltration in the marginal stroma and lymph follicles around tumors, which were found in stages I and II, were associated with favorable prognosis in giant cell carcinomas [25]. In our study, there was no association of the peritumoral lymphoplasmacytic infiltration and lymph follicle formation with good survival of pT1a PCs.

The prevalent hypothesis for the origin of the PC component is epithelial derivation with divergent mesenchymal dedifferentiation, so-called EMT, but it has not been well-defined [2,26,27]. Consistent with this view, we hypothesized that the coexisting adeno-, squamous, and large cell carcinoma portions are larger in smaller PCs, and become relatively smaller as the tumor increases in size. However, the results of our study revealed that only 3 (43%) of 7 p-T1a size ($\leq$20 mm in greatest dimension) tumors contained coexisting carcinoma portions, whereas the coexisting carcinoma portions were found in 23 (88.5%) of 26 tumors >20 mm and $\leq$50 mm in the greatest dimension, and in 19 (65.5%) of 29 tumors >50 mm in greatest dimension. Contrary to our hypothesis, the frequency and mean percentage of coexisting carcinoma portion were increased along to the entire tumor size. Furthermore, it was recently reported that the EGFR mutation was found in 3 (18%) of 17 patients with PC; these mutations were detected in the adenocarcinomatous component, but not in the pleomorphic component [4]. These findings suggest the possibility of a common stem cell or an independent multiclonal origin for pleomorphic and coexisting carcinoma components rather than an EMT-based derivation of the PC component from coexisting carcinomas.

In conclusion, we found that diffuse ZEB1 expression an immunohistochemical prognosticator in PC patients. Further investigation will be required to elucidate the mechanisms whereby ZEB1 expression leads to a poor prognosis. These results suggest that the ZEB1 gene may be useful as a new molecular target for therapy for patients with PC.

Conflicts of interest statement

All authors contributing to this work have no conflicts of interest to declare.

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