Prognostic impact of cancer stem cell-related markers in non-small cell lung cancer patients treated with induction chemoradiotherapy

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ABSTRACT

The expression of several cancer stem cell (CSC)-related markers has been confirmed in non-small cell lung cancer (NSCLC). The aim of this study was to clarify the clinical role of CSC-related markers in patients with NSCLC undergoing induction chemoradiotherapy (CRT). Fifty patients with clinically diagnosed N2 or N3 NSCLC who underwent induction CRT with docetaxel and cisplatin concurrently with thoracic radiation followed by surgery were examined in this study. The expressions of CSC related markers (CD133, ALDH1, ABCG2, and Bmi-1) were examined using immunohistochemical staining in surgically resected specimens. Among the 50 patients, 20 patients had no residual tumor cells in the resected specimen when examined pathologically; CSC-related marker expressions and their correlation to survival were evaluated in the other 30 patients. After a median follow-up period of 72 months, the 5-year overall survival rate of the patients with CD133-positive or ALDH1-positive specimens was significantly worse than that of the patients with both CD133-negative and ALDH1-negative expressions (44.9% vs. 90.0%, respectively; $P = 0.042$). In a multivariate analysis, CD133 and ALDH1 negativity ($P = 0.047$) and cN2-3 single station metastasis ($P = 0.03$) were significant independent prognostic factors for prolonged survival. The expressions of CSC-related markers after CRT were

significantly correlated with a poor prognosis in patients with NSCLC. The development of therapeutic strategies including adjuvant therapy that take CSC-related marker positivity into consideration is likely to be a key factor in further improvements of the prognosis of patients undergoing trimodality therapy.

1. INTRODUCTION

Lung cancer is the leading cause of death among patients with malignant tumors worldwide. For locally advanced non-small cell lung cancer (NSCLC), multimodal therapy including chemotherapy, radiotherapy and surgery can improve the survival of patients, compared with single-treatment modalities. Definitive chemoradiotherapy is one of the treatments of choice for locally advanced NSCLC, especially when N2 or N3 disease is apparent. While surgical resection after induction therapy is not currently considered an established standard approach, surgery after induction therapy is often performed by experienced institutions worldwide [1]. Stupp et al. recently reported an excellent outcome (5-year survival rate of 40%) for stage IIIB patients who were treated with docetaxel and cisplatin followed by accelerated radiotherapy and surgery [2]. We also reported a promising clinical outcome of trimodality therapy for NSCLC patients with stage III disease [3].

To further improve the outcome of induction therapy, prognostic factors for induction therapy needed to be identified. The histological response in resected specimens is usually examined, since a pathological complete response (pCR) or mediastinal downstaging are prognostic factors of induction therapy followed by surgical treatment. However, the rate of pCR or downstaging is approximately 30%, and

the exploration of novel prognostic factors may be useful [1-3].

Recently, cancer stem cells (CSCs), which are characterized by the capacity for self-renewal and pluripotency, have been attracting interest as a source of cancer cells [4]. Various molecules are being investigated as putative markers of CSCs in malignancies including lung cancer [5]. CD133, which was initially described as a surface antigen specific for human hematopoietic stem cells [6,7], is now being used to identify and isolate putative CSC populations from malignant tumors including cancers of the brain, prostate, liver, pancreas, and colon as well as melanomas [8-14]. CD133 has also been used to isolate cancer-initiating (stem) cells from lung cancer [15]; however, its clinical role in lung cancer remains unclear. As another candidate marker for CSCs, aldehyde dehydrogenase (ALDH), which is known to occur in brain tumors and breast cancer, is widely regarded as a surface marker of CSCs in lung cancer [16-18]. Serrano et al. reported that ALDH-positive lung cancer stem-like cells have longer telomeres but similar telomerase activity levels, compared with the non-CSC fraction [19].

A previous study reported that the expression of some CSC-related markers was related to a poor prognosis among patients with initially resected NSCLC, suggesting their potential use as prognostic markers such as CD133, ATP-binding cassette

superfamily G member 2 (ABCG2), Bmi-1, and Octamer-4 [20-22]. Indeed, CSCs are known to have drug or radiation-resistant features [23,24]. These reports suggest that the presence of residual CSC-like cells in specimens treated with induction CRT may indicate the resistance of the cancer cells and may be related to a poor prognosis.

In this study, we examined the expression of four CSC-related markers (CD133, ALDH1, ABCG2, and Bmi-1) using immunohistochemical (IHC) staining in surgically resected specimens that had been subjected to induction CRT and evaluated the prognostic impact of these CSC-related markers in viable cells after induction therapy.

2. MATERIALS AND METHODS

2.1. Patients, treatment plan and study design

A total of 50 patients with locally advanced N2 or N3 NSCLC who underwent induction chemotherapy concurrently with thoracic radiation followed by surgery between January 2000 and June 2009 at Okayama University Hospital were enrolled in this study. Twenty eight patients had pathological N2 disease, as confirmed by the mediastinoscopy. All the patients underwent a previously reported induction therapy regimen [3]. Briefly, the regimen consisted of docetaxel (40 mg/m^2) and cisplatin (40 mg/m^2) administered on days 1, 8, 29 and 36 plus concurrent thoracic irradiation at a

dose of 40 - 60 Gray. Following induction CRT, the response of the patients was evaluated using the results of chest radiography and computed tomography (CT) or ^{18}F-fluorodeoxyglucose positron emission tomography/CT. Patients without progressive disease were scheduled to receive surgery within 6 weeks of the completion of the induction therapy. The surgical procedure was determined based on the extent of the disease. A lobectomy was preferred; however, a bilobectomy, sleeve resection, or pneumonectomy was performed in patients requiring these procedures because of primary tumor or metastatic lymph node invasion. Postoperative adjuvant treatment was left to the physician's discretion, and 21 of the 50 patients received adjuvant chemotherapy. Study approval was obtained from the institutional review board, and informed consent was obtained from each of the enrolled patients.

2.2. IHC staining

Surgical specimens of the primary lesion were evaluated using IHC staining in the cases with residual tumor cells. Evaluations of pre-treatment biopsy samples of the primary lesion could not be performed because sufficient amounts of the samples necessary for the evaluation of CSC-related markers using IHC staining could not be obtained in many cases.

Surgical specimens were fixed in 10% formaldehyde, embedded in paraffin and cut into 4-μm-thick sections. The sections were deparaffinized in xylene and rehydrated in a graded series of ethanol. For antigen retrieval, the sections were heated in a microwave in 10 mM of sodium citrate (pH 6.0) for 30 minutes. The sections were incubated in 3.0% H_2O_2 solution for 10 minutes to block endogenous peroxidase activity. IHC staining was performed using the ImmPRESS Reagent Kit (Vector Laboratories, CA) according to the manufacturer's instructions. Following a blocking step with normal horse serum, the sections were incubated for 60 minutes with the primary antibodies at room temperature. The primary antibodies consisted of a mouse monoclonal anti-CD133 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany; diluted 1:50 in PBS), a rabbit polyclonal anti-ALDH1 antibody (Abcam, Cambridge, UK; diluted 1:400 in PBS), a mouse monoclonal anti-ABCG2 antibody (Abcam; diluted 1:400 in PBS), and a rabbit polyclonal anti-Bmi-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:50 in PBS). After a brief wash, the appropriate ImmPRESS Reagent (anti-mouse or anti-rabbit immunoglobulin) was added to the sections and incubated for 30 minutes. Antibody binding was detected using a ImmPACT DAB Peroxidase Substrate Kit (Vector Laboratories), and Mayers hematoxylin was used for counterstaining.

2.3. Assessment of IHC staining

Two investigators (KS and KI) who were unaware of the clinical data independently evaluated the marker staining under a light microscope at a x400 magnification. According to the immunostaining intensity, tumor cells with moderate or strong staining were considered positive, while tumor cells with no or weak staining were defined as negative. The IHC staining for CD133, ALDH1, ABCG2, and Bmi-1 was semi-quantitatively assessed based on the approximate percentage of positive cells over the total number of tumor cells and was determined according to previously described criteria as follows: CD133 negative $\leq$1%, positive >1% [21,25]; ALDH1 negative $\leq$10%, positive >10% [17]; ABCG2 negative $\leq$10%, positive >10% [21,26]; and Bmi-1 negative $\leq$5%, positive >5% [27]. Omission of primary antibody served as a negative control in each marker. The bronchial epithelial cells (in ALDH1, ABCG2, and Bmi-1) or macrophages (in ALDH1) were used as internal control (Supplementary fig. 1). In CD133, strongly stained sample was used as positive control.

2.4. Statistical analysis

In this study, the overall survival (OS) period was defined as the primary endpoint

and the disease-free survival (DFS) period was defined as the secondary endpoint. The OS and DFS were calculated from the date of initial treatment until the date of death or the last follow-up for OS and until confirmed disease recurrence or death for DFS.

The baseline characteristics of the patients were compared using the Wilcoxon rank sum test for continuous variables and the Fisher exact test for categorical variables, as appropriate. A univariate analysis of OS and DFS was performed using the Kaplan-Meier method with logrank testing, and a multivariate analysis was performed using the Cox proportional hazard model. All the data were analyzed using JMP, version 9.0.0 (SAS Institute Inc, Cary, NC). For each analysis, probability values of less than 0.05 were considered significant.

3. RESULTS

3.1. Expression of CSC related markers in NSCLC

Among the 50 surgically resected specimens, viable tumor cells remained in the specimens from 30 patients. Among these 30 patients, 9 patients (30%) were positive for CD133 expression, 18 patients (60%) were positive for ALDH1 expression, 14 patients (47%) were positive for ABCG2 expression, and 25 patients (83%) were positive for Bmi-1 expression. Details of each marker expression in each patient are

shown in Supplementary table 1. Examples of the IHC staining patterns are shown in Fig. 1, Supplementary fig. 1 and 2. The relations between the IHC staining patterns and the clinicopathological factors were examined (Table 1). With the exception of Bmi-1 expression, which was significantly higher in smokers than in non-smokers ($P = 0.011$), no significant associations were observed between the marker expression statuses and clinicopathological factors (age, sex, smoking history, histology, serum carcinoembryonic antigen [CEA] level, clinical or pathological stage, or cN2-3 station). Regarding each marker expression, ALDH1 expression correlated with Bmi-1 expression (Supplementary table 2).

3.2. Clinical outcomes and impact of CSC-related marker expressions

The relationships between the CSC-related marker statuses and the clinical outcomes were examined. After a median follow-up duration of 72 months, nine patients had died. Seventeen patients had experienced a disease relapse. The 3-year and 5-year OS rates were 71.4% (95% confidential interval [CI]: 85.1 - 52.2) and 61.5% (95% CI: 78.3 - 41.4), respectively. The 3-year and 5-year DFS rates were 52.1% (95% CI: 34.3 - 69.3) and 39.0% (95% CI: 22.6 - 58.4), respectively. The 5-year OS rate in the CD133-positive or ALDH1-positive patients was significantly poorer than that of

the patients who were negative for both CD133 and ALDH1 (44.9% [95% CI: 22.3 - 69.9] vs. 90.0% [95% CI: 53.3 - 98.6], respectively; *P* = 0.042) (Fig. 2). The relationships between other clinicopathological factors and survival were also examined. The selected clinicopathological factors were as follows: age, sex, smoking history, histology, CEA level, clinical disease stage (cStage), clinical N status (cN), clinical N2 or N3 metastasis station (cN2-3 station), and adjuvant chemotherapy (administered/not administered) (Table 2). Among them, the cN and cN2-3 station were significantly associated with the OS period. In a multivariate analysis, which included significant prognostic factors by the univariate analysis, CD133 and ALDH1 negativity (Hazard ratio [HR]: 0.16, 95% CI: 0.0086 - 0.98, *P* = 0.047) and single mediastinal nodal metastasis (not multinodal metastasis) (HR: 0.19, 95% CI: 0.028 - 0.91, *P* = 0.03) were significant independent prognostic factors of a prolonged survival (Table 3).

Postoperative adjuvant chemotherapy was performed in 15 of the 30 patients. Although the number was small, the CSC related marker status of the 15 patients who received adjuvant chemotherapy was not significantly related to the OS or DFS periods.

4. DISCUSSION

We showed that the positive expression of CD133 or ALDH1 is independent

predictor of disease relapse and a poor prognosis among patients who have received induction CRT using docetaxel and cisplatin. The positive expression of CD133 or ALDH1 in resected specimens may be an indicator of tumor resistance, indicating the failure to control preexisting micrometastasis or to prevent metastasis during or after CRT.

The four markers that were examined in this study have been previously reported as candidate CSC-related proteins in NSCLC. Tirino et al. reported that CD133-positive cells isolated from surgically resected specimens of previously untreated NSCLC were able to give rise to spheres and to act as tumor-initiating cells [28]. It has been also reported that CD133 positive endothelial progenitor cells are found in previously untreated frozen NSCLC tissue obtained by surgery and seem to be related to vasculogenesis [29]. On the other hand, Salnikov et al. reported that CD133 was indicative of a resistance phenotype but was not a prognostic marker for survival in surgically resected specimens of previously untreated NSCLC [30]. The authors showed a significant association between the expression of resistance-related proteins, such as glutathione S-transferase, thymidylate synthase, catalase, O^6-methylguanine-DNA methyltransferase, and p170, and CD133. In addition, Bertolini et al. reported that the cisplatin treatment of lung cancer cells in vitro resulted in enrichment of the

CD133-positive fraction after both acute cytotoxicity and in cells with a stable cisplatin-resistant phenotype [23]. These findings are consistent with our result, in which the expression of CD133 in surgically resected specimens after induction CRT was related to an unfavorable outcome. ABCG2, a member of the ATP binding cassette (ABC) transporter superfamily, is an important determinant of the side population (SP) phenotype [31]. SP cells with stem cell-like capabilities marked by ABCG2 have been found in a variety of hematologic and solid malignancies, as well as NSCLC [32,33]. Bmi-1 is a member of the Polycomb group family of proteins, which act as epigenetic chromatin modifiers [34,35]. Bmi-1 is known to be a key regulator in the self-renewal of stem cells [36], and a recent report has shown that the expression of Bmi-1 in surgically resected specimens was a significant prognostic factor of a poor outcome in lung adenocarcinoma [20]. In our study, no obvious relation was seen between the expression of ABCG2 or Bmi-1 and patient outcome. This discrepancy may be due to differences in the kinds of samples that were examined, since our specimens had been treated with CRT. In addition to the markers examined in the present study, other CSC-related markers have also been reported. Further study examining the impact of such markers on trimodality therapy is necessary.

Surgically resected specimens that had been treated with CRT were used in this

study. Regarding the clinical relevance, the expression status of CSC-related markers in post CRT specimens may be useful as biomarkers for the selection of adjuvant therapies after surgery. Since appropriate strategies for the treatment of CD133 or ALDH1-positive cases remain unknown at present, further investigation to establish appropriate adjuvant strategies is warranted. As other strategies, CSC-related markers could also be examined in enough amounts of specimens obtained before or after induction therapy to determine the contents of induction therapy or to determine the necessity of the further treatment, including surgery. However, based on the concept of CSC, the population of CSC-related marker positive cells may be very limited, and the possibility of misclassification, particularly the possibility of a false-negative diagnosis, is a concern. Indeed, the expression of CD133 was observed in a very limited portion of the surgically resected specimens in the present study.

What is the appropriate therapeutic strategy for tumors with positive CSC-related makers? In our knowledge, there is no relevant therapeutic strategy to specifically target cells with CSC-like phenotype in lung cancer. Of interest, histone deacetylase inhibitors are effective in chronic myelogenous leukemia stem cells appeared after acquisition of imatinib mesylate resistance [37].

In conclusion, the expression of CD133 or ALDH1 was significantly associated

with an unfavorable prognosis. Although a study involving a large number of patients is required before a definite conclusion can be made, our results suggest that the development of a therapeutic strategy that considers the expression of CSC-related markers may be a key to further improvements in the prognosis of patients undergoing trimodality therapy.

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CONFLICT OF INTEREST STATEMENT

None declared.

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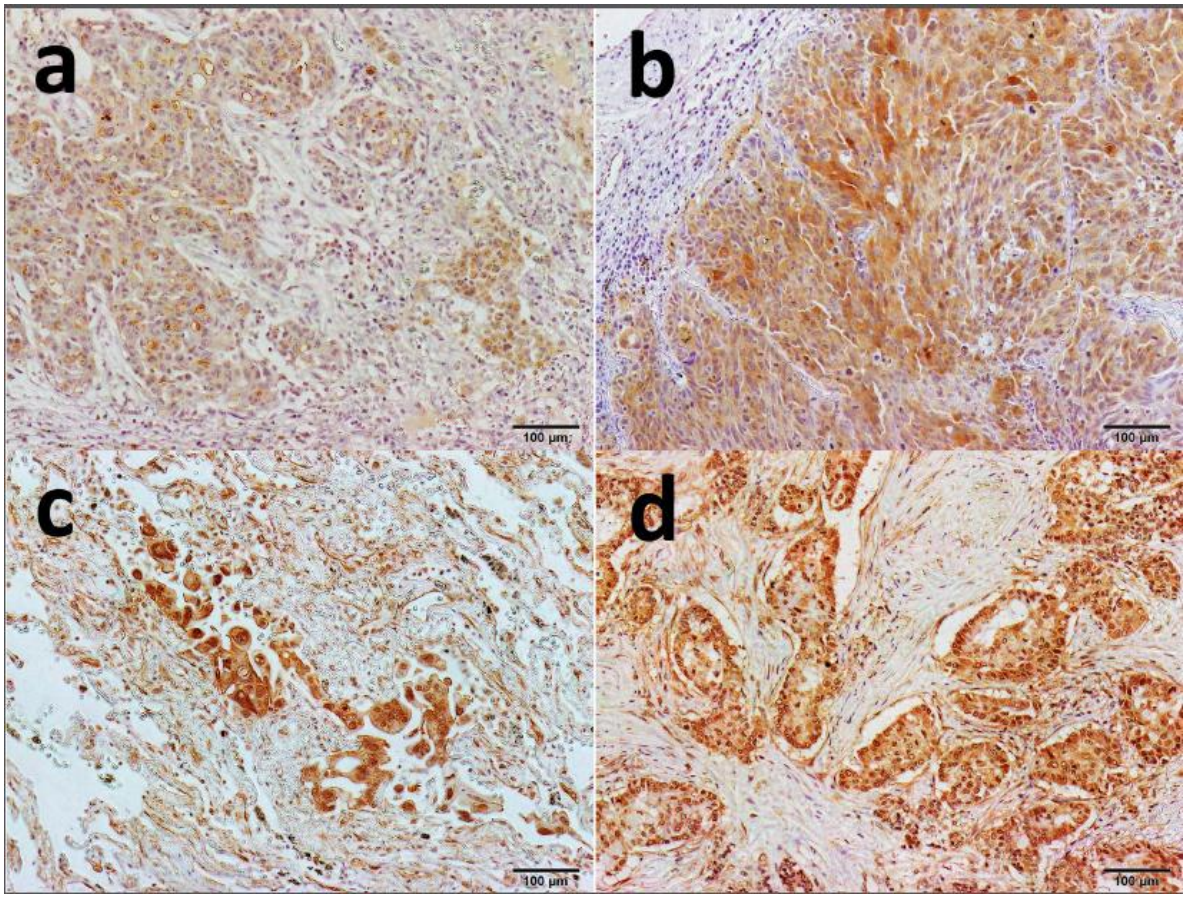
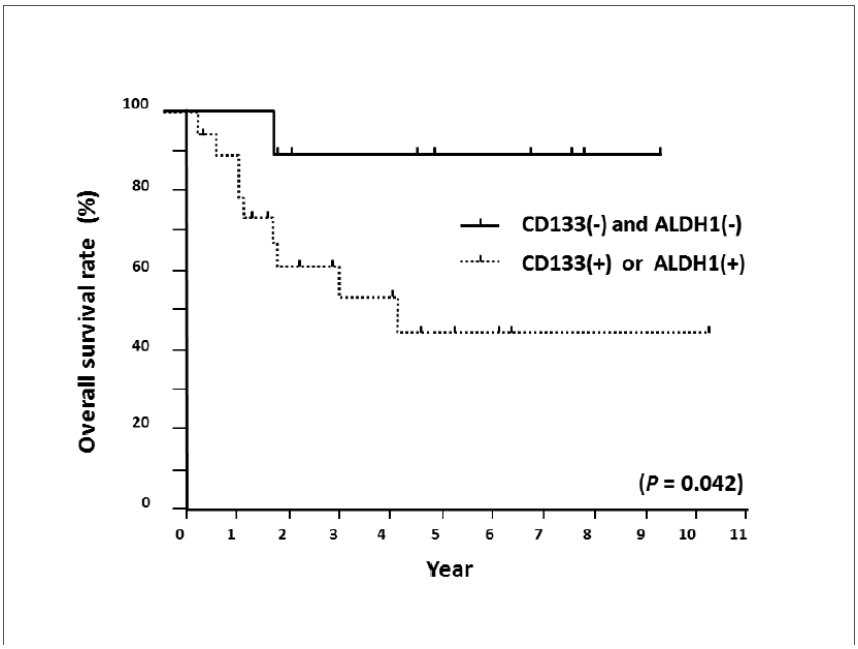


Figure 1. Representative examples of immunohistochemical staining for each of the markers in a surgical resected primary tumor. (a) CD133 staining; strong, 15 % positive, (b) ALDH1 staining; strong, 72 % positive (c) ABCG2 staining; strong, 60 % positive, (d) Bmi-1 staining; strong, 80 % positive



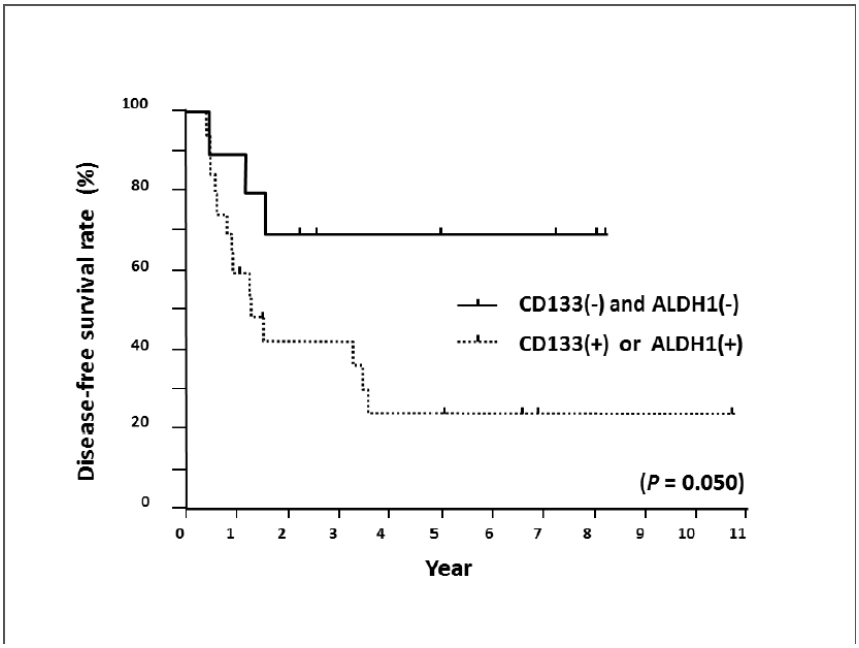


Figure 2. Kaplan-Meier curves showing the (a) OS and (b) DFS rates according to cancer stem cell related markers expression. The OS and DFS rates in the CD133-positive or ALDH1-positive patients were significantly poorer than that of the patients who were negative for both CD133 and ALDH1 ($P = 0.042$ and $P = 0.050$, respectively)

Table 1. Clinicopathological factors and CSC related markers expression

	CD133		ALDH1		ABCG2		Bmi-1	
Characteristics (n)	+ / -	*P*	+ / -	*P*	+ / -	*P*	+ / -	*P*
Age		0.43		0.71		0.27		1.00
< 60 (15)	6 / 9		10 / 5		9 / 6		13 / 2	
≥ 60 (15)	3 / 12		8 / 7		5 / 10		12 / 3	
Sex		0.68		1.00		0.44		0.14
Male (21)	7 / 14		13 / 8		11 / 10		19 / 2	
Female (9)	2 / 7		5 / 4		3 / 6		6 / 3	
Smoking history		0.37		0.21		0.85		0.011*
Non-smoker (8)	1 / 7		3 / 5		3 / 5		4 / 4	
Smoker (22)	8 / 14		15 / 7		11 / 11		21 / 1	
Histology		1.00		0.46		0.26		0.30
Ad (20)	6 / 14		13 / 7		11 / 9		18 / 2	
Non-Ad (10)	3 / 7		5 / 5		3 / 7		7 / 3	
CEA		1.00		0.71		0.27		0.33
<4.1 (15)	5 / 10		10 / 5		9 / 6		11 / 4	
≥4.1 (15)	4 / 11		8 / 7		5 / 10		14 / 1	
Clinical stage		0.20		0.14		1.00		1.00
IIIA (20)	8 / 12		14 / 6		9 / 11		17 / 3	
IIIB (10)	1 / 9		4 / 6		5 / 5		8 / 2	
cN2/3 station		1.00		0.71		0.72		1.00
Single (15)	4 / 11		8 / 7		6 / 9		12 / 3	
Multi (15)	5 / 10		10 / 5		8 / 7		13 / 2	
Recurrence		0.69		0.061		0.48		1.00
- (13)	3 / 10		5 / 8		5 / 8		11 / 2	
+ (17)	6 / 11		13 / 4		9 / 8		14 / 3	

Ad, adenocarcinoma

Table 2. 5-year overall survival and disease-free survival rate

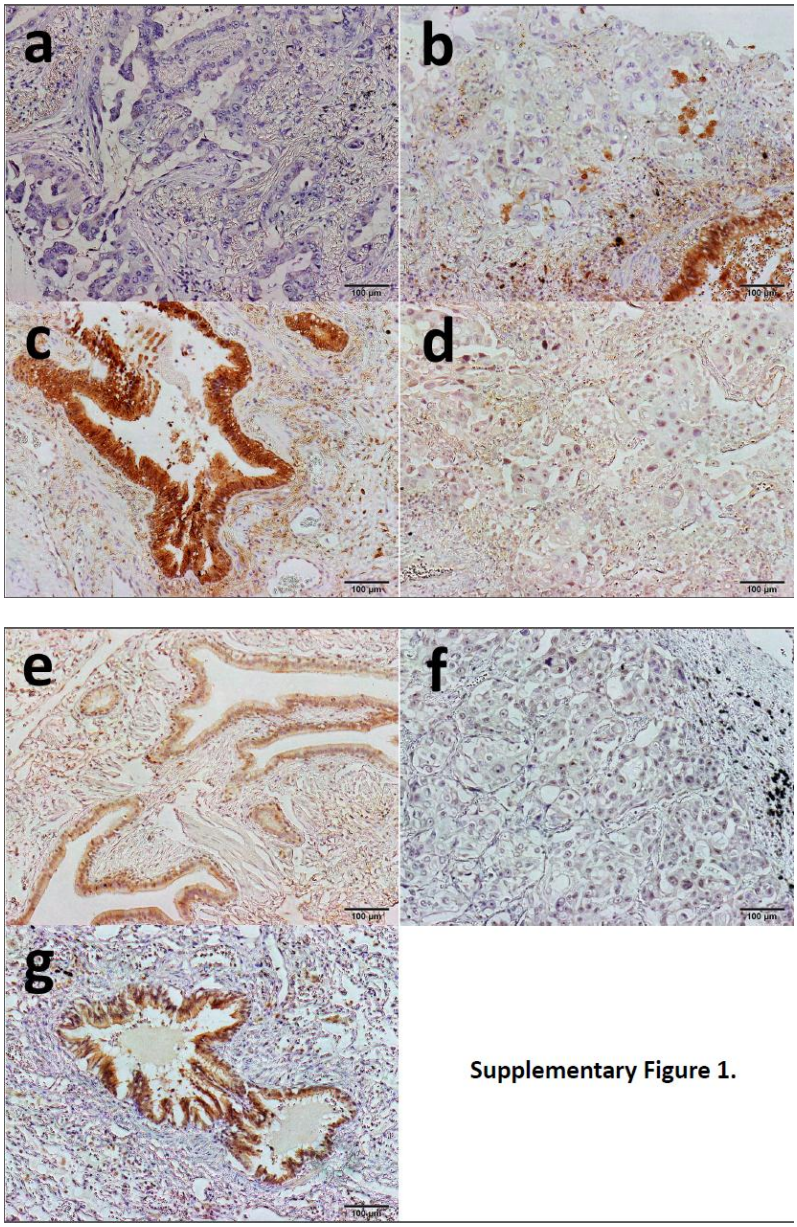
Subsets (n)	5-year OS (%)	*P*	5-year DFS (%)	*P*
Age, <60 (15) / ≥60 (15)	47.9 / 75.4	0.098	23.3 / 56.1	0.077
Sex, Male (21) / Female (9)	57.6 / 71.1	0.42	36.7 / 44.4	0.82
Smoking, Non-smoker (8) / Smoker (22)	72.9 / 57.6	0.43	50.0 / 35.4	0.76
Histology, Ad (20) / Non-Ad (10)	62.2 / 55.6	0.55	33.9 / 48.0	0.97
CEA, <4.1 (15) / ≥4.1 (15)	71.5 / 46.6	0.28	66.0 / 9.5	0.020*
Clinical stage, IIIA (20) / IIIB (10)	65.0 / 50.0	0.18	34.6 / 48.0	0.15
cN status, cN2 (26) / cN3 (4)	66.9 / 25.0	0.0098*	43.2 / 0.0	0.25
cN2-3 station, Single (15) / Multi (15)	85.7 / 37.7	0.014*	51.2 / 24.0	0.087
Adjuvant therapy, adm (15) / not adm (15)	55.0 / 70.7	0.69	26.7 / 55.6	0.24
CD133 expression, - (21) / + (9)	69.0 / 38.9	0.38	43.7 / 27.8	0.58
ALDH1 expression, - (12) / + (18)	75.0 / 50.9	0.33	65.6 / 24.4	0.073
ABCG2 expression, - (16) / + (14)	63.5 / 58.0	0.55	50.0 / 21.8	0.46
Bmi-1 expression, - (5) / + (25)	80.0 / 57.7	0.46	40.0 / 39.9	0.82
CD133 / ALDH1 expression,				
Both negative (10) / Either positive (20)	90.0 / 44.9	0.042*	70.0 / 24.6	0.042
CD133 / ABCG2 expression,				
Both negative (10) / Either positive (20)	78.6 / 51.7	0.26	60.0 / 27.1	0.29
CD133 / Bmi-1 expression,				
Both negative (5) / Either positive (25)	80.0 / 57.7	0.46	40.0 / 39.9	0.82
ALDH1 / ABCG2 expression,				
Both negative (7) / Either positive (23)	71.4 / 58.4	0.63	57.1 / 33.5	0.55
ALDH1 / Bmi-1 expression,				
Both negative (5) / Either positive (25)	80.0 / 57.7	0.46	40.0 / 39.9	0.82
ABCG2 / Bmi-1 expression,				
Both negative (4) / Either positive (26)	75.0 / 59.8	0.68	50.0 / 38.1	0.99

Ad, adenocarcinoma; adm, administered

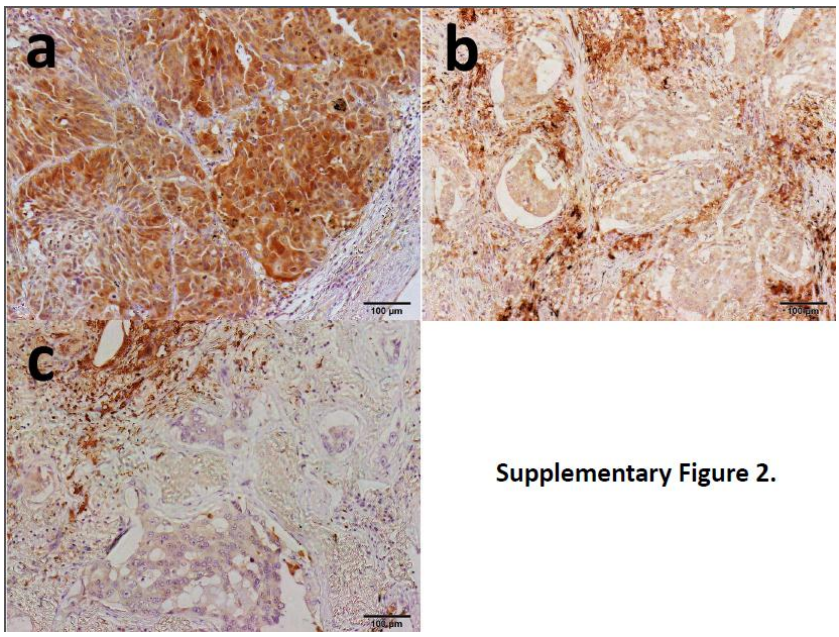
Table 3. Multivariate Cox proportional hazard model

Factors	Hazard ratio	*P*	95% CI
cN status, cN2 vs. cN3	0.68	0.61	0.16-3.43
cN2-3 station, Single vs. Multi	0.19	0.037*	0.028-0.91
CD133 / ALDH1 expression,			
Both negative vs. Either positive	0.16	0.047*	0.0086-0.98

CI, confidential interval



Supplementary figure 1. Negative slides and internal control for each of the markers. (a) CD133 negative staining, (b) ALDH1 negative staining, (c) ALDH1 internal control, (d) ABCG2 negative staining, (e) ABCG2 internal control, (f) Bmi-1 negative staining, (g) Bmi-1 internal control. ALDH1 is mainly present in the cytoplasm of the bronchial epithelial cells and macrophages as internal control (b and c; another view of the same slide). ABCG2 is mainly present in the cell membrane of the bronchial epithelial cells as internal control (d and e; another view of the same slide). Bmi-1 is mainly present in the cell membrane of the bronchial epithelial cells as internal control (f and g; another view of the same slide).



Supplementary figure 2. Immunohistochemical staining of ALDH1. (a) strong, (b) moderate, (c) no or weak staining in residual tumor cells after chemoradiotherapy.

Supplementary table1. Details of each marker expressions in each patient

Patient	CD133	ALDH1	ABCG2	Bmi-1	Number of positive factors
1	n	p	p	p	3
2	n	p	p	p	3
3	n	p	n	p	2
4	n	n	n	p	1
5	n	n	p	p	2
6	n	n	n	n	0
7	p	p	n	p	3
8	n	p	p	p	3
9	n	n	n	n	0
10	n	n	p	n	1
11	n	p	p	p	3
12	p	p	n	p	3
13	n	n	n	n	0
14	n	p	n	p	2
15	p	p	n	p	3
16	p	n	n	p	2
17	p	p	n	p	3
18	n	p	n	p	2
19	n	p	p	p	3
20	n	p	p	p	3
21	n	n	p	p	2
22	n	n	n	p	1
23	p	p	p	p	4
24	p	p	n	p	3
25	n	p	p	p	3
26	p	n	p	p	3
27	n	p	n	p	2
28	n	n	p	p	2
29	n	n	n	n	0
30	p	p	p	p	4

n, negative; p, positive

Supplementary table2. Correlation between each marker expression

	CD133	ALDH1	ABCG2	Bmi-1
CD133	-	0.25	0.44	0.29
ALDH1	-	-	0.72	0.0056*
ABCG2	-	-	-	0.34

P-values of two-sided Fisher's exact test