Aldehyde dehydrogenase 1 expression in cancer cells could have prognostic value for patients with non-small cell lung cancer who are treated with neoadjuvant therapy: identification of prognostic microenvironmental factors after chemoradiation

Yoshitaka Zenke[a,b,c], Genichiro Ishii[a], Yuichiro Ohe[b,c], Kaoru Kaseda[a,b], Tatsuya Yoshida[a,b], Shingo Matsumoto[b], Shigeki Umemura[b], Kiyotaka Yoh[b], Seiji Niho[b], Koichi Goto[b], Hironobu Ohmatsu[b], Takeshi Kuwata[b], Kanji Nagai[b], Atsushi Ochiai[a]

[a]Division of Pathology, Research Center for Innovative Oncology, National Cancer Center, Hospital East, Kashiwa, Chiba, Japan

[b]Division of Thoracic Oncology, National Cancer Center Hospital East, Kashiwa, Chiba, Japan

[c]Juntendo University Graduate School of Medicine, Tokyo, Japan

Correspondence to: Genichiro Ishii, MD, PhD or Atsushi Ochiai, MD, PhD, Pathology Division, Research Center for Innovative Oncology, National Cancer Center Hospital East, Kashiwa, Chiba, Japan 277-0882

E-mail: gishii@east.ncc.go.jp (G. Ishii), or aochiai@east.ncc.go.jp (A. Ochiai)

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Abstract

Prognostic factors for patients with non-small cell lung cancer (NSCLC) who have been treated with neoadjuvant therapy have not been fully assessed. The purpose of this study was to analyze prognostic biomarkers in NSCLC after treatment with neoadjuvant therapy, with special reference to the immunophenotypes of both the cancer cells and stromal cells. A total of 52 patients with NSCLC who were treated with neoadjuvant therapy followed by complete resection were included. We examined the expressions of 9 markers in the cancer cells and stromal cells. The 5-year disease-free survival rate of patients with high ALDH1 expression levels in their cancer cells was significantly lower than those with a low ALDH1 level (47.3% vs. 21.5%, respectively; p=0.023). The other molecules expressed in cancer cells did not exhibit any prognostic value. In NSCLC without neoadjuvant therapy (case control, n=104), expression of ALDH1 in cancer cells was not correlated with prognosis (p=0.507). A multivariate analysis identified ALDH1 expression in cancer cells as significantly independent prognostic factors for disease-free survival (p=0.045). The current study indicated that the immunophenotypes of ALDH1 in cancer cells could have prognostic value for patients with NSCLC who are treated with neoadjuvant therapy.

Key words: Non-small cell lung cancer, Neoadjuvant therapy, ALDH1, cancer-initiating cell, cancer stromal cell

1. Introduction

Lung cancer is the leading cause of death among patients with malignant tumors worldwide. Surgical resection is the standard treatment modality for early stages non-small cell lung cancer (NSCLC), but many patients develop local and distant recurrences and die.[1] For locally advanced NSCLC, multimodal therapy including chemotherapy, radiotherapy and surgery is often recommended to control and eliminate occult distant metastasis and to reduce and downstage the primary tumor and mediastinal metastasis, respectively. Induction chemotherapy followed by surgery has been demonstrated to improve survival in selected patients with NSCLC.[2-5] However, more recent studies have failed to confirm this data,[6, 7] and the issue remains controversial.

Although several randomized trials have reported that a pathological complete response (pCR) is a prognostic factor for chemoradiotherapy (CRT) followed by surgical treatment,[8, 9] biological predictive markers of NSCLC after treatment with CRT have not been fully assessed. Identifying biological predictive markers may help to distinguish patients who are likely to benefit from additional postoperative chemotherapy.

Carcinoma tissue is composed of cancer cells and stromal cells including cancer-associated fibroblasts (CAFs) and tumor-associated macrophages (TAMs).[10, 11] The cancer cells associate with CAFs and TAMs and together form the specific microenvironment of the cancer tissue. The biological and prognostic significance of the tumor microenvironment has been increasingly recognized[12].

Cancer-initiating cells (CICs) and the epithelial mesenchymal transition (EMT) are reportedly correlated with tumor progression and CRT resistance.[13, 14] Furthermore, the contribution of CAFs and TAMs to local

tumor-promoting effects and the resistance of cancer cells to chemotherapy have been recently reported. However, the biological characteristics of the cellular constituents within the tumor microenvironment after CRT are not fully understood.

The purpose of this study was to identify prognostic biological markers in patients treated with neoadjuvant therapy followed by surgical treatment by examining the immunophenotypes of both cancer cells and stromal cells, including CAFs and TAMs. We examined the expressions of 6 markers on cancer cells: geminin, cleaved caspase 3, E-cadherin, vimentin (an EMT-related marker), ALDH1 and CD44v6 (a CIC-related marker). To examine the prognostic value of CAFs, the expression levels of podoplanin and CD90 were examined, while the expression of CD204 was examined in TAMs.

2. Material and methods

2.1 Subjects

66 consecutively cases of patients with NSCLC who were treated with neoadjuvant chemotherapy, chemoradiotherapy, or radiotherapy followed by complete resection at our hospital between April 1992 and December 2009 were reviewed. The clinicopathological characteristics of the patients and the treatment results are summarized in Supplemental Table 1. Among the 66 surgically resected specimens, case with viable tumor cells remained in the specimens were included in this study (n=52) (Table 1). The median follow-up period was

56 months. Surgically resected 104 NSCLC patients without neoadjuvant therapy between April 1992 and December 2009 were also reviewed by matching for clinical stage and histopathology (Supplemental Table 2). The study was conducted with the approval of the Institutional Review Board of the National Cancer Center.

2.2 Neoadjuvant therapy

As neoadjuvant chemotherapy, 17 patients had received mitomycin, vindesine, and cisplatin, and 21 patients had received platinum-based combination chemotherapy, such as cisplatin plus vindesine, cisplatin plus vinorelbine, cisplatin plus docetaxel, cisplatin plus gemcitabine, or carboplatin plus paclitaxel. Only two patients had received docetaxel alone. The chemotherapy regimens used with radiotherapy were mitomycin, vindesine, and cisplatin or cisplatin plus vinorelbine. The median number of chemotherapy cycles was 2 (range: 1–4). The median total dose of radiotherapy was 45 Gy (range: 30–50 Gy).

2.3 Pathological studies

The surgically resected specimens were fixed in 10% formalin or absolute methyl alcohol and embedded in paraffin. The tumors were cut into 5 to 10-mm-thick slices, and serial 4-μm sections were stained with hematoxylin and eosin. All the slides containing the maximum surface area of the tumor in each case were reviewed. The representative pathologic findings for the cancer tissues after neoadjuvant therapy are shown in

96 Figure 1.

97 We also measured the area of the residual tumors (ART), which would reflect the residual tumor volume and

98 has been previously reported to have prognostic value in patients with neoadjuvant therapy, using a described

99 method previously.[15]

100

101 **2.4 Antibodies and immunohistochemistry**

102

103 The markers of cell proliferation and apoptosis used in the present study were geminin (clone EM6; Novocastra,

104 Newcastle-upon-Tyne, UK) and cleaved caspase 3 (polyclonal; Cell Signaling Technology, Danvers, MA, USA).

105 The markers of epithelial to mesenchymal transition (EMT)-related molecules were E-cadherin (clone 36; BD

106 Biosciences, San Jose, CA, USA), and vimentin (clone Vim 3B4; Dako Cytomation, Glostrup, Denmark). To

107 evaluate the expression of cancer stem cell-related molecules, we used ALDH1 (clone 44/ALDH; BD

108 Bioscience) and CD44v6 (clone VFF-7; Acris, Herford, Germany). To evaluate tumor promoting

109 cancer-associated fibroblasts (CAFs), we used podoplanin (clone D2-40; Signet, Princeton, NJ, USA) and CD90

110 (Anti-THY1; Atlas, Stockholm, SWEDEN). To evaluate activated macrophages, we used CD204 (clone

111 SRA-E5; Trans Genic, Hyogo, Japan).

112 Sections (4-μm each) were cut from the paraffin blocks and mounted on silanized slides. After antigen retrieval,

113 the slides were immersed in a 0.3% hydrogen peroxide solution

114 in methanol for 15 min to inhibit endogenous peroxidase activity. Individual slides were then incubated

115 overnight at 4℃ with different antibodies; after extensive washing with PBS, the smears were incubated with
116 EnVision (Dako, Glostrup, Denmark) for 1 hour at room temperature. The color reaction was developed for 3
117 min in 2% 3, 3'-diaminobenzidine in 50 mM Tris-buffer (pH 7.6) containing 0.3% hydrogen peroxide. Finally,
118 the sections were counterstained with Meyer's hematoxylin, dehydrated and mounted.
119
120 **2.5 Immunohistochemical scoring**
121
122 All the stained tissue sections were scored semiquantitatively and were evaluated independently under a light
123 microscope by two pathologists (Y.Z. and G.I.) who had no knowledge of the patients' clinicopathological data.
124 The labeling scores for the tumor cells were calculated by multiplying the percentage of positive tumor cells per
125 lesion (0%–100%) by the staining intensity level (0 = negative; 1 = weak; 2 = strong). We selected the median
126 score to define high and low staining.
127 For geminin and cleaved caspase 3, the number of positive tumor cells per 100 tumor cells was counted. For
128 CD 204-positive TAMs, the number of positive infiltrating cells was counted under a microscopic at × 400 (area
129 = 0.0625 mm2), as previously reported.[16] We selected the median number of positive cells to define the high and
130 low group. For the CAFs, according to the definition used in a previous study, cases with positive-stained
131 spindle-shaped cells accounting for more than 10% of the cells in the cancer stroma were identified as the high
132 group.[17]
133

2.6 Statistical analysis

Disease-free survival (DFS) was defined as the time from surgery to tumor recurrence, death or the date of the last follow-up. The survival curves were estimated using the Kaplan-Meier method, and the differences in survival between the subgroups were compared using the log-rank test. A multivariate analysis was conducted using the Cox proportional-hazard model. *P* values of less than 0.05 were considered to be significant. The statistical analysis software (JMP, Version 9) was used to perform the analyses.

3. Results

3.1. Patient characteristics

Table 1 shows the clinicopathological characteristics of the patients who received neoadjuvant therapy. Forty patients received chemotherapy, while 12 patients received chemoradiotherapy before surgery.

A univariate analysis of the clinicopathological factors was performed to identify factors influencing the disease-free survival, and the log-rank test was used to compare the two groups. Among the clinical factors, older age (≥65 years) was significantly correlated with a shorter DFS (p=0.008). Larger ART (ART>400 mm²) was also significantly correlated with a shorter DFS (p=0.035) (Table 2). There were not the correlations between clinical response rate and disease-free survival (p=0.54).

3.2. Immunohistochemical staining of the cancer cells and prognostic impact

Univariate analyses were performed according to the Cox proportional hazard model to determine the prognostic value of the expression of each molecule in cancer cells (Table 3a). The expression statuses of geminin, cleaved caspase 3, E-cadherin, vimentin, and CD44v6 in the cancer cells had no prognostic impact.

On the other hand, ALDH1 expression displayed prognostic significance. Figure 2 shows the representative results of ALDH1 expression in cancer cells. The 5-year disease-free survival rate of cases with a high ALDH1 expression level was 21.5%, while that of the cases with a low ALDH1 expression level was 47.3%. A high ALDH1 expression level in the cancer cells was significantly correlated with a shorter DFS (p=0.02). The Kaplan-Meier curve for DFS according to the ALDH1 expression status in the cancer cells is shown in Figure 3A.

3.3. Immunohistochemical staining of cancer associated fibroblasts (CAFs) and tumor-associated macrophages (TAMs) and their prognostic impact

The 5-year DFS rates of patients with low podoplanin and CD90 levels in CAFs were 37.9% and 33.8%, while those of patients with high podoplanin and CD90 levels in CAFs was 29.1% and 37.5%, respectively. The 5-year DFS rate of the patients with low CD204 levels in TAMs was 38.4%, while that of the patients with high CD204 levels in TAMs was 29.0%.

None of the molecules examined in the CAFs and TAMs had any prognostic impact (*P*=0.90, *P*=0.75, *P*=0.98) (Table 3b).

3.4 Prognostic impact of ALDH1 expression in NSCLC without neoadjuvant therapy

We examined the ALDH1 expression levels in 104 surgically resected NSCLC specimens from patients who did not undergo neoadjuvant therapy (Supplemental Table 2). The 5-year DFS rate of the patients with a high ALDH1 expression level was 48.3%, while that of the cases with a low ALDH1 expression level was 59.8% (Figure 3B). However, the difference was not significant (*P*=0.507).

3.5 Multivariate analyses to identify factors significantly associated with the prognosis

A multivariate analysis using the Cox proportional hazard model was performed to determine the prognostic usefulness of conventional pathological factors and the immunohistochemical staining of cancer cells, CAFs, TAMs and ART (Table 4). A high ALDH1 expression level in cancer cells and an ART>400 mm² were identified as significantly independent prognostic factors for DFS (*p*=0.045, *p*=0.011, respectively).

Discussion

This is the first report that examined the prognostic significance of biological markers in NSCLC after neoadjuvant therapy, focusing on the characteristics of both cancer cells and stromal cells including CAFs and TAMs. In the current study, we clearly showed that a high ALDH1 expression level in cancer cells was an independent predictor of the DFS. Although previous studies have reported the prognostic significance of CAFs and TAMs in lung cancer patients who did not receive CRT.[16, 18] none of the molecules examined in the present study had any prognostic impact.

Generally, CIC and EMT characteristics are known to have drug or radiation-resistant features.[13, 19] Shien et al. reported that NSCLC patients who had undergone induction CRT and who exhibited positivity for CIC-related molecules (CD133-positive or ALDH1-positive) had a significantly poorer prognosis.[20] On the other hand, Shintani et al. reported that the DFS rate of patients with EMT marker-positive tumor cells was significantly lower than that of patients with EMT marker-negative tumor cells among NSCLC patients who had undergone CRT.[14] Since it was assumed that these cells had already metastasized systemically before or during CRT, these results and our current results suggest the possibility that the remaining CIC and/or EMT related marker positive cancer cells might be responsible for the development of distant metastasis in patients with induction CRT.

Aldehyde dehydrogenase 1 (ALDH1) belongs to the aldehyde dehydrogenase superfamily which is responsible for the oxidation of aldehydes to their corresponding carboxylic acids.[21] ALDH1 can serve as a cancer stem/initiating cell marker in several types of cancers.[22-24] In NSCLC, the prognostic value of the ALDH1 expression level has been controversial.[25-27] We examined the prognostic value of ALDH1 in 104 cases of NSCLC without neoadjuvant therapy (Figure 3B). However, the expression level of ALDH1 in the cancer cells

was not correlated with the prognosis. These results suggested that the ALDH1 expression level might be a prognostic marker only in NSCLC patients who have undergone neoadjuvant therapy. One possible reason of this discrepancy might be explained by the hypothesis that within the original tumor microenvironment, ALDH1-negative cancer cells also have a cancer initiating capacity. Prasmickaite et al. reported that both ALDH-positive and ALDH-negative melanoma cells demonstrated similarly high abilities for clone formation *in vitro* and tumor initiation *in vivo* when isolated from melanoma xenografts.[28] Therefore, ALDH-positive and ALDH-negative cancer cells may have similarly metastatic capacity in NSCLC patients without neoadjuvant therapy. However, drug sensitivity may be different and ALDH1-negative cancer cells may disappear by treatment, and only ALDH1-positive cancer cell remained. The elucidation of the molecular mechanisms that are involved is needed for further *in vitro* or *in vivo* studies.

Previous studies have reported that podoplanin positive CAFs and CD204-positive TAMs were correlated with a poor prognosis in patients with lung adenocarcinoma and squamous cell carcinoma.[11, 16, 18, 29] However, in the current study, CAFs and TAMs did not have any prognostic impact. These results suggested the possibility that tumor-promoting stromal cells do not function in the cancer microenvironment during CRT.

We previously reported that the area residual tumor (ART) is a novel histopathological evaluation method for predicting the outcome of patients with NSCLC who are treated with neoadjuvant therapy.[15] ART was defined as an estimator of the residual quantity of tumor. In this study, the results of a multivariate analysis showed that a high ALDH1 expression level in cancer cells and the ART were independent prognostic factors. According to subgroup analyses combining the ALDH1 expression level in cancer cells and the ART, the 5-year DFS of each

group was 62.3%, 40.0%, and 8.8%, respectively. ALDH1-positivity and an ART>400 mm² (5-year DFS, 8.8%) was associated with a significantly shorter DFS time than ALDH1-negativity and an ART≤400 mm²(5-year DFS, 62.3%) (p=0.005) (Supplemental Figure 1). Therefore, to examine the biomarkers in patients who have received CRT, it is very important to perform both quantitative and qualitative analyses of the residual tumor cells.

In conclusion, the presence of ALDH1-positive cancer cells was an independent recurrence predictor in patients who received neoadjuvant therapy, while CAFs and TAMs did not provide any predictors. Although prospective studies with a larger number of patients are required to confirm the prognostic significance of ALDH1 expression in cancer cells in validation populations with neoadjuvant therapy, our results suggest that the immunophenotypes of ALDH1 expression can serve as a guide to additional treatment after surgical resection in patients who received neoadjuvant therapy.

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version

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Figure legends

Figure. 1. Histologic features of non-small cell lung cancer treated with neoadjuvant therapy followed by surgical resection. (A): Squamous cell carcinoma: the cancer cells did not show any obvious histologic changes after chemoradiotherapy. (B): Degenerated cancer cells showing a bizarre nucleus. (C): Form cell infiltration around necrotic foci. (D): Stromal hyalinosis.

Figure. 2. ALDH1 staining in cancer cells. (A): High ALDH1 expression in adenocarcinoma. (B): Negative ALDH1 expression in adenocarcinoma. (C): High ALDH1 expression in squamous cell carcinoma. (D): Negative ALDH1 expression in squamous cell carcinoma.

Figure. 3. Kaplan-Meier disease-free survival curve for patients with and those without neoadjuvant therapy according to the ALDH1 expression level in cancer cells. (A): Kaplan-Meier disease-free survival curve for patients with neoadjuvant therapy. High expression, dotted line; Low expression, solid line. (B): Kaplan-Meier disease-free survival curve for patients without neoadjuvant therapy (case control). High expression, dotted line; Low expression, solid line.

Supporting Information

Supplemental Figure. 1. Kaplan–Meier disease-free survival according to ALDH1 expression and ART. Group (A): ALDH1 negative expression in cancer cells/ART≦400 mm²; Group (B): ALDH1 positive expression in cancer cells/ART≦400 mm² or ALDH1 negative expression in cancer cells/ART>400 mm²; Group (C): ALDH1 positive expression in cancer cells/ ART>400 mm².

Supplemental Table 1. Clinicopathological characteristics of the patients received neoadjuvant therapy (n=66)

Supplemental Table 2. Clinicopathological characteristics of the patients without neoadjuvant therapy (n=104) (case control)

Figure. 1.

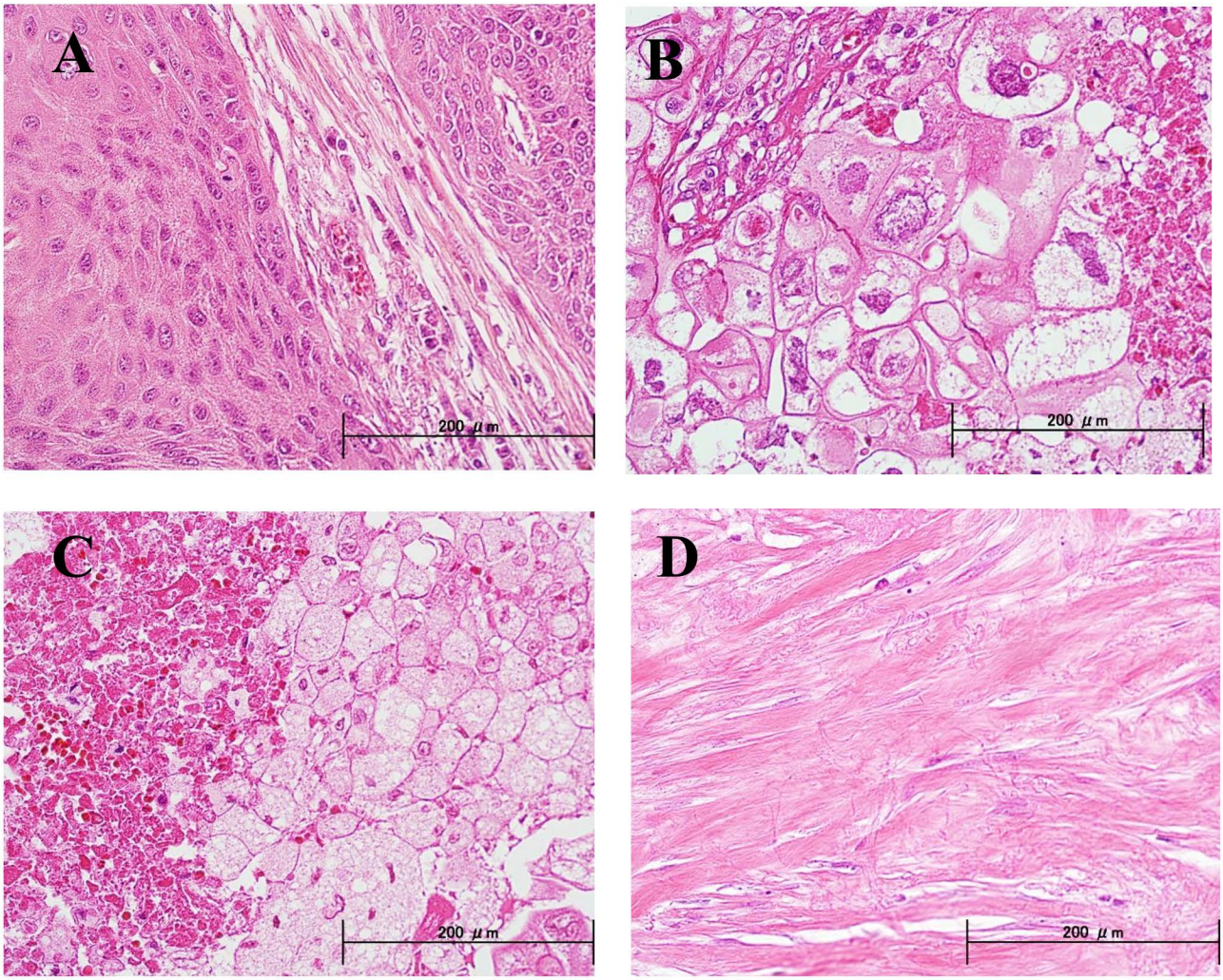

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Figure. 2.

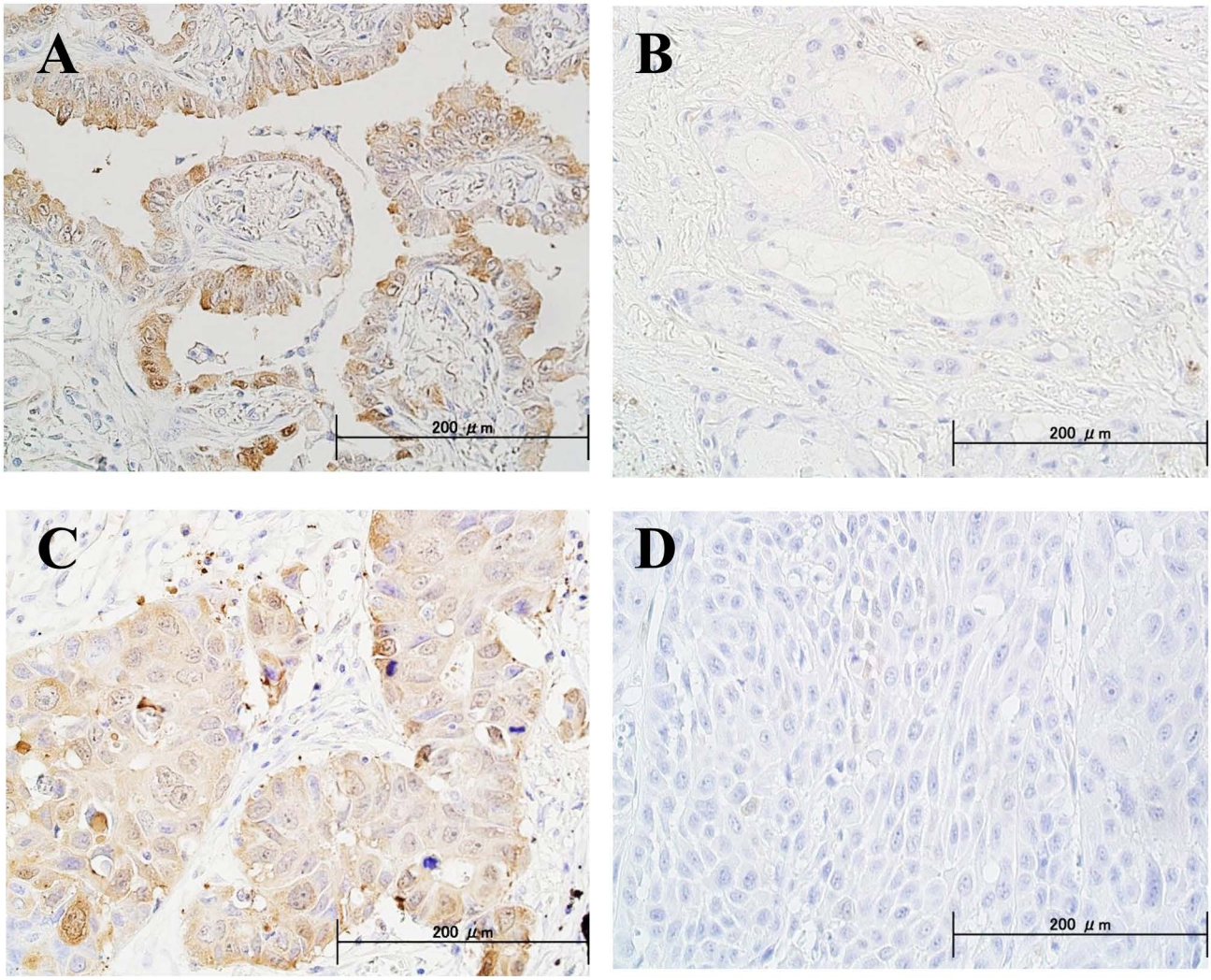

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Figure. 3A.

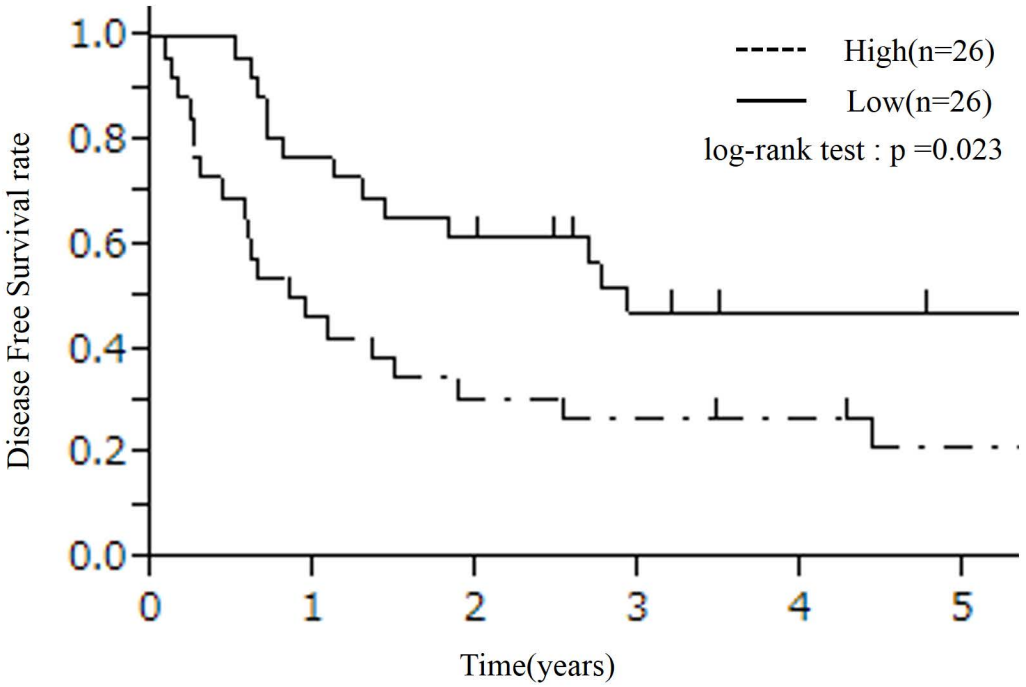


Figure. 3B.

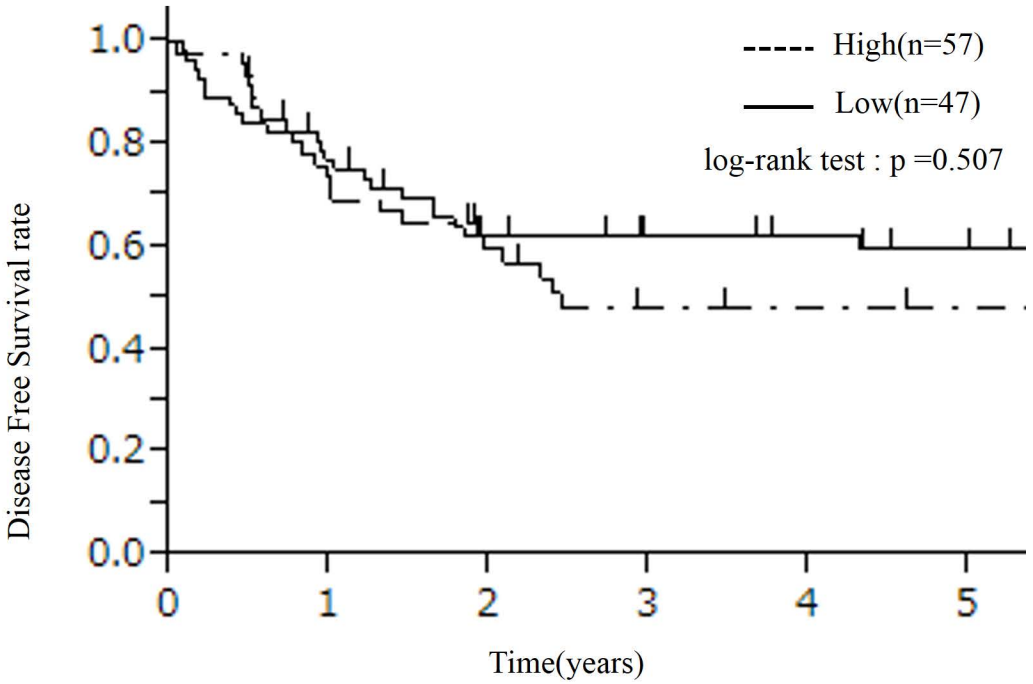


Table 1. Clinicopathological characteristics of the patients received neoadjuvant therapy (n=52)

Characteristic	Number of patients
Gender	
Male/Female	44/8
Age (yr)	
Median(range)	64(32-76)
Smoking history	
Non-smoker	34
Smoker	18
Histology	
Adenocarcinoma	26
Squamous cell carcinoma	19
Large cell carcinoma	2
Others	5
Clinical stage	
I/II/III/IV	5/24/20/3
c-T:T1/T2/T3/T4	0/17/28/7
c-N:N0/N1/N2/N3	32/6/11/3
Pathological stage	
I/II/III/IV	9/23/20/0
yp-T:T1/T2/T3/T4	5/17/29/1
yp-N:N0/N1/N2/N3	29/11/12/0
Neoadjuvant therapy	
Chemotherapy	40
Chemotherapy+radiotherapy	12
Clinical response	
Complete response	1
Partial response	26
Stable disease	22
Progression disease	3
Vascular invasion	
v(-)/v(+)	21/31
Lymphatic invasion	
ly(-)/ly(+)	36/16
Pleural invasion	
pl(-)/pl(+)	21/31

Table 2. Univariate analysis of clinicopathological factor for disease-free survival

Prognostic Factor	Hazard Ratio	95%CI	P-value
Age			
≦65 vs >65	2.50	1.26-5.01	0.008‡
Gender			
male vs female	1.73	0.69-3.79	0.22
Smoking history			
Non-smoker vs Smoker	1.60	0.80-3.12	0.17
Therapy			
chemotherapy vs chemo+radiotherapy	1.08	0.49-2.70	0.85
Clinical response			
CR,PR vs SD,PD	1.23	0.41-1.60	0.54
T status			
pT1-2 vs pT3-4	1.18	0.60-2.40	0.62
Nodal Status			
pN0 vs pN1-2	1.79	0.87-3.43	0.11
Histology			
adenocarcinoma vs Others	1.32	0.67-2.67	0.41
Vascular invasion			
v(-) vs v(+)	1.71	0.85-3.65	0.13
Lymphatic pemeation			
ly(-) vs ly(+)	1.05	0.52-2.25	0.87
Pleural invasion			
pl(-) vs pl(+)	1.11	0.56-2.25	0.76
ART (mm^2)			
≦400 vs ＞400	2.14	1.05-4.72	0.03‡

ART: Area of the residual tumor
Log-rank test was used in comparison between the two groups.（‡＜0.05）

Table 3a. Univariate analysis of immunohistochemical staining of cancer cells

Antibodies	High (No. of Patients)	Low	5-year Disease-free survival rate (%)	P-value
Proliferation and apoptosis				
geminin	28	24	High: 30.3 Low: 39.7	0.51
cleaved caspase 3	26	26	High: 30.2 Low: 38.7	0.37
EMT related molecules				
E-cadherin	26	26	High: 38.0 Low: 30.5	0.98
vimentin	13	39	High: 27.6 Low: 36.8	0.79
Stem cells related molecules				
ALDH1	26	26	High: 21.5 Low: 47.3	0.02‡
CD44v6	26	26	High:29.9 Low:38.4	0.71

Table 3b. Univariate analysis of immunohistochemical staining of stromal cells

Antibodies	High (No. of Patients)	Low	5-year Disease-free survival rate (%)	P-value
Cancer-associated fibroblasts				
podoplanin	29	23	High: 29.1 Low: 37.9	0.90
CD90	8	44	High: 37.5 Low: 33.8	0.75
Tumor-associated macrophages				
CD204	26	26	High: 29.0 Low: 38.4	0.98

Log-rank test was used in comparison between the two groups.（‡＜0.05）

Table 4. Multivariate analysis of pathological prognostic factors who received neoadjuvant therapy

Variable	unfavorable	Hazard Ratio	95%CI	P-value
Geminin	High	0.81	0.35-1.79	0.60
Cleaved caspase 3	High	0.62	0.23-1.70	0.35
E-cadherin	Low	1.20	0.45-3.15	0.70
Vimentin	High	2.11	0.71-6.12	0.17
ALDH1	High	2.26	1.01-5.32	0.04‡
CD44v6	High	1.24	0.52-2.97	0.61
Podoplanin	High	0.90	0.26-3.00	0.86
CD90	Low	1.27	0.40-3.64	0.65
CD204	High	0.96	0.34-2.72	0.95
v	positive	2.15	0.76-6.19	0.14
ly	positive	1.81	0.69-4.74	0.22
pl	positive	1.55	0.46-5.40	0.47
ART	>400	4.68	1.41-17.3	0.01‡

ART: Area of the residual tumor (mm^2)

Multivariate analysis was conducted using the Cox proportional-hazard model :（‡＜0.05 ）,95%Cl, 95% confidence interval

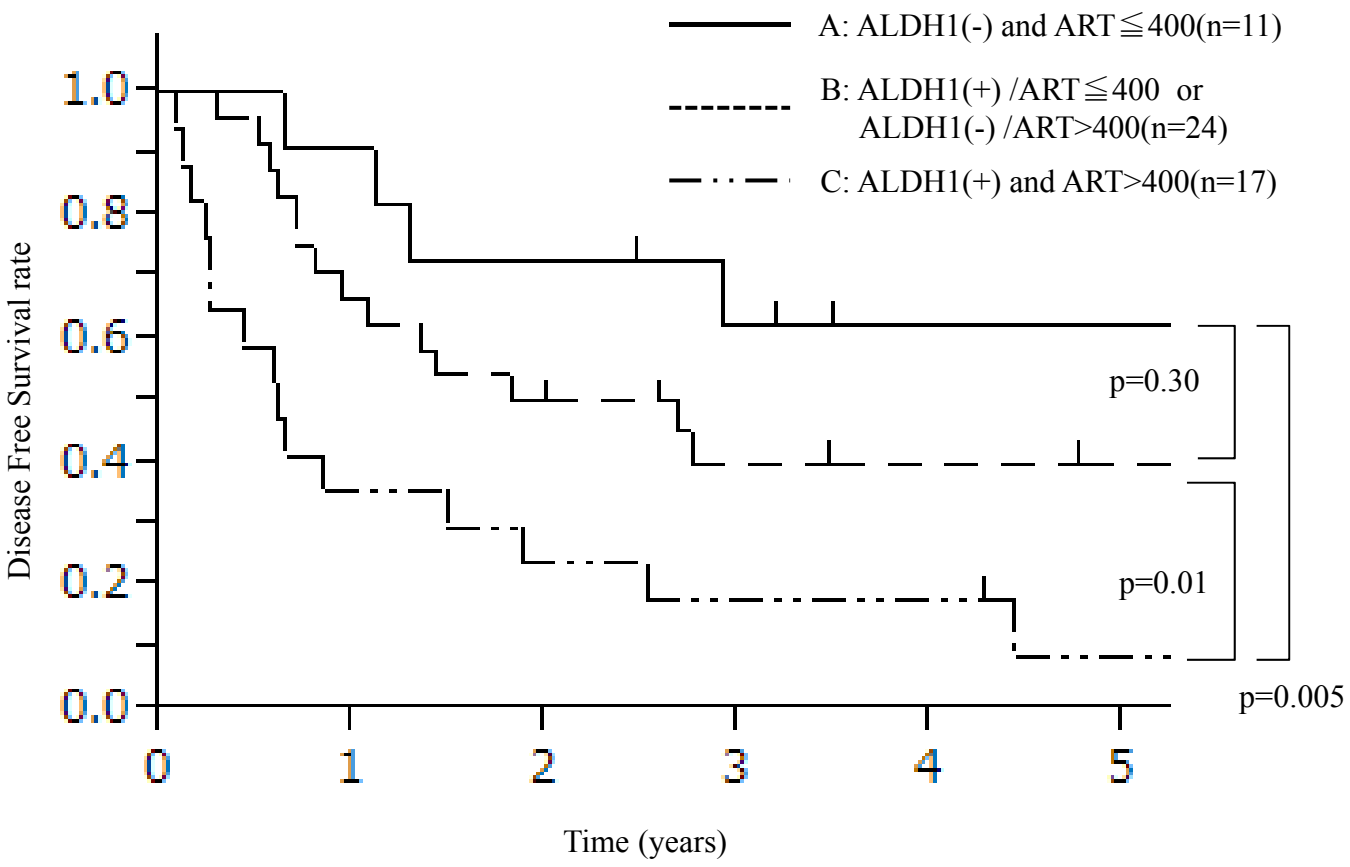
Supplemental Table 1. Clinicopathological Characteristics of the patients received neoadjuvant therapy (n=66)

Characteristic	Number of patients
Gender	
Male/Female	49/17
Age (yr)	
Median(range)	61(32-76)
Histology	
Adenocarcinoma	35
Squamous cell carcinoma	19
Large cell carcinoma	4
Others	8
Clinical stage	
I/II/III/IV	8/31/24/3
c-T:T1/T2/T3/T4	2/20/36/8
c-N:N0/N1/N2/N3	41/8/14/3
Histopathological evaluation	
CR	11
non-CR	55
Neoadjuvant therapy	
Chemotherapy	49
Chemotherapy+radiotherapy	14
Radiotherapy	3

Supplemental Table 2. Clinicopathological characteristics of the patients without neoadjuvant therapy (n=104) (case control)

Characteristic	Number of patients
Gender	
Male/Female	78/26
Age (yr)	
Median(range)	65(38-86)
Smoking history	
Non-smoker	29
Smoker	75
Histology	
Adenocarcinoma	56
Squamous cell carcinoma	38
Large cell carcinoma	4
Others	6
Clinical stage	
I/II/III/IV	21/48/35/0
c-T:T1/T2/T3/T4	18/35/47/4
c-N:N0/N1/N2/N3	61/37/6/0
Vascular invasion	
v(-)/v(+)	42/62
Lymphatic invasion	
ly(-)/ly(+)	72/32
Pleural invasion	
pl(-)/pl(+)	51/53

Supplemental Figure. 1.



Supplemental Figure. 1. Kaplan–Meier disease-free survival according to ALDH1 expression and ART. Group (A): ALDH1 negative expression in cancer cells/ART≦400 mm²; Group (B): ALDH1 positive expression in cancer cells/ART≦400 mm² or ALDH1 negative expression in cancer cells/ART>400 mm²; Group (C): ALDH1 positive expression in cancer cells/ ART>400 mm².