

Cancer stem cell-related marker expression in lung adenocarcinoma and relevance of histologic subtypes based on IASLC/ATS/ERS classification

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Background: The cancer stem cell (CSC) theory has been proposed to explain tumor heterogeneity and the carcinogenesis of solid tumors. The aim of this study was to clarify the clinical role of CSC-related markers in patients with lung adenocarcinoma and to determine whether each CSC-related marker expression correlates with the histologic subtyping proposed by the International Association for the Study of Lung Cancer (IASLC), the American Thoracic Society (ATS), and the European Respiratory Society (ERS) classifications.

Methods: We reviewed data for all 103 patients in whom complete resection of adenocarcinoma had been performed. Expression of CSC-related markers, ie, aldehyde dehydrogenase 1A1 (ALDH1A1), aldo-keto reductase 1C family member 1 (AK1C1), and 1C family member 3 (AK1C3), was examined using immunostaining on whole-mount tissue slides, and the tumors were reclassified according to the IASLC/ATS/ERS classification.

Results: ALDH1A1 expression was observed in 66.0% of tumors, AK1C1 in 62.7%, and AK1C3 in 86.1%. Immunoreactivities with the frequency of mean expression of ALDH1A1 in papillary predominant adenocarcinoma were significantly higher than those of solid predominant adenocarcinoma ($P<0.05$). Papillary predominant adenocarcinoma had significantly lower expression of AK1C1 when compared with noninvasive or solid predominant adenocarcinomas ($P<0.05$). On multivariate analysis, larger tumor size (hazards ratio 1.899, $P=0.044$), lymph node metastasis (hazards ratio 2.702, $P=0.005$), and low expression of ALDH1A1 (hazards ratio 3.218, $P<0.001$) were shown to be independently associated with an unfavorable prognosis.

Conclusion: Immunohistochemistry of ALDH1A1 expression is strongly associated with prognosis. Expression of each CSC-related marker varies according to subtype, suggesting that a comprehensive histologic subtyping approach in the IASLC/ATS/ERS classification provides new molecular biology insights into the genesis of lung adenocarcinoma according to CSC theory.

Keywords: cancer stem cell marker, adenocarcinoma, ALDH1A1, AK1C1, AK1C3, prognosis

Introduction

Lung cancer is the most lethal of all cancers, and adenocarcinoma (ADC) is the most common histopathologic type of lung cancer worldwide.¹ Major advances in ADC management have resulted from the understanding of molecular biology, development of molecular targeting agents, and identification of biomarkers for targeted treatment. However, there exists a widely divergent clinical, radiologic, molecular and pathologic spectrum in lung ADC. In this context, the International Association for the Study of Lung Cancer (IASLC), the American Thoracic Society (ATS), and the European Respiratory Society (ERS) have proposed a new subclassification of lung ADCs that relies on the predominant structural morphology.² Histologic subtyping according to

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the IASLC/ATS/ERS classification has been reported to have a strong relationship with prognosis in several studies.³⁻⁷

Cancer stem cell (CSC) theory has been proposed to explain tumor heterogeneity and the carcinogenesis of solid tumors, including lung cancer.⁸ CSCs, a very small population of specialized cells, have potential for self-renewal and extensively proliferative characteristics that sustain tumor formation.^{8,9} Various molecules are being investigated as putative markers of CSCs. In this study, we focused on aldehyde dehydrogenase 1A1 (ALDH1A1), aldo-keto reductase 1C family member 1 (AK1C1), and 1C family member 3 (AK1C3), which have been previously identified as labeling CSCs in breast, colon, prostate, and lung cancer.¹⁰⁻¹⁵ In particular, ALDH1 has already been evaluated as an effective prognostic marker in lung cancer.^{12,15,16}

In the current study, we attempted to determine whether expression of each CSC-related marker is correlated with the IASLC/ATS/ERS classification, and whether expression of CSC-related markers has any bearing on overall survival.

Materials and methods

Patients

The subjects recruited for this study consisted of 103 patients with lung ADC who underwent complete surgical resection at Tokyo Medical University Hospital between December 1999 and January 2002. All of these patients underwent complete lobar resection and systematic mediastinal lymph node dissection. We excluded patients who had undergone preoperative chemotherapy or radiotherapy. Diagnoses were made according to the criteria of the current World Health Organization classification for lung cancer and the IASLC/ATS/ERS international multidisciplinary classification of lung ADC.² The 7th edition International Union Against Cancer/American Joint Committee on Cancer TNM classification was applied to all ADCs.¹⁷ Data collection and analyses were approved, and the need to obtain written informed consent from each patient was waived by the Tokyo Medical University institutional review board.

Clinical characteristics were retrieved from the clinical records available. The following clinicopathologic factors were assessed retrospectively in relation to immunohistochemical analysis: age, gender, smoking history, pathologic staging, tumor size, pathologic nodal involvement, grade of differentiation, vascular invasion, and pleural invasion.

Histopathology

After the specimens were fixed with formalin and embedded in paraffin, serial 4 μ m sections were stained with

hematoxylin and eosin and by the Alcian Blue-periodic acid-Schiff method to visualize cytoplasmic mucin and by the Elastica van Gieson method to visualize elastic fibers. All slides were evaluated by three of the authors (YS, MN, JM) together using a multiheaded microscope and discussed until consensus was achieved.

All tumor areas were evaluated on the slides. If several tumor foci were present, all foci were included in the analysis. Evaluation was done according to the criteria of the IASLC/ATS/ERS classification, recording the percentage of each histologic component in 5% increments: adenocarcinoma in situ (Figure 1A), minimally invasive adenocarcinoma (Figure 1B), lepidic and acinar (Figure 1C), solid (Figure 1D), papillary, micropapillary (Figure 1E), and mucinous predominant (Figure 1F). The predominant pattern was defined as the pattern with the largest area percentage.

Immunohistochemistry

Expression of three CSC-related proteins was tested with the following commercially available antibodies according to the respective manufacturer's protocols: monoclonal rabbit anti-ALDH1A1 antibody (Abcom Japan, Tokyo, Japan), polyclonal anti-AK1C1 antibody (GeneTex, Irvine, CA, USA), and monoclonal anti AK1C3 antibody (Sigma Japan, Tokyo, Japan). Sections were briefly incubated with xylene, rehydrated with graded ethanol solutions, and incubated with methyl alcohol containing 3% hydrogen peroxide to remove endogenous peroxidase activity. After washing thoroughly with phosphate-buffered saline, sections were incubated with adequately diluted primary antibodies and then with Histofine[®] simple stain (Nichirei Bioscience, Tokyo, Japan), and finally visualized with products of the peroxidase and diaminobenzidine reaction.

Antibody binding was microscopically recognizable as brown cytoplasmic staining. We categorized immunoreactivity by the percentage of the immunopositive area. In tumor cells of interest, when more than 5% of the staining extensiveness showed an unequivocally strong reaction with an antibody, the tumor was classified as positive.

Statistical analysis

Overall survival was measured from the date of surgery to the date of death from any cause or the date on which the patient was last known to be alive. Survival curves were plotted according to the Kaplan–Meier method and compared using the log-rank test. Categorical comparisons were performed using the Pearson chi-squared test. Multivariate analysis was performed using the Cox proportional hazards model. A

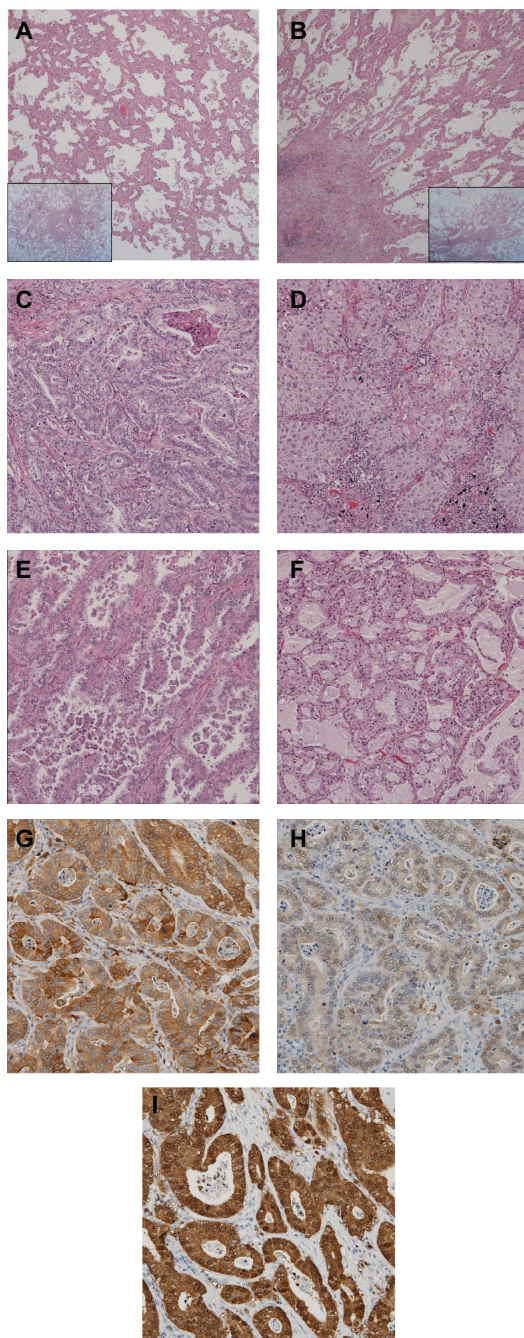


Figure 1 Images of predominant growth patterns and immunohistochemical staining of ALDH1, AK1C1, and AK1C3. (A) Adenocarcinoma in situ, (B) minimally invasive adenocarcinoma, (C) acinar, (D) solid, (E) micropapillary, and (F) mucinous predominant adenocarcinoma. Also shown are immunohistochemical staining images for (G) ALDH1A1, (H) AK1C1, and (I) AK1C3. These slides correspond to the acinar predominant adenocarcinoma shown in Figure 1C.

Abbreviations: ALDH1A1, aldehyde dehydrogenase 1A1; AK1C1, aldo-keto reductase 1C family member 1; AK1C3, aldo-keto reductase 1C family member 3.

one-way analysis of variance, with the Tukey-Kramer multiple comparison post hoc test, was used to allow for unequal sample sizes and determine whether there was a significant difference between the mean immunohistochemical extensiveness according to the predominant histologic pattern of

ADC. A *P*-value of less than 0.05 was considered to indicate a statistically significant difference. The Stat-view 5.0 software package was used to perform the statistical analysis (SAS Institute Inc., Cary, NC, USA).

Results

Predominant growth pattern and ALDH1A1, AK1C1, and AK1C3 expression

All resected specimens were reclassified according to the IASLC/ATS/ERS classification. Representative results are shown in Figure 1. Reclassification of the 103 specimens resulted in three adenocarcinoma in situ (2.9%, Figure 1A), six minimally invasive adenocarcinoma (5.8%, Figure 1B), and 94 invasive ADCs (91.3%). Invasive ADCs were further divided into: lepidic predominant, eight (7.8%); papillary predominant, 39 (37.9%); acinar predominant, ten (9.7%, Figure 1C); solid predominant, 27 (26.2%, Figure 1D); and micropapillary predominant, five (4.9%, Figure 1E). Specific ADC subtypes included four invasive mucinous ADCs (3.9%, Figure 1F) and one enteric ADC (1.0%). Representative examples of immunohistochemical stains for ALDH1A1, AK1C1, and AK1C3 are shown in Figure 1G–I, respectively. This corresponds to the acinar predominant ADC shown in Figure 1C. These CSC-related markers were expressed mainly in the cytoplasm and membrane of the tumor cells. Tumor cells in 68 (66.0%) of the 103 specimens were positive for ALDH1A1, tumor cells in 64 (62.7%) were positive for AK1C1, and tumor cells in 87 (86.1%) were positive for AK1C3.

Correlation between CSC-related marker expression and predominant ADC subtypes

We investigated the correlation between expression of ALDH1A1, AK1C1, and AK1C3 and each predominant subtype. Figure 2 presents the immunohistochemical extensiveness results for adenocarcinoma in situ–minimally invasive ADC (noninvasive ADC), lepidic, acinar, solid, papillary, micropapillary, and mucinous ADC. Immunoreactivities with the percentage of immunopositive areas show that the mean expression of ALDH1A1 in papillary predominant ADCs was significantly higher than that in solid predominant ADCs (Figure 2A, $P < 0.05$). On the other hand, Figure 2B shows that papillary predominant ADCs had significantly lower expression of AK1C1 compared with noninvasive or solid predominant ADCs ($P < 0.05$). No significant

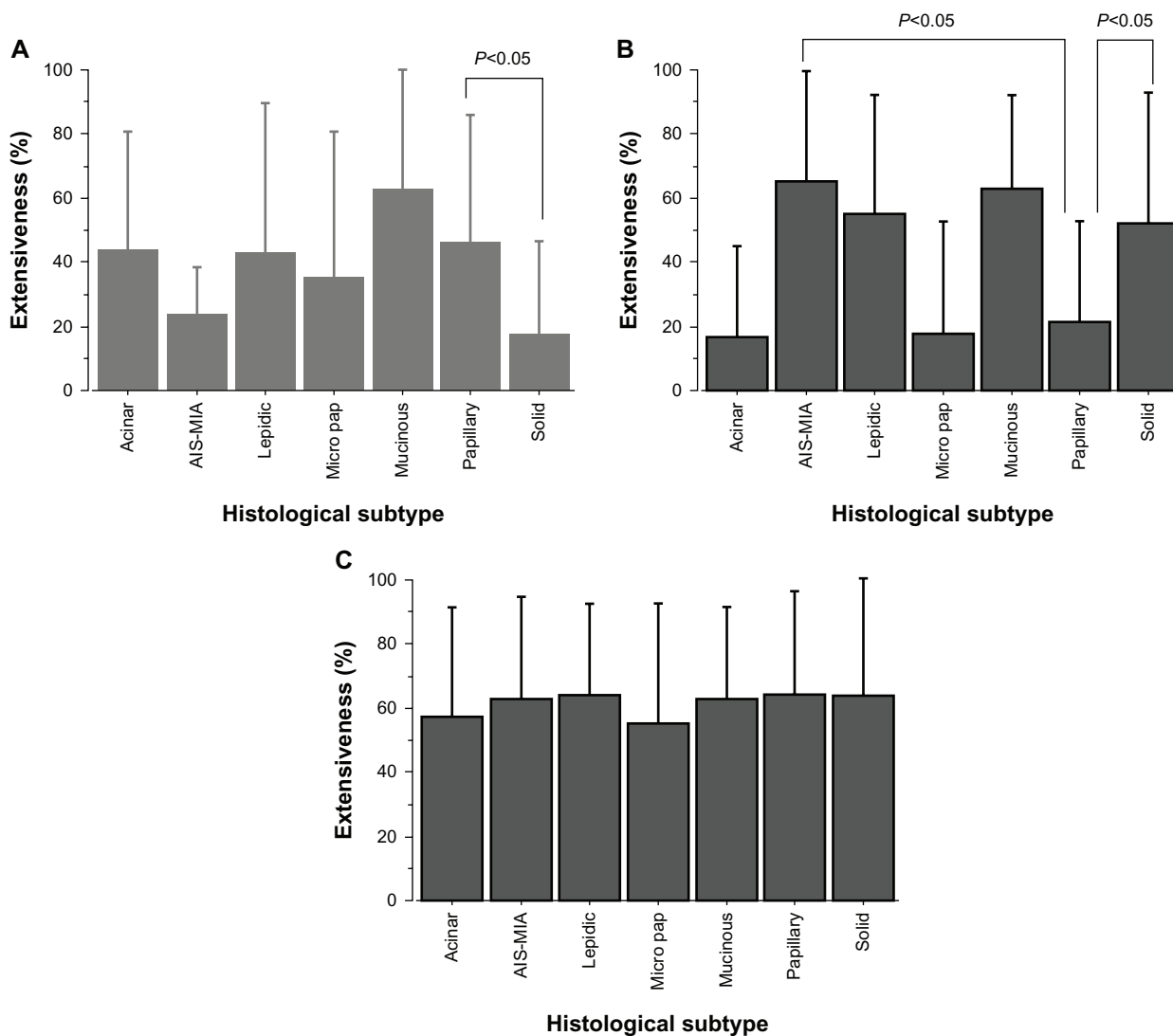


Figure 2 Cancer stem cell-related marker expression in each histologic subtype of lung adenocarcinoma. An overview of the correlation between ALDH1A1, AK1C1, and AK1C3 expression and predominant subtypes is provided. The bars reflect the mean \pm standard error of the mean for extensiveness of the immunopositive area for each of the growth patterns. **(A)** Mean expression of ALDH1A1 in papillary predominant adenocarcinomas is significantly higher than that in solid predominant adenocarcinomas ($P < 0.05$). **(B)** Papillary predominant adenocarcinomas had significantly lower expression of AK1C1 compared with noninvasive or solid predominant adenocarcinomas ($P < 0.05$). **(C)** No significant difference in AK1C3 expression was found for any predominant subtype.

Abbreviations: AIS, adenocarcinoma in situ; MIA, minimally invasive adenocarcinoma; ALDH1A1, aldehyde dehydrogenase 1A1; AK1C1, aldo-keto reductase 1C family member 1; AK1C3, aldo-keto reductase 1C family member 3.

difference in AK1C3 expression was found between any of the predominant subtypes (Figure 2C).

Patient characteristics and survival analyses

The median follow-up for survivors was 8.9 years. Table 1 shows the 5-year overall survival proportions according to clinicopathologic characteristics in 103 patients with lung ADC. On univariate analysis, pathologic stage, tumor size, lymph node involvement, histologic vascular invasion, pleural invasion, and ALDH1A1 immunoreactivity status were found to be significantly associated with survival outcome. The 5-year overall survival proportions

of patients with ALDH1A1-positive status and ALDH1A1-negative status were 77.9% and 62.2%, respectively. Patients with an ALDH1A1-positive status had longer overall survival than those with an ALDH1A1-negative status ($P = 0.002$, Figure 3), whereas staining with AK1C1 and AK1C3 had no prognostic significance ($P = 0.249$ and $P = 0.113$, respectively). A multivariate Cox proportional hazards model demonstrated that larger tumor size (hazards ratio 1.899, $P = 0.044$), lymph node metastasis (hazards ratio 2.702, $P = 0.005$), and low expression of ALDH1A1 immunoreactivity (hazards ratio 3.218, $P < 0.001$) were independently associated with unfavorable overall survival (Table 2).

Table I Patient characteristics and univariate analysis of overall survival

Variable	Cases, n (%)	5-year OS rate	P-value
Overall	103	72.6%	
Age (years, median 65)			
<65	50 (49)	73.6%	0.416
≥65	53 (51)	71.7%	
Gender			
Male	57 (55)	68.0%	0.226
Female	46 (45)	78.3%	
Smoking status			
Smoker	59 (57)	67.8%	0.458
Never smoker	44 (43)	79.2%	
p Stage			
I	78 (76)	83.2%	(Stage I versus II–IV) <0.001
II	12 (12)	66.7%	
III	11 (11)	27.3%	
IV	2 (2)	50.0%	
Tumor size			
≤3.0 cm	61 (59)	80.0%	0.006
>3.0 cm	42 (41)	61.9%	
Lymph node metastasis			
Absent	82 (80)	81.5%	<0.001
Present	21 (20)	38.1%	
Differentiation			
Well or moderate	72 (70)	76.2%	0.304
Poor	31 (30)	61.3%	
IASLC/ATS/ERS classification			
AIS	3 (3)	100%	
MIA	6 (6)	100%	
Lepidic	8 (8)	87.5%	
Papillary	39 (38)	64.1%	
Acinar	10 (10)	100%	
Micropapillary	5 (5)	40.0%	
Solid	27 (26)	66.7%	
Mucinous	4 (4)	100%	
Other (enteric)	1 (1)	100%	
Vascular invasion			
Absent	43 (42)	81.1%	0.001
Present	56 (58)	64.3%	
Pleural invasion			
Absent	82 (80)	79.3%	0.005
Present	21 (20)	45.7%	
Adjuvant chemotherapy			
With	40 (39)	75.0%	0.337
Without	63 (61)	71.1%	
ALDH1A1 expression			
<5% positive cells	35 (34)	62.2%	0.002
≥5% positive cells	68 (66)	77.9%	
AK1C1 expression			
<5% positive cells	38 (37)	71.1%	0.249
≥5% positive cells	64 (63)	74.7%	
AK1C3 expression			
<5% positive cells	14 (14)	61.9%	0.113
≥5% positive cells	87 (86)	74.7%	

Abbreviations: AIS, adenocarcinoma in situ; MIA, minimally invasive adenocarcinoma; OS, overall survival; IASLC, International Association for the Study of Lung Cancer; ATS, American Thoracic Society; ERS, European Respiratory Society; ALDH1A1, aldehyde dehydrogenase IA1; AK1C1, aldo-keto reductase IC family member 1; AK1C3, aldo-keto reductase IC family member 3.

Correlation between clinicopathologic features and ALDH1A1 expression

ALDH1A1 expression status was the most powerful prognostic indicator in this cohort. Therefore, we examined correlations between ALDH1A1-positive cases and clinicopathologic features (Table 3). The ALDH1A1-positive cases were significantly associated with less poorly differentiated ADC ($P=0.013$). No other clinicopathologic factors were correlated with these cases.

Discussion

We set out to determine whether ALDH1A1 immunoreactivity status was the most powerful independent prognostic factor for overall survival in lung ADC, and expression of each CSC-related marker varied in histologic subtype according to the IASLC/ATS/ERS classification. To the best of our knowledge, the association between CSC-related marker expression and the IASLC/ATS/ERS classification has not been previously investigated in patients for all stages of lung ADC.

The three markers examined in this study have been previously reported as candidate CSC-related markers in different types of tumors. ALDH1 is a detoxifying enzyme responsible for oxidation of intracellular aldehydes. The ALDH isoform, ALDH1A1, has been shown to play a role in drug resistance, and its activity has been used to identify stem-like subsets in human hematopoietic cancers and other solid tumors.^{18–25} Previously published studies reported that overexpression of ALDH1A1 correlated with poor prognosis in lung cancer.^{12,15} Theoretically, a high proportion of CSCs in the tumor should be associated with an unfavorable prognosis. However, our results show that increased expression of ALDH1A1 correlated with more favorable overall survival, so are very much in conflict with the above mentioned literature. One of the reasons for this discrepancy may lie in the methodologic differences or different cutoff values used for distinguishing between positive and negative CSC-related marker expression. Our method was based on evaluating whole-mount tissue slides and 66% of tumor specimens were ALDH1A1-positive, whereas the results of the previous studies based on tissue microarrays showed only 29%–45% of tumor samples to be positive with a mixture of ADCs and other histology.^{12,15} Although the current study population was too small to draw any statistically definite conclusions, the methodology of using tissue microarray may prevent detailed observation and increase the rate of false-negative results. Kahlert et al reported that low immunohistochemical expression of ALDH1 in pancreatic cancer was associated with

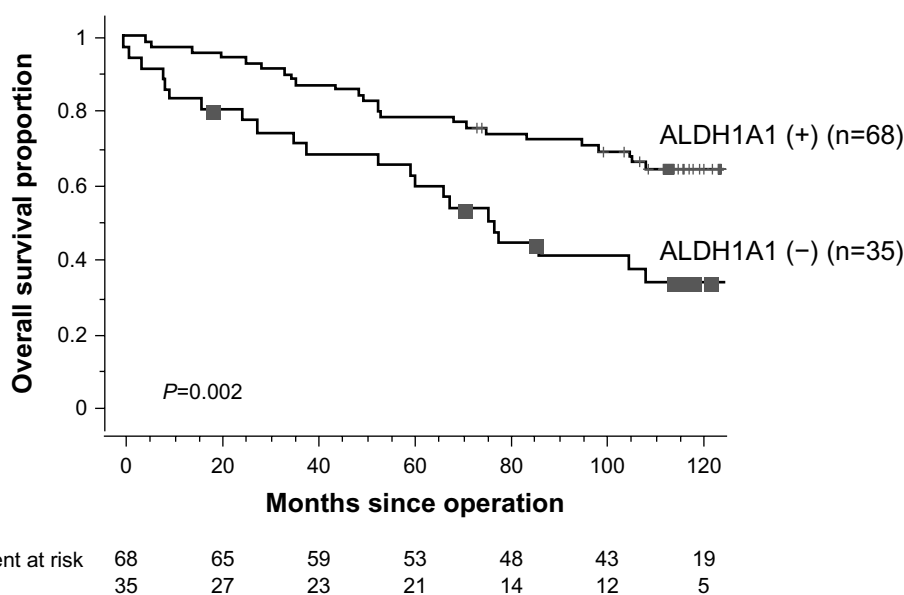


Figure 3 Kaplan–Meier overall survival curves by ALDH1 expression. Five-year overall survival for ALDH1A1-positive and ALDH1A1-negative patients was 77.9% and 62.2%, respectively ($P=0.002$).

Abbreviation: ALDH1A1, aldehyde dehydrogenase 1A1.

poor survival,²⁶ and Chang et al demonstrated that ALDH1 expression correlated with a favorable prognosis in ovarian cancer,²⁷ being in line with our study results. It seems possible that one CSC marker from one organ or specific histology is not necessarily useful for identifying CSCs from other organs or histology. In lung ADC, ALDH1 may not be purely a CSC marker but the prognostic marker playing some pivotal role in biologic tumor behavior.

ALDH1A1-positive ADCs demonstrated a significant correlation with less poorly differentiated ADCs, and the frequency of ALDH1A1-positive tumors in papillary predominant ADCs was higher than in solid predominant ADCs.

Table 2 Multivariate analysis of overall survival

Variable	Hazard ratio	95% CI	P-value
Tumor size			
≤3.0 cm	1	1.017–3.546	0.044
>3.0 cm	1.899		
Lymph node metastasis			
Absent	1	1.354–5.392	0.005
Present	2.702		
Vascular invasion			
Absent	1	0.897–3.868	0.095
Present	1.863		
Pleural invasion			
Absent	1	0.873–3.831	0.110
Present	1.829		
ALDH1A1 expression			
<5% positive cells	3.218	1.674–6.188	<0.001
≥5% positive cells	1		

Abbreviations: ALDH1A1, aldehyde dehydrogenase 1A1; CI, confidence interval.

These results suggest that ALDH1A1 may play some role in essential morphogenic functions in lung ADC, and ALDH1A1-positive tumors may indicate lower biological aggressiveness.

Aldo-keto reductase (AKR) enzymes comprise a functionally diverse gene family.²⁸ In humans, four AKR1C isoforms have been identified. Of these, AK1C1 and AK1C3 are known to be cytosolic oxidoreductases that are involved in reduction of progesterone to the inactive form, 20- α hydroxyprogesterone, and the metabolism of steroids and prostaglandins with multispecificity, respectively.^{29–31} Although neither CSC-related immunoreactivity marker had prognostic significance, predominantly solid ADCs had significantly higher expression of AK1C1 when compared with acinar or papillary predominant ADCs.

The prognostic value of the IASLC/ATS/ERS classification of lung ADC has been validated in several retrospective studies.^{3,5–7} Yoshizawa et al recently reported a significant correlation between *EGFR* mutations and adenocarcinoma in situ/minimally invasive adenocarcinoma/lepidic/papillary subtypes, and *KRAS* mutation and mucinous subtypes.⁶ Kadota et al demonstrated that immunoreactions of thyroid transcription factor-1 differ depending on the predominant structural subtype.³² In the current study, the histologic subtype appeared to be correlated with specific CSC-related marker expression. The novel classification of lung ADC has important implications, not just for predicting patient prognosis, but also for genetic alterations and molecular biology, and

Table 3 Correlation between ALDH1A1 expression and clinicopathologic characteristics

Variable	ALDH1A1 expression		P-value
	<5% positive cells	≥5% positive cells	
Overall	35	68	
Age (years, median 65)			
<65	18	32	
≥65	17	36	0.674
Gender			
Male	18	39	
Female	17	29	0.567
Smoking status			
Ever smoker	20	39	
Never smoker	15	29	0.984
p Stage			
I	24	54	
II	3	9	(I versus II–IV)
III	7	4	
IV	1	1	0.224
Tumor size			
≤3.0 cm	21	40	
>3.0 cm	14	28	0.908
Lymph node metastasis			
Absent	25	57	
Present	10	11	0.139
Differentiation			
Well or moderate	19	53	
Poor	16	15	0.013
IASLC/ATS/ERS classification			
AIS	1	2	
MIA	0	6	
Lepidic	4	4	
Papillary	10	29	
Acinar	2	8	(AIS-MIA versus invasive ADC)
Micropapillary	2	3	0.129
Solid	15	12	
Vascular invasion			
Absent	12	31	
Present	23	33	0.174
Pleural invasion			
Absent	28	54	
Present	7	14	0.944
Adjuvant chemotherapy			
With	15	25	
Without	20	43	0.548

Abbreviations: ADC, adenocarcinoma; AIS, adenocarcinoma in situ; MIA, minimally invasive adenocarcinoma; IASLC, International Association for the Study of Lung Cancer; ATS, American Thoracic Society; ERS, European Respiratory Society; ALDH1A1, aldehyde dehydrogenase IA1.

plays a pivotal role as a common language between oncologists/pulmonologists, pathologists, radiologists, molecular biologists, and thoracic surgeons.

In conclusion, although this study is limited because the number of patients was relatively small, the expression of ALDH1A1 is an independent predictor of overall survival.

Moreover, the frequency of ALDH1A1-positive ADCs that were papillary predominant was higher than for solid predominant, and AK1C1 expression was found to be significantly lower in papillary predominant ADCs than in noninvasive or solid predominant ADCs, suggesting that the comprehensive histologic subtyping approach in the IASLC/ATS/ERS classification provides new molecular biology insights regarding CSC theory.

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Disclosure

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

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