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ALDH7A1 expression is associated with recurrence in patients with surgically resected non-small-cell lung carcinoma

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Abstract

Aim—The purpose of this study was to describe the prognostic significance of ALDH7A1 in surgically treated non-small-cell lung carcinoma. (NSCLC).

Materials & methods—We immunohistochemically analyzed ALDH7A1 expression in surgically resected NSCLC from 89 patients using a tissue microarray.

Results—ALDH7A1 staining was positive in 43 patients and negative in 44 patients, with two tumor sections missing. For stage I NSCLC patients, ALDH7A1 positivity was associated with decreased recurrence-free and overall survival. Multivariate analysis demonstrated that ALDH7A1-expressing NSCLC tumors had a significantly higher incidence of lung cancer recurrence compared with patients with ALDH7A1-negative tumors, although there was no association with overall survival.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Conclusion—For patients with NSCLC, low ALDH7A1 expression was associated with a decreased incidence of cancer recurrence. Specifically in stage I patients, negative staining for ALDH7A1 was associated with improved recurrence-free and overall survival, suggesting a predictive role in surgically treated patients.

Keywords

ALDH family; ALDH7A1; biomarker; non-small-cell lung carcinoma; recurrence

Lung cancer is the leading cause of cancer mortality in the USA [1], with the vast majority (~80%) comprising non-small-cell lung carcinoma (NSCLC). Surgical resection is often the initial treatment strategy in stage I, stage II and selected stage III disease [2]. In addition to surgical resection, adjuvant therapy plays an important role in reducing recurrence as well as improving overall survival (OS).

One of the overarching challenges in this disease setting has been the characterization of novel biomarkers to stratify patients and tailor therapy. Specifically, markers of cancer stem cells (CSCs) – cells with inherent tumorigenic and self-propagating potential – have been an area of great interest. This had led to the identification of several proteins, including CD133 and ALDH1A1 [3].

The aldehyde dehydrogenase (ALDH) superfamily of enzymes catalyze the oxidation and detoxification of a wide variety of endobiotic and xenobiotic aldehyde compounds into their corresponding weak carboxylic acids [4]. Aldehyde compounds, such as those induced during chemotherapy, are highly reactive and capable of inducing a variety of cellular damage, including the formation of potentially mutagenic DNA adducts. Thus, overexpression of ALDH enzymes contributes to therapeutic resistance through their metabolic activity.

Several ALDH isoforms have already been identified as biomarkers in a variety of cancers, including lung cancer. Notably, ALDH1A1 expression is associated with decreased cancer-specific survival in NSCLC [5], and decreased overall and disease-free survival in ovarian cancer [6]. These findings have prompted the investigation of other ALDH isoforms.

Recently, van den Hoogan *et al.* reported that ALDH7A1 is highly expressed in prostate cancer cell lines and primary human prostate cancer tissue. In addition, the matched bone metastases of those primary tumors expressing ALDH7A1 also demonstrated elevated ALDH7A1 expression, suggesting that high ALDH activity may predict for tumor initiation and metastasis initiation in prostate cancer [7]. Furthermore, ALDH7A1 was discovered to be upregulated in human papilloma virus-immortalized cervical epithelial cells and nodular melanoma [8,9]. Additionally, upregulation of ALDH7A1 was shown to effect permissive G₁/S cell-cycle progression through interaction with cyclin A [10]. Given its role in cell-cycle regulation and chemotherapy metabolism, the present study details our analysis of the role of ALDH7A1 as a prognostic biomarker for surgically resected NSCLC patients.

Materials & methods

Patients

After institutional review board approval and according to the approved Vanderbilt Institutional Review Board protocol (IRB#: 010178) previously described [11], we searched the Vanderbilt University Hospital (TN, USA) and the Nashville Veterans Administration Hospital (TN, USA) database for patients treated with surgical resection for NSCLC between 1996 and 2002. Patients with incompletely resected lung cancer tumors (R1 or R2) were excluded from the analysis; only complete (R0) resections were used in this study.

A total of 89 patients were identified, and clinical data were obtained from the tumor registry and electronic medical records at Vanderbilt Medical Center and the Nashville Veterans Administration Medical Center. The patient follow-up data used in this study was derived from Vanderbilt's Lung Specialized Programs of Research Excellence database. The data managers followed all of the patients for survival, progression and recurrence.

Tissue microarray

For eligible patients, hematoxylin and eosin pathology slides were reviewed to verify the diagnosis of NSCLC and select blocks for immunohistochemical staining. At least two separate representative areas of the tumor were chosen for tissue microarray construction using a Beecher Instruments Manuel Tissue Arrayer (model MTA-1; Estigen Tissue Science, Tartu, Estonia). The pathologist was blinded to the corresponding clinical outcomes.

Immunohistochemistry

Suitable tissue sections of 3–5 µm were heated for 5–10 min at 58°C, deparaffinized by placement into three xylene baths (10 min each), hydrated through a series of ethanols of decreasing concentration and placed in a buffer bath of Tris-buffered saline. Endogenous peroxide was quenched using Dako Peroxidase Blocking Reagent (catalog no. S2001; Dako, Glostrup, Denmark) and nonspecific staining blocked using Dako Serum-Free Protein Block (catalog no. X0909) according to the manufacturer's protocol. Optimal slide pretreatment and primary antibody dilution were predetermined by running a preliminary series of positive-control slides using a variety of pretreatments with several dilutions in each pretreatment. Optimal staining for ALDH7A1 (catalog no. ab53278; Abcam, Cambridge, UK) was obtained by using a citrate antigen retrieval and a dilution of 1:1000 (antigen retrieval was carried out by placing slides in the citrate buffer – Dako catalog no. S1700 or a 1x solution of Dako catalog no. S1699 – and placing in a preheated vegetable steamer for 25 min, followed by a 15-min cool down at room temperature and appropriate buffer washes). Primary antibody was applied at room temperature for 1 h. All further steps and chromagen development were carried out according to the manufacturer's instructions – Dako EnVision™ + System-HRP (catalog no. K4010). Slides were counterstained using a Gills #1 hematoxylin, rinsed appropriately and dehydrated through a series of ethanols of increasing concentration into xylene. A nonaqueous cover slip material was used (Permount; Thermo Fisher Scientific, MA, USA).

Three separate tumor samples from each case were examined by immunohistochemistry on the tissue microarray sections and scored by a lung pathologist (R Eisenberg). The reviewers were blinded to the original diagnosis and the patient outcomes at the time of review. Staining was assessed in four to five high-powered fields at 200 × magnification. ALDH7A1 immunoreactivity was evaluated semiquantitatively based on the intensity of staining. It was scored as 1+ (weak), 2+ (moderate) or 3+ (intense). The reviewers also determined the percentage of area stained with ALDH7A1. The labeling score was determined by multiplying intensity score by the percentage area stained positive (intensity percentage area with positive ALDH7A1 staining). The mean staining score for the three tissue sections was entered for statistical analyses. Samples with no staining or weak staining were considered negative, and samples with moderate-to-intense staining were considered positive (as defined below).

Statistical analysis

The primary analysis focused on detecting the association between ALDH7A1 staining and OS/recurrence-free survival (RFS). The staining score was categorized into two groups as weak/negative staining (score ≤ 100) and strong/positive staining (score >100). This threshold was determined by visually determining a clear positive stain supported by histograms of the range of scores. Overall survival was calculated from the date of diagnosis to the date of death or last follow-up. RFS was calculated from the date of diagnosis to the date of recurrence or last follow-up; data were censored for patients that were alive (recurrence free) at their last follow-up visit. Kaplan–Meier survival curves were calculated for the subgroups of potential risk factors and were compared using the log-rank test. The multivariable analysis was completed using the Cox proportional hazards model to estimate hazard ratios and confidence intervals of ALDH7A1 staining with adjustments for disease stage and histological type. Demographic frequencies between weak and strong staining groups were compared using the Fisher exact test. All p-values are based on two-tailed tests and differences were considered statistically significant when $p < 0.05$. Analyses were performed with use of the SAS system version 9.2 and R version 2.9.2 (SAS Institute, Inc., NC, USA).

Results

Clinical characteristics

Demographic and clinical characteristics of the 89 patients in this study are detailed in Table 1. A total of 64% of the cohort were male; 89% were Caucasian and 11% were African–American. A total of 63% of patients had stage I disease, 13% had stage II disease and 24% had stage III disease. There was equal representation of the various NSCLC histologies, including 36% of the patients with adenocarcinoma, 39% with squamous cell carcinoma and 25% with other, including bronchioloalveolar carcinoma ($n = 5$), large cell carcinoma ($n = 5$) and poorly differentiated NSCLC ($n = 12$). A total of 13 patients received adjuvant radiation, 14 patients received adjuvant chemotherapy, ten patients received adjuvant chemoradiation and 52 patients received no adjuvant therapy at the discretion of the treating medical/radiation oncologists. There was no difference in failure rate for these groups (31, 29, 20 and 26% recurrence rate, respectively).

ALDH7A1 staining in NSCLC

Tissue microarray slides containing the tumors of 89 patients with surgically resected NSCLC were immunohistochemically stained with a previously published and validated anti-ALDH7A1 antibody [7]. We observed positive ALDH7A1 staining in 43 tumor samples and negative staining in 44 samples; two tumor sections were missing (Table 1). As shown in Table 1, significant associations were seen between positive ALDH7A1 staining and the histologic classification ($p = 0.0291$). Analyses indicated no significant association between ALDH7A1 staining and gender, race or pathologic stage. There were significant associations between positive ALDH7A1 staining and survival ($p = 0.0158$), and between positive ALDH7A1 staining and lung cancer recurrence ($p < 0.001$).

Positive ALDH7A1 staining is associated with decreased RFS in NSCLC

To determine whether ALDH7A1 expression correlates with prognosis in patients with surgically resected NSCLC, we analyzed the associations between ALDH7A1 immunoreactivity and OS and RFS. Table 2 displays the results of our univariate analysis using the log-rank test. As expected, lower pathologic stage correlated with an improvement in OS, and was significantly associated with longer RFS ($p < 0.001$). In addition, histologic type was significantly associated with RFS ($p = 0.02024$; lung adenocarcinoma has a higher association with recurrence), but not with OS ($p = 0.9564$). Patients whose tumors stained positive for ALDH7A1 had a statistically significant reduction in RFS compared with negative ALDH7A1 staining ($p < 0.001$). There was also a trend toward decreased OS in positive ALDH7A1-staining tumors ($p = 0.1216$). Analyses indicated that gender and race had no significant association with either OS or RFS. Kaplan-Meier curves for OS and RFS, comparing positive versus negative ALDH7A1-staining tumors (Figure 1A & B), pathologic stage (Figure 1C & D) and histologic type (Figure 1E & F) are shown.

Given that the majority of our patients had stage I NSCLC, we analyzed ALDH7A1 staining in this cohort of patients. Statistical analysis revealed that the OS for stage I patients with positive ALDH7A1 staining was significantly decreased compared with ALDH7A1-negative stage I patients ($p = 0.025$). Similarly, the analysis showed that ALDH7A1-positive stage I patients had significantly shorter RFS relative to stage I patients with negative ALDH7A1 staining ($p = 0.019$). These results are shown in Figure 1G&H.

To determine the independent predictive value of ALDH7A1 staining in NSCLC patients, a Cox proportional hazards model was used for multivariate analysis, as seen in Table 3. Positive staining of ALDH7A1 was significantly associated with decreased RFS ($p = 0.0025$), but not OS. The hazard ratio for recurrence in ALDH7A1-positive tumors was 6.24 (95% CI: 1.82–16.9). Neither tumor histology or stage showed a significant association with OS. However, both lung adenocarcinoma and 'other' histology had increased recurrence relative to squamous cell carcinoma of the lung ($p = 0.0118$ and $p = 0.0128$, respectively). Additionally, stage II disease was significantly associated with increased recurrence relative to stage I disease ($p < 0.001$). There was no significant association with increased recurrence in stage III versus stage I lung cancer patients ($p = 0.1709$); however, this may be related to the relatively small patient sample size. Smoking status was not significantly associated with either OS or RFS, while performance status was associated with OS but not RFS.

Discussion

In recent years, the field of lung cancer has witnessed an explosion of novel biomarkers that are both prognostic and predictive. ERCC1 and RRM1 levels have been integrated into chemotherapy trials to assess response to therapy. Identification of *EGFR* mutational status has led to the development of targeted therapies, such as erlotinib and cetuximab, while the *EML4-ALK* translocation has led to the use of crizotinib. These new biomarkers have the potential to allow optimal stratification of patients for therapy, both on and off trial. The goal of this study was to determine the role ALDH7A1, a marker of CSCs, as a biomarker in patients with completely resected NSCLC.

CSCs are able to undergo self-renewal, proliferation and differentiation into phenotypically diverse malignant cell populations, thereby initiating and driving carcinogenesis [12,13]. In addition, CSCs have been shown to exhibit chemo- and radio-resistance [14–16]. Therefore, much effort has been directed at targeting CSCs to overcome therapeutic resistance and improve clinical outcomes. Several biomarkers of CSCs have been identified in lung cancer, including CD133 [17,18] and ALDH1 [5,6]. Lung CSCs enriched with CD133 are highly tumorigenic and exhibit resistance to cisplatin therapy [19]. In addition, CD133 expression in NSCLC has been associated with increased recurrence [20]. Similarly, high ALDH activity in epithelial cancers confers a chemoresistant, tumorigenic and metastatic phenotype [6,21,22]. Accordingly, ALDH1A1 expression is associated with decreased OS in stage I NSCLC [5,23]. Similarly, our study demonstrated that positive ALDH7A1 expression was associated with decreased OS in stage I patients and decreased RFS in the entire cohort. Therefore, ALDH7A1 expression has the potential to be a clinically useful biomarker in NSCLC.

ALDH7A1 is a member of the ALDH superfamily. The primary function of these enzymes is the detoxification of endobiotic and xenobiotic aldehyde compounds into their corresponding weak carboxylic acids [4]. Aldehydes are highly reactive and can form adducts that mediate DNA damage and inactivation of enzymes. ALDH7A1 is localized in the cytosol, nucleus and mitochondria, and has been shown to attenuate both oxidative [24] and hyperosmotic stress-induced apoptotic cell death through metabolism of osmolyte precursors [25].

ALDH7A1 has recently been shown to be upregulated in human prostate cancer *in vitro* and in human tissue samples, including matched metastases [7]. High ALDH7A1 expression in these prostate cancer cell lines was associated with enhanced tumor-initiating and metastasis-initiating potential. Subsequent studies by van den Hoogen *et al.* indicated that ALDH7A1 plays an important role in the initiation of bone metastasis formation in prostate cancer [26]. Indeed, knockdown of ALDH7A1 resulted in a decrease in prometastatic factors, including N-cadherin, twist and osteopontin. Knockdown of ALDH7A1 also resulted in a decrease in CSC surface marker expression [26].

ALDH7A1 has also been demonstrated to be upregulated in human papilloma virus-immortalized human cervical cells treated with the nicotine-derived carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, a known carcinogen derived from cigarette

smoke [8]. In this context, it is suggested that ALDH7A1 may play a role in the malignant transformation of human papilloma virus-immortalized cervical cells to cervical carcinoma through metabolism of 4- (methylnitrosamino)-1-(3-pyridyl)-1-butanone. The role of 4- (methylnitrosamino)-1-(3-pyridyl)-1-butanone in the upregulation of ALDH7A1 may be consistent with data that has shown that cigarette smoking increases ALDH family activity in preinvasive lesions leading to invasive squamous cell carcinoma of the lung [21]. The same study also found that increased ALDH activity may occur as a step in the transformation of lung adenocarcinoma. Rose *et al.* showed that ALDH7A1 is overexpressed in nodular melanoma relative to superficial spreading melanoma in a multivariate model [9]. Overexpression of ALDH7A1 in nodular melanoma, the most aggressive form of melanoma, may contribute to the highly carcinogenic nature of this cancer.

Recent evidence suggests that ALDH7A1 may have an important role in cell growth. Chan *et al.* demonstrated that ALDH7A1 is constitutively expressed throughout the cell cycle in HEK293 and WRL68 cells, with notable upregulation of intranuclear ALDH7A1 at the G₁/S phase transition [10]. As seen in Figure 2, staining of ALDH7A1 in our study is ubiquitous in positively stained tumor samples and is not restricted to an intranuclear location. The role for ALDH7A1 in cell-cycle progression is unclear, but may involve its role in the oxidation of various aldehydes. Oxidative stress induces the production of reactive oxygen intermediates that cause the peroxidation of lipids, thereby increasing the level of aldehydes. Aldehydes can be directly toxic through the formation of adducts that damage DNA and inactivate enzymes [27]. Indeed, oxidative stress is known to be greatest during the G₁/S phase transition [28,29]. As such, the upregulation of ALDH7A1 during the G₁/S phase transition may correspond to the increased need for the metabolism of reactive aldehydes during that stage. Chan *et al.* also reported that knockdown of ALDH7A1 results in a decrease in cyclin A, a protein required for G₁/S cell-cycle progression. Therefore, enhanced ALDH7A1 expression in human cancers may be more permissive to cell-cycle progression, thereby aiding in carcinogenesis.

Overexpression of ALDH7A1, as well as other ALDH enzymes, may provide new therapeutic targets in addition to its potential as a biomarker. High ALDH activity is associated with chemoresistant and metastatic phenotypes [6,7,21,22]. CSCs have been shown to exhibit increased quiescence [30,31], resulting in suboptimal responses to currently available therapies that primarily target highly proliferative cells. Novel approaches specifically targeting CSCs may provide a key to the successful treatment of solid tumors, including lung cancer. Tetraethylthiuram disulfide (disulfiram, also known as Antabuse®; Teva Pharmaceutical Industries, Inc., Petach Tikva, Israel) and diethylaminobenzaldehyde, both ALDH inhibitors, have been shown to decrease proliferation and motility in lung cancer cells *in vitro* [22]. Similarly, inhibition of ALDH activity using diethylaminobenzaldehyde has been demonstrated to sensitize cyclophosphamide-resistant sarcoma cells *in vitro* [32].

Given patients with positive ALDH7A1 staining are at higher risk of relapse and more likely to have chemoresistant tumors, development of anti-ALDH7A1 (among other ALDH enzymes) therapies may prove to significantly enhance the therapeutic potential of chemotherapy and radiation through abrogation of therapeutic resistance. Further research is

necessary to adequately assess the potential of ALDH7A1 as a therapeutic target in lung cancer. Additionally, because cancer recurrence relies on the presence of tumorigenic cells, there may be value in exploiting ALDH7A1 expression in lung cancer to monitor treatment response and for detection of potentially occult tumorigenic cell populations post-treatment.

There are some potential limitations in our study. Although we focused on completely resected NSCLC, we did not analyze the impact of postoperative chemotherapy or specific chemotherapy agents in our cohort. Given that the majority of our patient cohort had stage I disease and that surgery monotherapy can be curative in patients with stage I NSCLC, this may explain the difference in OS noted for this subgroup as opposed to the entire cohort. While our findings are hypothesis generating, they need to be confirmed in an independent, blinded, patient cohort. Further work is necessary to validate ALDH7A1 staining in NSCLC and increase the significance of our findings.

Conclusion

In summary, our results suggest that ALDH7A1 is an independent prognostic biomarker for OS and recurrence in patients with surgically resected stage I NSCLC. Furthermore, high ALDH7A1 expression was associated with decreased RFS. This novel finding is the first to demonstrate an association between ALDH7A1 staining and prognosis in lung cancer. Future research, including larger prospective studies, are necessary to better elucidate the value of ALDH7A1 staining in predicting recurrence in NSCLC and to validate the findings of this study. In addition, future studies should determine the role of ALDH7A1 in therapeutic resistance, carcinogenesis and metastasis, as well as the potential of targeting ALDH enzymes in the treatment of lung cancer.

Future perspective

Molecular biomarkers have altered the landscape of lung cancer over the past few years, with both prognostic and predictive biomarkers altering treatment regimens and the standard of care. Within the next 5–10 years, there will be further expansion of this field with a refinement of biomarkers to identify CSC populations both temporally and spatially. Specific targeting of this class of cells will allow for more efficacious therapies with improved side-effect profiles and therapeutic indices, and will result in clinically meaningful improvements in locoregional control, distant control and ultimately OS.

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Executive summary

- ALDH7A1 staining occurs in approximately 50% of non-small-cell lung carcinoma (NSCLC) specimens.
- The adenocarcinoma subtype has a higher incidence of ALDH7A1 positivity than squamous histology.
- Positive ALDH7A1 staining is associated with decreased recurrence-free survival in NSCLC.
- ALDH7A1 expression is associated with decreased recurrence-free survival in patients with completely surgically resected NSCLC.
- For patients with stage I disease, ALDH7A1 staining was associated with both decreased overall survival as well as recurrence-free survival.
- Positive ALDH7A1 staining may indicate the necessity of adjuvant therapy.
- These findings are hypothesis generating and further studies are necessary to validate this work.

Figure 1. Kaplan-Meier survival curves for non-small-cell lung carcinoma patients

Overall and recurrence-free survival are shown for: (A & B) Pos versus Neg ALDH7A1-stained tumors; (C & D) disease stage; (E & F) histology; (G & H) Pos versus Neg ALDH7A1 staining in stage I non-small-cell lung carcinoma, p-values for each variable were calculated using the log-rank test. Adeno: Adenocarcinoma subtype; Neg: Negative; Pos: Positive; Squam: Squamous subtype.

Figure 2. ALDH7A1 staining in non-small-cell lung carcinoma

Three lung cancer samples were obtained from tumor blocks for each of the 89 patients and were stained with an anti-ALDH7A1 antibody. Representative images for 0, 1 +, 2+ and 3+ staining intensities are shown (magnification: 200×).

Table 1

Patient characteristics.

Characteristics	ALDH7A1 staining			Total (n = 89)	p-value [†]
	Negative (n = 44)	Positive (n=43)	Missing (n=2)		
Gender; n (%)					
Female	14(32)	17(40)	1 (50)	32 (36)	0.5065
Male	30(68)	26 (60)	1 (50)	57 (64)	
Race; n (%)					
African-American	5 (11)	5 (12)	0 (0)	10 (11)	1.0000
Caucasian	39 (89)	38 (88)	2 (100)	79 (89)	
Disease stage; n (%)					
Stage	29 (66)	25 (58)	2 (100)	56 (63)	0.7448
Stage II	5 (11)	7 (16)	0 (0)	12 (13)	
Stage III	10 (23)	11 (26)	0 (0)	21 (24)	
Histological type; n (%)					
Adenocarcinoma	11 (25)	20 (47)	1 (50)	32 (36)	0.0291
Squamous	23 (52)	11 (26)	1 (50)	35 (39)	
Other	10 (23)	12 (28)	0 (0)	22 (25)	
Death; n (%)					
Survival/censored	14 (32)	4 (9)	1 (50)	19 (21.35)	0.0158
Death	30 (68)	39 (91)	1 (50)	70 (78.65)	
Recurrence; n (%)					
No	40 (91)	21 (49)	2 (100)	63 (71)	<0.001
Yes	4 (9)	22(51)	0 (0)	26 (29)	

[†] p-value is for comparison of positive and negative ALDH7A1 staining.

Table 2

Univariate analysis of prognostic factors.

Factor	Patients [†] (n)	3-year overall survival		3-year recurrence-free survival	
		% (95% CI)	p-value [‡]	% (95% CI)	p-value [‡]
Gender					
Female	32	42 (25–59)	0.6404	68 (49–87)	0.2787
Male	57	47 (35–59)		72 (59–85)	
Race					
African-American	10	30 (2–58)	0.8489	NA	0.6269
Caucasian	79	47 (36–57)		72 (61–84)	
Disease stage					
Stage	56	55 (43–68)	0.1589	84 (73–95)	<0.001
Stage II	12	17 (0–38)		26 (0–65)	
Stage III	21	30 (12–49)		40 (11–70)	
Histological type					
Adenocarcinoma	32	37 (20–53)	0.9564	53 (31–75)	0.0203
Squamous	35	50 (35–65)		86 (73–99)	
Other	22	40 (20–60)		63 (39–86)	
ALDH7A1 staining					
Negative	44	49 (34–65)	0.1216	87 (74–99)	<0.001
Positive	43	40 (25–54)		49 (32–67)	

[†]Analyses were limited to those patients for whom data were available.

[‡]p-values for each variable were calculated by the log-rank test

NA: Not applicable.

Table 3

Multivariate analysis for prognostic factors.

Factor	Death		Recurrence	
	<i>Hazard ratio (95% CI)</i>	<i>p-value</i> [†]	<i>Hazard ratio (95% CI)</i>	<i>p-value</i> [†]
Positive vs negative ALDH7A1	1.30(0.77–2.19)	0.3337	5.55(1.82–16.9)	0.0025
Adenocarcinoma vs squamous	1.45(0.79–2.66)	0.2278	5.07(1.43–17.9)	0.0118
Other vs squamous	1.07(0.56–2.01)	0.8434	5.11 (1.41–18.4)	0.0128
Stage II vs stage	1.83(0.91–3.66)	0.0895	8.48(2.56–28.2)	<0.001
Stage III vs stage	0.724(0.39–1.36)	0.3159	1.67(0.67–4.16)	0.2737
Smoker vs nonsmoker	3.87(0.5–29.95)	0.514	2.01 (0.25–16.24)	0.514
Karnofsky Performance Status	1.08(1.02–1.15)	0.007	1.05(0.94–1.18)	0.411

[†]p-values were derived from the Cox proportional hazards model with simultaneous inclusion of age, age × age, and all factors shown.