Clinical significance of ALDH1 combined with DAPI expression in patients with esophageal carcinoma

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Abstract. Esophageal carcinoma is the most common type of tumor, with the incidence in China accounting for 50% of cases worldwide and the majority of patients not surviving due to tumor recurrence. According to the cancer stem cell theory, tumor development and recurrence is due to the excitation of a cancer stem cell. Aldehyde dehydrogenase 1 (ALDH1) is an appropriate marker for cancer stem cells and the present study aimed at determining the function of ALDH1 in human esophageal carcinoma. Indirect fluorescence antibody staining was used to investigate the association between the level of ALDH1 protein expression and clinicopathological parameters, including sex, age, vein invasion, degree of tumor cell differentiation and clinical stage. DAPI was used to stain the nuclei of tumor cells and exclude non-tumor cells. The results of the present study revealed that ALDH1 expression was associated with the level of tumor cell differentiation, tumor-node-metastasis stage and lymphatic invasion. In addition, increased expression of ALDH1 was identified in esophageal carcinoma tissues compared with in healthy esophageal tissues. Therefore, ALDH1 may be used as a parameter for the pathology of esophageal carcinoma.

Introduction

Esophageal carcinoma is the most common type of cancer worldwide with ~482,300 novel cases and 406,800 mortalities reported annually (1). In China, the incidence of esophageal carcinoma accounts for 50% of cases worldwide and esophageal carcinoma is the fourth leading cause of malignant tumor-associated mortality, with 95% of esophageal cancer cases diagnosed as squamous cell carcinoma (2). Surgery is the optimal treatment for patients with esophageal carcinoma (3,4); however, the majority of patients do not survive due to tumor recurrence, in spite of radical resection and extended lymph node dissection having been performed. A number of factors affect tumor recurrence including age, sex, local tumor stage, tumor location, degree of cell differentiation, lymph node metastases or vascular involvement (5).

Aldehyde dehydrogenase 1 (ALDH1) is a detoxifying enzyme which responds to the oxidation of intracellular aldehydes (6,7). The function of ALDH1 is to oxidize intracellular aldehydes and therefore confer resistance to alkylating agents (8). As a modulator of cell viability, ALDH1 converts retinol into retinoic acid, which serves an important function in the early differentiation of stem cells (9). Murine, human hematopoietic, neural stem and progenitor cells have been identified to exhibit increased ALDH1 activity, and ALDH1 activity is a commonly used marker for healthy and malignant stem cells (10,11). Previous immunohistochemistry results have demonstrated that ALDH1 expression was limited in healthy tissue, but was markedly increased in malignant tissue, including breast, lung and colorectal cancer (12-14). However, whether the expression of ALDH1 is associated with the differentiation of tumor cells in esophageal carcinoma remains unknown. Therefore, the present study aimed at identifying whether ALDH1 expression exhibited an association with patients with esophageal cancer using fluorescent immunostaining.

In 1994, Grimason *et al* (15) used the fluorogen DAPI to interact with the nuclei of sporulated oocysts in conjunction with a fluorescein isothiocyanate-conjugated anti-cryptosporidium monoclonal antibody and used fluorescence microscopy to visualize the oocyst nuclei, which enabled improved observation. DAPI is a non-cytotoxic dye that does not affect cell viability (16). DAPI is able to be combined with cellular DNA, permeate through the membrane of cell, rapidly enter the nucleus of living cells and bind with DNA to form a DAPI-DNA complex. The wavelengths of the complex for excitation and emission are 360 and 460 nm, respectively.

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Under the excitation of an ultraviolet ray, DAPI exhibits blue fluorescence, therefore under a fluorescence microscope a blue nucleus may be observed. The formula of DAPI is $C_{16}H_{15}N_5$ and the molecular mass is 277,324 Da (17).

In the present study, DAPI was used to non-specifically stain the nuclei of the tumor cells. Subsequently, ALDH1-specific fluorescent staining was used on the cancer stem cell cytoplasm and a merged image was developed. The aim of the present study was to use indirect fluorescent immunostaining to identify whether the expression of ALDH1 may be a notable clinicopathological prognostic factor for human esophageal carcinoma.

Materials and methods

Patients and tissues. Specimens of human esophageal squamous cell carcinomas were obtained from the Department of Pathology, The First Hospital of Qiqihar (Qiqihar, China) between January 2010 and January 2014. Prior to surgery no patients had received any therapy, including chemotherapy or radiation. Of the 50 specimens, 10 cases were well-differentiated, 20 were moderately differentiated and 20 cases were poorly differentiated squamous tumor cells. In addition, healthy esophageal tissues were obtained from the same cohort of patients, but these were obtained from a distant location from the esophageal carcinoma (≥5 cm). All tissues (thickness, 4 μm) were fixed in formalin, embedded in paraffin. When required, the tissues were deparaffinized and dehydrated in 10% formalin at 27˚C for 5 min. The present study was approved by the Ethical Committee of the First Hospital of Qiqihar. Additionally, written informed consent was obtained from all participating patients. All tissues were evaluated by two pathologists individually.

Indirect fluorescent immunostaining. Indirect fluorescent immunostaining was performed as described previously (18). First, DAPI (dilution, 1:500; cat no. D9564; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used to non-specifically stain the nuclei of cancer cells (15). The cells were then incubated at 37˚C with fluorophore I-labeled IgG (dilution, 1:20; catalog no. HZ3387121; excitation, 360 nm; emission, 460 nm; EarthOx Life Sciences, Millbrae, CA, USA) for 30 min. Sections were rinsed with TBS-Tween-20 (TBST) three times and incubated at 4˚C with an antibody against ALDH1 (dilution, 1:400; cat no. HZ3487111; EarthOx Life Sciences) overnight. Subsequently, sections were incubated at 37˚C with fluorophore II-labeled IgG (dilution, 1:50; cat no. HZ3387125; excitation, 490 nm; emission, 520 nm; EarthOx Life Sciences) for 30 min. Sections were rinsed with TBST three times and coverslips were placed on the slides. Finally, fluorescence microscopy (magnification, x200; Nikon Eclipse 80i; Nikon Corporation, Tokyo, Japan) was used to observe the results.

Evaluation of labeling. Evaluation of the expression of ALDH1 was performed by two pathologists independently. Imaging analysis of ALDH1 expression was performed in one selected area per case. Cases exhibiting ≥20% positive cells were classified as significant and the remaining cases were classified as negative ALDH1 expression (19).

Table I. Patient characteristics.

Clinical data	n (%)
Total	50
Male	33 (66)
Female	17 (34)
Age, years	
≥60	27 (54)
<60	23 (46)
Median	52.3 (35-70)
Differentiation	
Well	10 (20)
Moderate	20 (40)
Poor	20 (40)
TNM stage	
I/II	23 (46)
III/IV	27 (54)
Lymph node metastasis	
Positive	28 (56)
Negative	22 (44)
Vein invasion	
Positive	26 (52)
Negative	24 (48)

Table II. Comparison between ALDH1-positive expression in esophageal cancer and healthy esophageal tissues.

Group	Total	ALDH1-positive, %	χ^2	P-value
Esophageal cancer tissue	50	23 (46)		
Healthy esophageal tissue	50	8 (16)	5.259	<0.05

ALDH1, aldehyde dehydrogenase.

Statistical analysis. All data were analyzed using SPSS software (version 12.0; SPSS, Inc., Chicago, IL, USA). The association between the expression of ALDH1 and the clinicopathological parameters was evaluated using the χ^2 test. In addition, the expression of ALDH1 between esophageal squamous cell carcinoma and healthy esophageal tissues were analyzed using the χ^2 test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Patient characteristics. In the present study, a total of 50 patients were included. A total of 33 were male (66%) and 17 were female (34%), a ratio of 1.94:1. The patient median age was 52.3 years (range, 35-70 years), with 27 (54%) and 23 (46%) patients aged ≥60 and <60 years, respectively. All patients were diagnosed with human esophageal squamous

Table III. Association between ALDH1-positive expression and the clinicopathological features of human esophageal carcinoma.

Clinicopathological feature	n	Positive	Negative	Positive rate, %	χ^2	P-value
Sex						>0.05
Male	33	13	20	39.4	1.705	
Female	17	10	7	58.8		
Age, years						>0.05
≥60	27	13	14	48.1	0.109	
<60	23	10	13	43.5		
Histological grade						<0.05
Well	10	2	8	20	11.554	
Moderate	20	6	14	30		
Poor	20	15	5	75		
TNM stage						<0.05
I/II	23	6	7	26.1		
III/IV	27	17	10	63.0	6.798	
Lymphatic invasion						<0.05
Positive	28	18	10	64.3	8.567	
Negative	22	5	17	22.7		
Vein invasion						>0.05
Positive	26	11	15	42.3	0.290	
Negative	24	12	12	50.0		

ALDH1, aldehyde dehydrogenase; TNM, tumor-node-metastasis.

cell carcinoma, and classified as exhibiting well-, moderately or poorly differentiated tumor cells. A total of 10 (20%), 20 (40%) and 20 (40%) of patients exhibited well-, moderately and poorly differentiated tumor cells, respectively. In addition, patients were staged according to the tumor-node-metastasis (TNM) classification (20). A total of 23 (46%) patients were at TNM stages I/II and 27 (54%) were at stages III/IV. Lymphatic and vein invasion was observed in 28 (56%) and 26 (52%) patients, respectively (Table I).

Expression of ALDH1 in healthy esophageal tissues and esophageal carcinoma tissues. Expression of ALDH1 was identified in the cytoplasm of esophageal carcinoma tissues and a limited number of healthy esophageal tissues. Human esophageal carcinoma tissues exhibited markedly increased expression levels of ALDH1 protein, compared with that of healthy esophageal tissues. In addition, compared with healthy esophageal tissues, human esophageal carcinoma tissues exhibited significantly increased expression levels of ALDH1 protein (χ^2=5.259; P<0.05). Of the 50 healthy controls, ALDH1 activity was identified in ~16% of esophageal cells. However, in the 50 esophageal cancer tissues, positive ALDH1 incidence was 46%. The results are presented in Table II.

Association between the expression of ALDH1 and the clinicopathological features of esophageal carcinoma. First, DAPI was used to stain the cancer cell nuclei, which excluded non-tumor cells. The association between ALDH1 protein expression and the clinicopathological features of human esophageal squamous cell carcinoma are summarized in Table III. No significant difference was identified between the expression of ALDH1 and sex, age or vein invasion. However, ALDH1 expression was identified to be associated with the level of differentiation of the tumor cells. It was revealed that, as the level of differentiation of tumor cells decreased, the positive rate of ALDH1 expression increased. Compared with well- and moderately differentiated tumor cells (Figs. 1 and 2), the ALDH1 intensity was markedly increased in poorly differentiated malignant tumor cells (Fig. 3). All images were stained individually and finally merged. A positive association was identified between the differentiation of tumor cells and the positive expression of ALDH1 (χ^2=11.554; P<0.05). ALDH1-positive expression was only observed in 2/10 (20%), 6/20 (30%) and 15/20 (75%) of the well-, moderately and poorly differentiated cases, respectively. In addition, patients of stages III/IV esophageal carcinoma exhibited an increased expression rate of ALDH1 (63.0%), compared with those of stages I/II (26.1%) (χ^2=6.789; P<0.05). Furthermore, in the cases of lymphatic invasion, the positive rate of ALDH1 expression (64.3%) was increased, compared with that of the cases without lymphatic invasion (22.7%; χ^2=8.567; P<0.05).

Discussion

Human esophageal cancer is a life-threatening disease worldwide. Although radical surgery may be performed, the 5-year survival rate rarely exceeds 30%. A number of patients with the early-stage disease exhibit an increased risk of disease recurrence following treatment (21). Esophageal carcinomas are divided into the adenocarcinoma and squamous cell

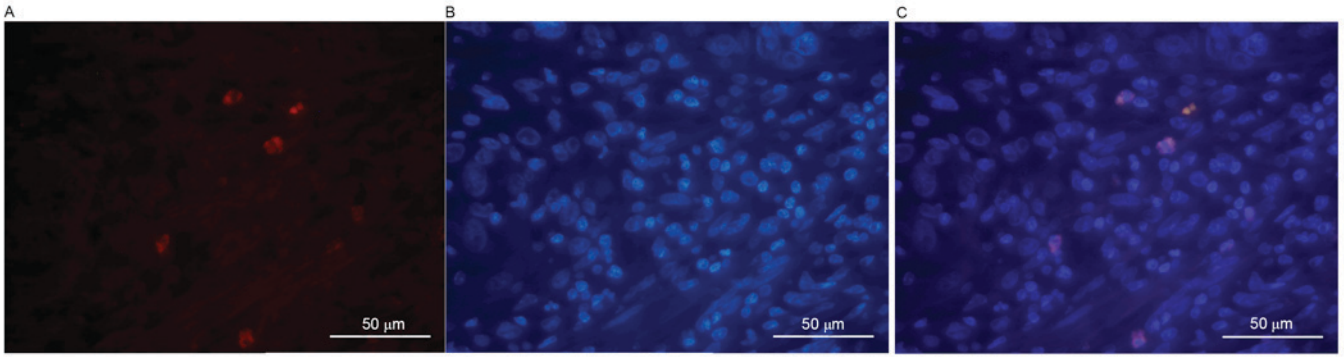

Figure 1. ALDH1 combined with DAPI expression in well-differentiated cells of esophageal carcinoma tissues. (A) ALDH1 expression. (B) DAPI expression. (C) Merged image of A and B (magnification, x200). ALDH1, aldehyde dehydrogenase.

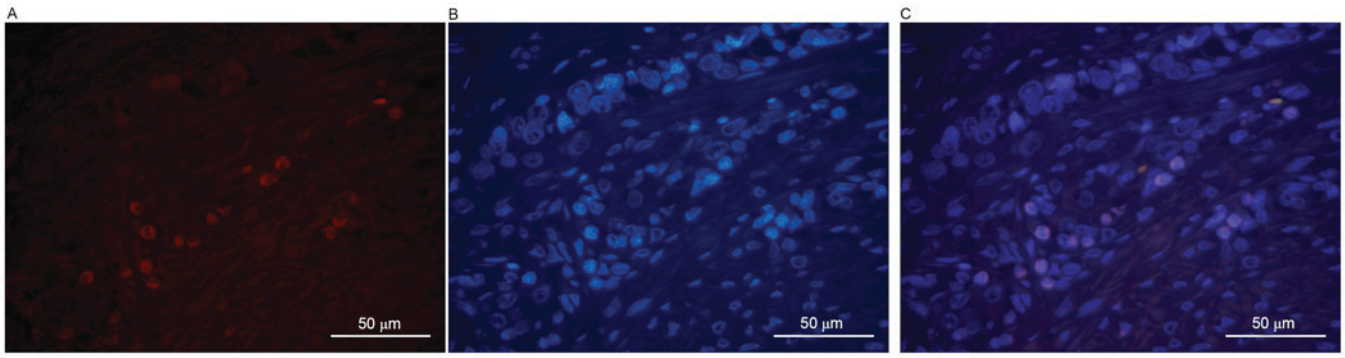

Figure 2. ALDH1 combined with DAPI expression in moderately differentiated cells of esophageal carcinoma tissues. (A) ALDH1 expression. (B) DAPI expression. (C) Merged image of A and B (magnification, x200). ALDH1, aldehyde dehydrogenase.

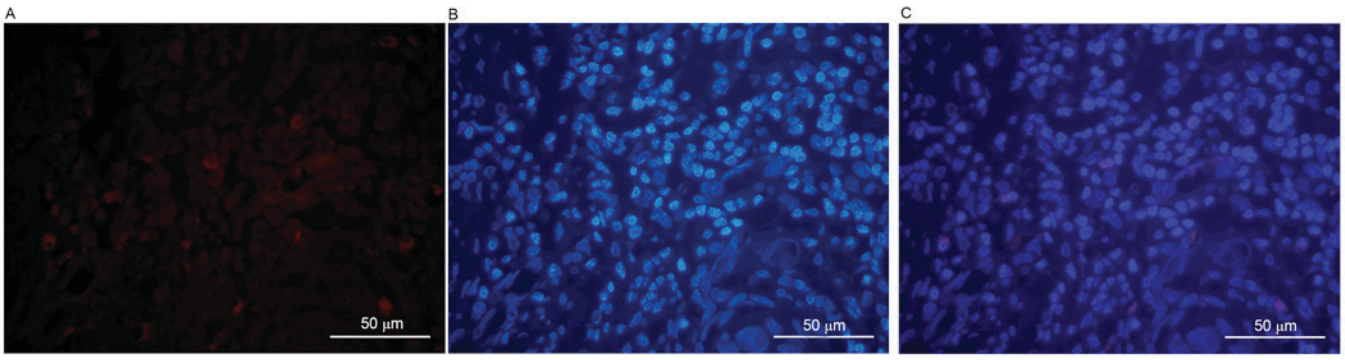

Figure 3. ALDH1 combined with DAPI expression in poorly differentiated cells of esophageal carcinoma tissues. (A) ALDH1 expression. (B) DAPI expression. (C) Merged image of A and B (magnification, x200). ALDH1, aldehyde dehydrogenase.

carcinoma histological subtypes; the latter accounts for the majority of esophageal carcinoma cases. Increased incidence of esophageal cancer is due to obesity, smoking, consumption of hot beverages and red meat, increased alcohol intake and a decreased intake of fresh vegetables or fruit (22). The morbidity of squamous cell carcinoma is increasing in developing countries, particularly in China (23).

According to the cancer stem cell theory, malignant tumors develop from a cancer stem cell, which has the capability of pluripotency and self-renewal (24). ALDH1, a detoxifying enzyme responsible for the oxidation of intracellular aldehydes, is a marker of cancer stem cells (10). The results of the present study demonstrated that, compared with healthy esophageal tissues, esophageal cancer tissues expressed an increased positive rate of ALDH1 (χ^2=5.259; P<0.05). In addition, an association between the differentiation degree of tumor cells and the expression of ALDH1 was identified. Using indirect immunofluorescence staining, it was demonstrated that an increased degree of differentiation of the tumor cells was associated with decreased expression of ALDH1 (χ^2=11.554; P<0.05). Furthermore, TNM stages III/IV exhibited an increased positive expression of ALDH1, compared with that of TNM stages I/II (χ^2=6.789; P<0.05). Additionally, it was identified that positive rates of ALDH1 expression were

increased in cases of lymphatic invasion, compared with that in the tissues without lymphatic invasion (χ^2=8.567; P<0.05). In the present study, DAPI was used to non-specifically stain the nuclei of tumor cells to exclude the impact of non-tumor cells.

The expression of ALDH1 was associated with the clinicopathological characteristics of human esophageal squamous cell carcinoma. ALDH1 may serve an important function in the process of human esophageal cancer. However, whether ALDH1 may be used alone to identify cancer stem cells and whether the prognosis of patients may be predicted on the basis of ALDH1 expression requires additional studies.

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