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Original Article

Overexpression of ALDH1A1 is associated with poor prognosis and therapeutic effects of sulforaphane in esophageal cancer

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Abstract: Human esophageal cancer is one of the most common causes of cancer death in China. Tumor recurrence and metastasis are the main reasons for the failure in cancer treatments. Increasing evidences indicated that cancer stem cell (CSC) are thought to be responsible for tumor initiation and progression. Therefore, therapies targeting cancer stem cells offer a promising strategy for cancer treatment. In this study, we not only evaluated ALDH1A1 level in esophageal cancer and stromal cells, but also studied therapeutic effects of Sulforaphane on ALDH1A1+ cancer stem like cells. Immunohistochemical analysis indicated that 66% (66/100) primary lesions exhibited positive staining for tumor cell ALDH1A1 expression. For another, strong stromal ALDH1A1 expression was also observed in 56% (56/100) cases. Clinicopathological relevance analysis showed ALDH1A1 expression in tumor cells was significantly correlated with lymph node metastasis, Stage and poor prognosis. While high stromal expression of ALDH1A1 was significantly associated with better overall survival. According to the efficacy of Sulforaphane on inhibiting CSC, we examined whether it could eradicate the ALDH1A1-positive esophageal cancer stem like cells. MTS assay showed that Sulforaphane decreased esophageal cancer growth, with an IC50 of 10 μmol/L for TE-13 and OE-33. At 5 μmol/L concentration, Sulforaphane eliminated the ALDH1A1-positive CSCs by 86% in the TE13 cell line, and by 68% in the OE33 cells. In conclusion, overexpression of ALDH1A1 in esophageal cancer cells is associated with poor prognosis. Meanwhile, Sulforaphane could be used to target ALDH1A1 positive cancer stem-like cells for cancer treatment.

Keywords: Esophageal cancer, aldehyde dehydrogenase 1A1, cancer stem-like cells, sulforaphane, prognosis

Introduction

Human esophageal carcinoma is one of the most common cause of cancer death worldwide [1]. Cancer recurrence and distant metastasis are the leading cause of death in esophageal cancer. Cancer stem cells (CSCs) have been identified in many human tumors, and may be responsible for cancer initiation and progression [2]. Aldehyde dehydrogenase 1A1 (ALDH1A1) has been regarded as a possible candidate CSC marker in various solid cancers including esophageal cancer [3]. Furthermore, ALDH1A1 positive esophageal cancer cells also possessed the capability of drug resistance and invasion [4]. Therefore, targeting cancer stem cells therapies might offer a promising strategy for esophageal cancer treatment.

Sulforaphane (SF), a natural compound found in broccoli sprouts, has been proved to effectively eradicate cancer stem cells in different types of cancer. In breast cancer, SF could suppress mammosphere formation in vitro and tumor growth in vivo by inhibiting the ALDH-positive cancer stem cell population [5]. Similarly, SF could reverse chemoresistance by eradicate the pancreatic CSCs [6, 7]. Although these studies support that SF may possess therapeutic potential against CSCs, the efficacy of SF on Esophageal cancer stem cell (ECSC) still remains largely unknown.

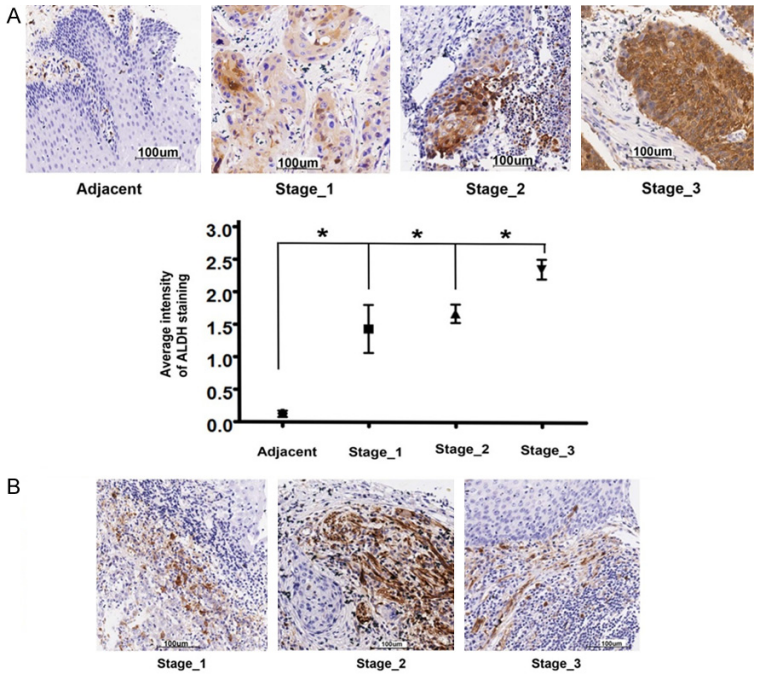

Figure 1. Immunohistochemical analysis of ALDH1A1 expression in esophageal cancer tissues. A. ALDH1A1 staining in esophageal cancer with different stages. B. ALDH1A1 staining in stromal cells.

In this study, we studied the clinical relevance of ALDH1 levels in parenchymal and stromal cells for esophageal cancer patients. Moreover, we examined the efficacy of Sulforaphane to inhibit the ALDH1A1-positive CSCs by in vitro methods.

Methods

Immunohistochemistry and tissue microarray assay

The commercial tissue array containing 100 cases of ESCC patients were constructed by Shanghai Biochip Co. Ltd. as described [8]. For all the specimens, clinicopathological information (age, gender, pathology, differentiation, TNM stage, and follow-up data) was available. The expression of ALDH1A1 in the tissues was evaluated by immunohistochemical staining with specific antibodies. Standard Avidin-biotin complex peroxidase immunohistochemical staining was performed. Briefly, after deparaffinizationin xylene and graded alcohols, heated antigen retrieval was done in citrate buffer (10 mmol/L pH 6.0) by water-bath kettle heating for 30 min. Endogenous peroxidase was blocked in 0.3% hydrogen peroxide for 10 min. Nonspecific binding was blocked by incubation in 10% normal animal serum for 10 min. Sections were incubated at 4°C for 24 h with primary antibodies including polyclonal antibody against ALDH1A1 (Abcam, ab52492). Protein expression levels were scored by staining intensity and the percentage of immunoreactive cancer cells. Tissues with no staining were rated as 0, with faint staining or moderate to strong staining in 5% of cells as 1, with moderate staining or strong staining in 5% to 40% of cells as 2, and with strong staining in > 40% of cells as 3. Esophageal cancer tissues that registered levels 0 and 1 were defined as negative for expression, whereas samples at levels 2 or 3 were defined as positive.

MTS cell proliferation assay

MTS assay was applied to assess viability of cancer cell after treatment with sulforaphane. Cell lines were plated at a density of 3,000 cells per well in 96-well plates and allowed to adhere overnight. Cells were then incubated with SF in increasing concentrations for a period of 72 hours. Proliferation was determined by MTS assay according to manufacturer's instruction. Reduction of tetrazolium compound MTS into a soluble formazan product with absorbance at 490 nm is directly proportional to number of viable cells in culture. Pharmacodynamic modeling was performed using a nonlinear, variable slope model in GraphPad Prism (GraphPad Software).

Aldefluor assay

Aldefluor assay was used to evaluate the effect of sulforaphane on esophageal cancer stem cells in vitro as described before [9]. Aldefluor assay was done according to the manufacturer's guidelines (StemCell Technologies). Single cells obtained from cell cultures were incubated in an Aldefluor assay buffer containing an ALDH substrate, bodipy-aminoacetaldehyde (1 μmol/L per 1,000,000 cells), for 40 to 50 minutes at 37°C. As a negative control, a fraction of cells from each sample was incubated under identical condition in the presence of the ALDH inhibitor diethylaminobenzaldehyde (DEAB).

Table 1. Correlation between ALDH1A1 expression and clinicopathological characteristics in 100 Esophageal cancer cases

	ALDH1A1		*P*-value
	Negative	Positive	
Gender (Male:Female)	19:15	48:18	0.090
Age	65.85±8.704	65.00±9.67	0.667
Tumor size (cm)			0.910
< 10 cm^3	21	40	
> 10 cm^3	13	26	
Grade			0.807
1	25	47	
2+3	9	19	
Depth of invasion			0.800
T1+T2	5	11	
T3+T4	29	55	
Lymph node involvement			0.002
N0	23	23	
N1+N2	11	43	
Stage			0.002
1+2	24	25	
3	10	41	
ALDH1A1 Staining location			0.001
Nuclear	25	26	
Nuclear+cytoplasm	9	40	
ALDH1A1 Stromal staining			0.405
Negative	13	31	
Positive	21	35	

Flow cytometry was used to measure ALDH-positive cell population.

Statistical analysis

The SPSS 15 software package (SPSS, Inc., Chicago, IL) was used for statistical analysis. The association between the markers and clinicopathologic features was analyzed using χ^2-test or two-sided t-test as appropriate. Kaplan-Meier analysis was adopted to evaluate the effects of ALDH1A1 expression on the overall survival (OS) of patients with esophageal cancer. Statistical differences were determined using two-tailed Student's t test. Data are presented as mean ± SD ($n \geq 3$).

Results

ALDH1A1 expression in primary esophageal cancer tissues

The expression of ALDH1A1 in esophageal cancer tissues was analyzed by immunohistochemistry. The results showed that 66% (66/100) primary lesions exhibited positive staining for ALDH1A1, and strong stromal expression was also observed in 56% (56/100) cases. There was no detectable staining of ALDH1A1 in the adjacent normal tissues (**Figure 1A**). Statistical analysis indicated that ALDH1A1 expression in esophageal cancer was significantly correlated with lymph node metastasis (P=0.002) and Stage (P=0.002) (**Table 1**). There were no statistically significant association between ALDH1A1 expression and other clinical parameters such as gender, age, tumor size, grade, and Depth of invasion. Taken together, the results demonstrated that high levels of ALDH1A1 were significantly associated with cancer progression, which can also be released into tumor surroundings.

Association of ALDH1A1 expression with prognosis in primary esophageal cancer

Based on the expression pattern of ALDH1A1, we analyzed the impact of ALDH1A1 expression in cancer cells or stromal cells on esophageal cancer patient's survival. Kaplan-Meier survival analysis demonstrated that overexpression of ALDH1A1 in esophageal cancer cells was significantly shorter than that of patients with negative expression ones (P=0.008, **Figure 2A**). While, we also observed that stromal expression of ALDH1A1 in primary tumors indicated better overall survival (P=0.022, **Figure 2B**).

Sulforaphane inhibits esophageal cancer cell growth in vitro

Sulforaphane was previously shown to inhibit cancer cell growth in different types of cancer [9, 10]. We first investigated the anti-proliferative effects of Sulforaphane on two human esophageal cancer cell lines TE-13 and OE-33 by MTS assay. Cells were treated with increasing concentrations of Sulforaphane for 72 hours and the ratio of viable cells of treatment relative to control is plotted in **Figure 3A**. The

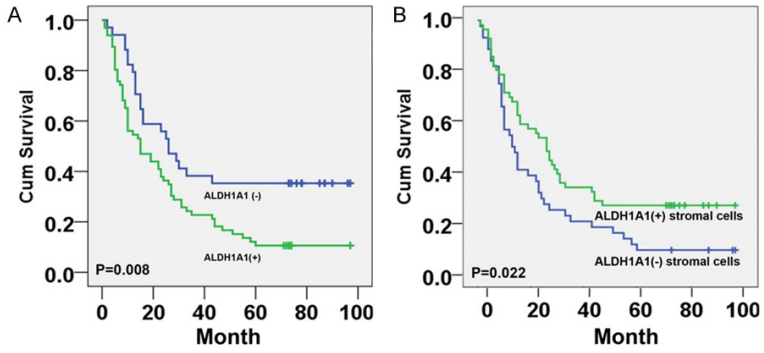

Figure 2. Survival curves for esophageal cancer using the Kaplan-Meier method and the log-rank test. A. Overall survival curves for patients with negative ALDH1A1 expression (blue line) and positive ALDH1A1 in esophageal cancer cells (green line). B. Overall survival curves for patients with negative ALDH1A1 expression (blue line) and positive ALDH1A1 expression in stromal cells (green line).

results demonstrated that cell proliferation gradually inhibited as the concentration of Sulforaphane increased, with an IC50 of 10 μmol/L for TE-13 and OE-33.

Sulforaphane inhibits ALDH1A1-positive esophageal cancer stem cells in vitro

It has been reported that cell population with high ALDH1A1 enzymatic activity has been shown to enrich cancer stem cells in esophageal cancer [2]. Then, we examined whether sulforaphane could inhibit the ALDH1A1-positive esophageal cancer cells. Aldefluor assay indicated that 1 μmol/L sulforaphane significantly decreased the ALDH-positive population of TE13 cells, whereas 5 μmol/L sulforaphane could reduce 86% ALDH1A1-positive cell population. Similarly, sulforaphane also inhibited the ALDH-positive cells in OE33 cells. The inhibition rate was 18% under 1 μmol/L sulforaphane and 68% under 5 μmol/L sulforaphane, respectively (**Figure 3B**).

Discussion

Esophageal cancer is one of the most common causes of cancer death worldwide, especially popular in China [11]. The poor prognosis for esophageal cancer was largely due to recurrence and metastasis [12]. Cancer stem cell (CSC) is believed to possess the self-renewal capacity and can give rise to the heterogeneous lineages of daughter cancer cells [13]. It has been reported that esophageal cancer tissues contained the CSC, and ALDH1A1 has been regarded as a candidate cancer stem cell marker. Other people also reported that level of ALDH1A1-expressing cells in ESCC was correlated with worse clinical outcome of patients [2].

In the current study, we detected the level of ALDH1A1 in esophageal cancer tissues. The results indicated that 66% (66/100) primary lesions exhibited positive staining for tumor cell ALDH1A1 expression, and significantly correlated with lymph node metastasis (P=0.002) and Stage (P=0.002). It has been reported that stromal expression of ALDH1A1 was associated with reduced cancer progression in breast cancer [14]. Then, we detected the ALDH1A1 level in stromal cells. Noticeably, strong stromal expression was also observed in 56% cases, but it did not correlate with any of the clinicopathological parameters examined. In this study, we also found that tumor cell expression of ALDH1A1 was significantly associated with poor overall survival. Consistent with previous studies, we also found that stromal ALDH1 staining in primary tumors indicated better overall survival. It is possible that ALDH1 has been involved in the biosynthesis of retinoic acid, which could inhibit the growth of cancer cells [15]. According to previous studies, there is a discrepancy on the location of ALDH1A1 and their clinical significance. In this study, we found that the staining of ALDH1A1 was mostly accumulated in the cancer cell nucleus in early stage, while nucleus and cytoplasmic staining became predominant in late stage cancer.

According to CSC theory, it is possible to develop other novel agents targeting CSCs to eradicate cancer. SF has been proved to effectively abrogate CSCs in breast cancer [5] and pancreatic cancer [16]. To verify whether SF could inhibit the growth of esophageal cancer stem cell, we employed SF to treat two esophageal cancer cell lines containing ALDH1A1 positive cell population in vitro. MTS assay showed that SF inhibited esophageal cancer cell growth in vitro, with an IC50 of 10 μmol/L for TE-13 and OE-33.

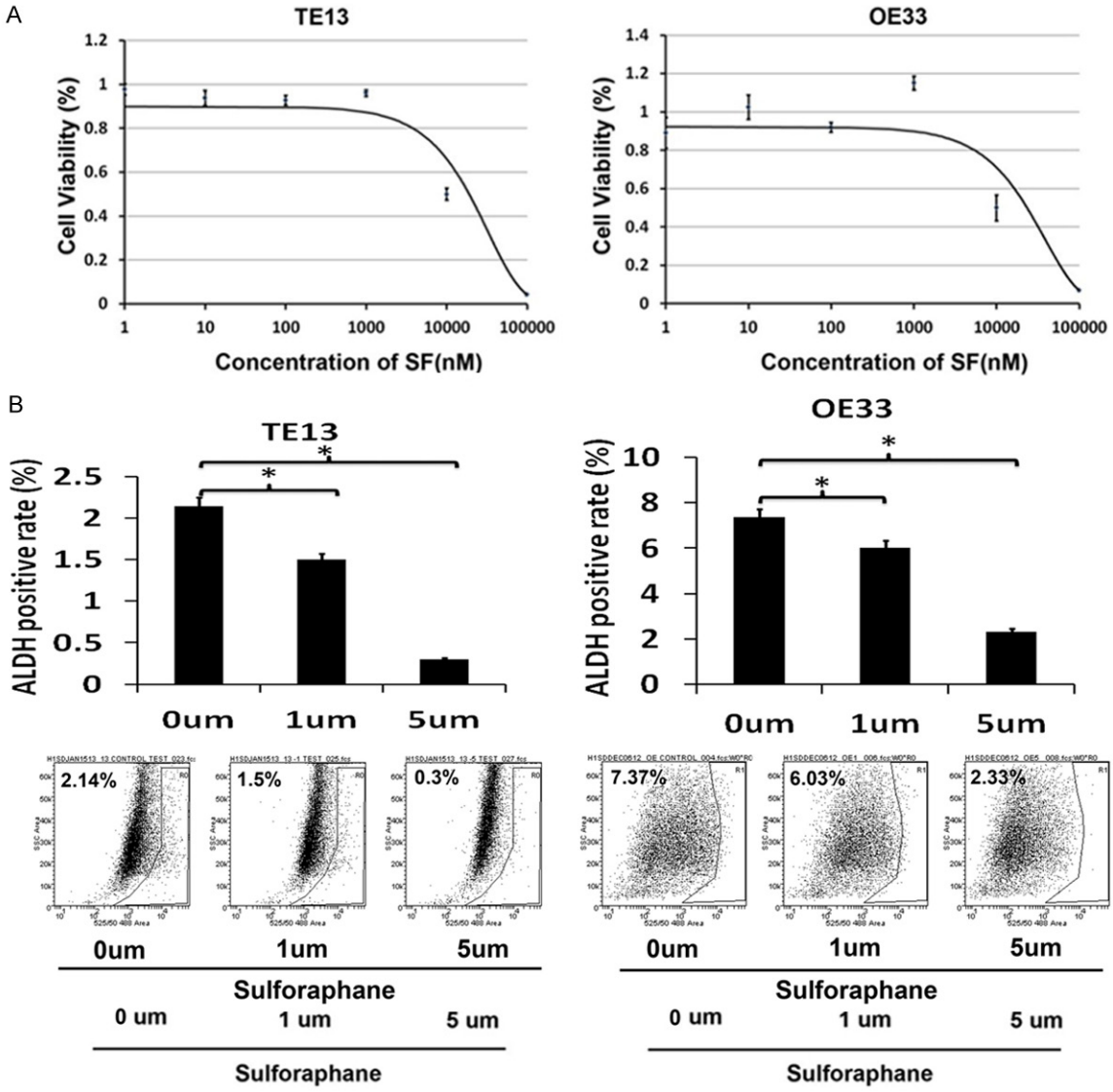

Figure 3. Sulforaphane inhibited proliferation and ALDH-positive cell population in esophageal cancer cells. A. The anti-proliferative effect of Sulforaphane on TE13 and OE33 cells was measured by MTS assay. B. Sulforaphane decreased the percentage of ALDH-positive cells.

To further analyze the efficacy of SF against cancer stem cell, we employed the Aldefluor assay to evaluate the ability of SF to target esophageal cancer stem cells. The results demonstrated that 5 μmol/L SF could significantly decrease the ALDH1A1-positive CSCs by 86% in the TE13 cell line, and by 68% in the OE33 cells.

In conclusion, ALDH1A1 overexpression in cancer cells was closely associated with poor clinical outcome in esophageal carcinoma. And, Sulforaphane could be used to target ALDH1A1 positive cancer stem-like cells for esophageal cancer treatment.

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Disclosure of conflict of interest

None.

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