

Research Paper

Increased Expression of Eps15 Homology Domain 1 is Associated with Poor Prognosis in Resected Small Cell Lung Cancer

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Abstract

One of the great challenges of small cell lung cancer (SCLC) treatment is identifying patients at high risk for recurrence after surgical resection and chemotherapy. We examined Eps15 homology domain 1 (EHD1) protein expression in paraffin sections of 85 resected SCLC tissues, metastatic lymph nodes and normal bronchial epithelial tissues using immunohistochemistry to study the correlation between EHD1 expression and patient clinicopathological features. Within these variables, disease free survival (DFS) analyzed by the log-rank test was constructed using the multivariate Cox proportional hazards regression model and Kaplan–Meier analysis. Immunohistochemistry results showed that EHD1 protein was significantly increased in SCLC tissues compared with normal tissues ($P < 0.001$). Moreover, EHD1 expression was positively correlated with tumor size ($P = 0.019$). Multivariate Cox proportional hazards model analysis showed that EHD1 expression ($P = 0.047$; HR, 1.869; 95% CI, 1.008–3.466) and American Joint Committee on Cancer (AJCC) status ($P < 0.001$; HR, 1.412; 95% CI, 1.165–1.711) were independent prognostic indicators of DFS. In conclusion, these data demonstrated a remarkable correlation between the cytoplasmic expression of EHD1 protein and adverse prognosis in patients receiving early-stage cisplatin treatment for resected SCLC.

Key words: Eps15 homology domain 1; Disease-free survival; Small cell lung cancer; Survival; Immunohistochemistry

Introduction

Lung cancer is the most common neoplasm worldwide, with approximately 1.6 million new cases and 1.4 million deaths occurring each year [1, 2]. Small cell lung cancer (SCLC), which originates from neuroendocrine-cell precursors, accounts for up to 15% of all newly diagnosed lung cancers [3, 4]. SCLC is also the most aggressive subtype of lung cancer, with a high risk for early locoregional and distant metastases and a post-treatment relative 5-year survival rate of only 6.4% [5]. To date, platinum-based chemotherapy has been the principal treatment for

SCLC patients at initial diagnosis [6-8]. However, due to primary or secondary resistance to chemotherapy, most SCLC patients rarely survive beyond 2 years from the time of diagnosis [9-11].

Mammalian Eps15 homology domain 1 (EHD1) is found on the chromosomal band 11q13, which has been found to be amplified or rearranged in plenty of carcinomas including lung, breast, head and neck and associated with poor prognosis [12-17]. EHD1 plays an important role in the regulation of various cellular events [18], including the recycling of proteins to the

plasma membrane [19]. Notably, one of the most important proteins regulated in this manner are the $\beta 1$ integrins [20], which bind to the extracellular matrix (ECM) and stimulate signaling pathways that influence proliferation, apoptosis, cell spreading, migration, invasion and metastasis [21-23]. The results of recent studies suggest that EHD1's role in vesicle trafficking may also be related to cancer invasion and metastasis [24, 25].

A positive correlation between the expression of EHD1 and poor survival has been observed in non-small cell lung cancer (NSCLC) [26, 27]. The aim of this study was to investigate the ability of EHD1 to serve as a prognostic marker of SCLC. To assess this prognostic capacity, the expression of EHD1 protein in tumor, metastatic lymph node and normal bronchial epithelial tissues from 85 resected SCLC patients was measured using immunohistochemistry (IHC) and correlated with certain clinicopathological features of patients.

Materials and methods

Tissue samples and patients

To assess the prognostic capacity of EHD1 for SCLC, formalin-fixed, paraffin embedded (FFPE) SCLC tumor, lymph node metastases and normal bronchial epithelial tissues were collected from 85 SCLC patients who underwent surgery between January 2008 and November 2011. Inclusion criteria were provided to be SCLC by pathology reports and included from stage I to stage IIIA. No patient received chemotherapy or radiotherapy prior to surgery. Four to six cycles of platinum-based adjuvant chemotherapy were administered to all patients. All cases representing a spectrum of SCLC were retrieved from Harbin Medical University Cancer Hospital. Exclusion criteria were stage IV of disease and history of other cancers. For each patient, each tissue specimen type was resected during the same surgical procedure. Primary cancers were evaluated in accordance with the 7th edition of the American Joint Committee on Cancer (AJCC) staging system (TNM). All patients were followed until death or the study closing date (January 21, 2014). The median follow-up time for survivors was 48.70 months (range 3.00-65.70 months). The study was approved by the Ethical Committee of Harbin Medical University in Harbin, China. Informed consent was obtained from all patients. All investigators involved in the study, apart from the study statistician, were blinded to patient outcome throughout all laboratory analyses.

Immunohistochemistry

To detect the expression of EHD1 in FFPE sections, IHC was performed as described. The tissue sections were first dried at 70°C for 3 h. After deparaffinization and hydration, sections were washed in phosphate-buffered saline (PBS; 3 × 3 min). Endogenous peroxidase was quenched with 3% H₂O₂ for 15 min. After washing in distilled water, sections were washed in PBS (3 × 5 min). Antigen retrieval was performed in citrate buffer (pH 6.0). Each section was then treated with 300-500 ml EHD1 rabbit polyclonal antibody (Abcam, Cambridge, UK, ab75886, diluted at 1:200) solution overnight at 4°C. The sections were incubated with peroxidase-conjugated streptavidin for 30 min, and the reaction products were visualized with diaminobenzidine as a chromogen and counterstained with commercial hematoxylin. The percentage of positive cells was determined by counting 500 cells in five random selected fields per section. IHC staining was scored based on intensity, as follows: 0 (no staining), 1 (weak staining = light yellow), 2 (moderate staining = yellow brown) and 3 (strong staining = brown). The percentage (0-100%) of the extent of reactivity was scored as follows: 0 (no positive tumor cells), 1 (fewer than 10% positive tumor cells), 2 (10-50% positive tumor cells) and 3 (greater than 50% positive tumor cells). Next, the cytoplasmic expression score was obtained by multiplying the intensity and reactivity rate values. Scores of < 4 were classified as low expression, and the remainders were classified as high expression. Two blinded independent observers interpreted all slides.

Statistical analysis

All analyses were performed using statistical software (SPSS 13.0 for Windows; SPSS, Inc., Chicago, IL, USA). Differences were considered statistically significant when *P* values were < 0.05. Correlation of EHD1 expression levels between tissue types and correlation of EHD1 expression levels in tissues with clinicopathological features of patients were assessed using chi-square tests. Disease-free survival (DFS) were calculated from the date of surgery resection to the date of last follow-up or relapse. Cumulative DFS curves were plotted by the Kaplan-Meier method, and the relationship between each of the variables and survival was assessed by log-rank test in univariate analysis. The covariates with *P* ≤ 0.15 in univariate analyses were adopted into multivariate analyses. The parameters were then tested by the multivariate Cox proportional hazards model, which was performed to identify independent variables for predicting survival. Risk ratios and their 95% confidence intervals (CIs) were recorded for each factor.

Results

EHD1 protein expression in tumor, lymph node metastases and normal bronchial epithelial tissues

Using IHC, EHD1 protein expression was examined in tumor, lymph node metastases and normal bronchial epithelial tissues of 85 SCLC patients. The frequency of positive staining for EHD1 was 58.82% (50/85) in the SCLC samples, which was significantly higher ($P < 0.001$) than that in the normal tissues (21.18%) (Table 1). The cytoplasmic staining patterns observed for EHD1 were consistent with data from our previous studies (Fig. 1) [26, 27].

Association of EHD1 protein expression and clinicopathological features

Correlation of EHD1 expression levels with a range of clinicopathological features of patients, including age, gender, smoking history, adjuvant radiotherapy history, tumor histology, tumor size, presence of lymph node metastasis (LNM) and AJCC stage in SCLC patients (Table 2), was assessed. High expression of EHD1 in the cytoplasm was only positively correlated with tumor size ($P = 0.019$). No such significant correlations between EHD1 and other clinicopathological features were found in this study.

Univariate and multivariate Cox regression analysis of potential prognostic indicators of DFS in SCLC patients

In all patients, AJCC stage and EHD1 overexpression were significantly associated with DFS based on univariate Cox regression models. Other features showed no major prognostic value. Multivariate Cox proportional hazards model analysis of the same set of patients showed that AJCC stage ($P < 0.001$; HR, 1.412; 95% CI, 1.165-1.711) and EHD1 ($P = 0.047$; HR, 1.869; 95% CI, 1.008-3.466) were independent prognostic indicators of DFS in SCLC patients (Table 3).

Table 1. Expression of EHD1 in small cell lung cancer, lymph node metastases and normal bronchial epithelial tissues

Tissue	Normal ^b	P	Tissue	Lymph node ^c	P
Small cell lung cancer ^a			Small cell lung cancer ^a		
		<0.001			0.539
Low	35 (41.18%)	67 (78.82%)	35 (41.18%)	17 (47.22%)	
High	50 (58.82%)	18 (21.18%)	50 (58.82%)	19 (52.78%)	

note: [0 (negative) \leq score \leq 1+] and [2+ \leq score \leq 3+] represent low negative and strong positive staining of EHD1, respectively. All the cut off points contributed to acquiring the optimum balance ratio between negative and positive. ^a Lung primary tissues; ^b Normal bronchial epithelial tissues; ^c Lymph node metastases.

Table 2. The correlation between clinicopathological features and the expression of EHD1

Variables	EHD1 (n=85) ^a		P
	Low	Strong	
Age			0.364
≤60	27(31.76%)	39(45.88%)	
>60	10(11.76%)	9(10.59%)	
Gender			0.521
male	19(22.35%)	28(32.94%)	
female	18(21.18%)	20(23.53%)	
Smoking history			0.546
never	1(1.18%)	4(4.71%)	
ever	20(23.53%)	25(29.41%)	
unknown	16(18.82%)	19(22.35%)	
Adjuvant radiotherapy			0.828
Yes	10(11.76%)	14(16.47%)	
No	27(31.76%)	34(40.00%)	
Tumor histology			0.462
pure	35(41.18%)	43(50.59%)	
combined	2(2.35%)	5(5.88%)	
Tumor Size			0.019*
≤3	14(16.47%)	25(29.41%)	
>3	24(28.24%)	22(25.88%)	
Nodal			0.290
negative	22(25.88%)	23(27.06%)	
positive	15(17.65%)	25(29.41%)	
AJCC Stage			0.781
I	17(20.00%)	20(23.53%)	
II	11(12.94%)	13(15.29%)	
III	9(10.59%)	15(17.65%)	

note: [0 (negative) \leq score \leq 1+] and [2+ \leq score \leq 3+] represent low negative and strong positive staining of EHD1, respectively. All the cut off points contributed to acquiring the optimum balance ratio between negative and positive. * $P < 0.05$. ^a Three EHD1 samples were excluded because of damage to or cytolysis of the paraffin block.

Table 3. EHD1 expression in primary tumor tissues as an independent prognostic factor for DFS in resected small cell lung cancer patients

Variables	Univariable ^a		Multivariable ^b	
	HR (95% CI)	P value	HR (95% CI)	P value
Age	1.004 (0.966-1.043)	0.840		0.155
Gender	1.364 (0.771-2.411)	0.286		0.250
Smoking history	0.766 (0.468-1.287)	0.326		0.854
Adjuvant radiotherapy	1.252 (0.933-1.679)	0.134		0.066
Tumor histology	0.695 (0.259-1.865)	0.470		0.331
AJCC stage	1.439 (1.182-1.752)	<0.001	1.412 (1.165-1.711)	0.001
EHD1 expression	1.958 (1.060-3.615)	0.032	1.869 (1.008-3.466)	0.047

note: CI, confidence interval; HR, hazard ratio; DFS, disease free survival. ^a Variables were adopted for their prognostic significance ($P < 0.05$) in univariate analysis using forward, stepwise selection (forward likelihood ratio). ^b A Cox proportional hazards regression model was used for multivariate analysis.

Kaplan-Meier survival curve analysis of DFS in SCLC patients and correlation of EHD1 expression

Kaplan-Meier survival curves stratified for EHD1 expression are shown in Fig. 2a. EHD1 expression showed significant effects on DFS ($P = 0.029$), specifically that high EHD1 expression was associated with poor DFS. Additionally, two subgroups were

classified according to AJCC stage status (Fig. 2b, 2c). Accordingly, the prognosis for patients with low expression of EHD1 was significantly better ($P = 0.009$) than for patients with high expression of EHD1 in

stage I SCLC, whereas EHD1 expression was not a significant predictor of DFS in stage II and IIIA SCLC ($P = 0.552$).

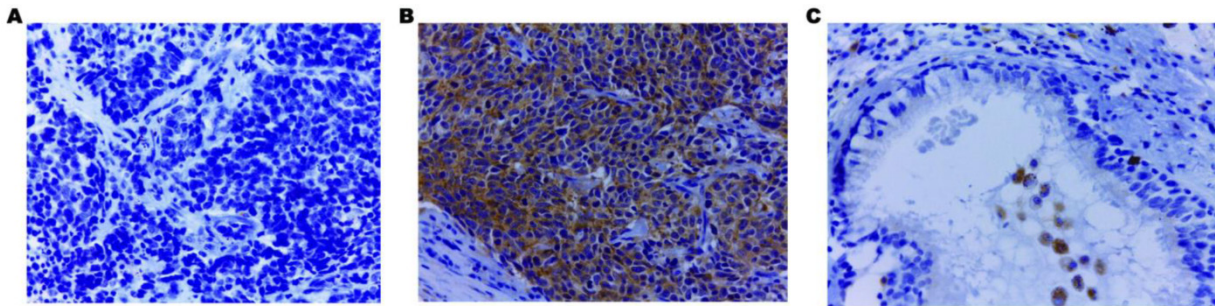


Fig. 1 Immunohistochemical staining of EHD1 in FFPE tissue samples ($\times 400$). (A) Cytoplasmic EHD1-negative specimen (SCLC). (B) Cytoplasmic EHD1 high-expression specimen (SCLC). (C) Cytoplasmic EHD1 low-expression specimen (normal bronchial epithelial tissue).

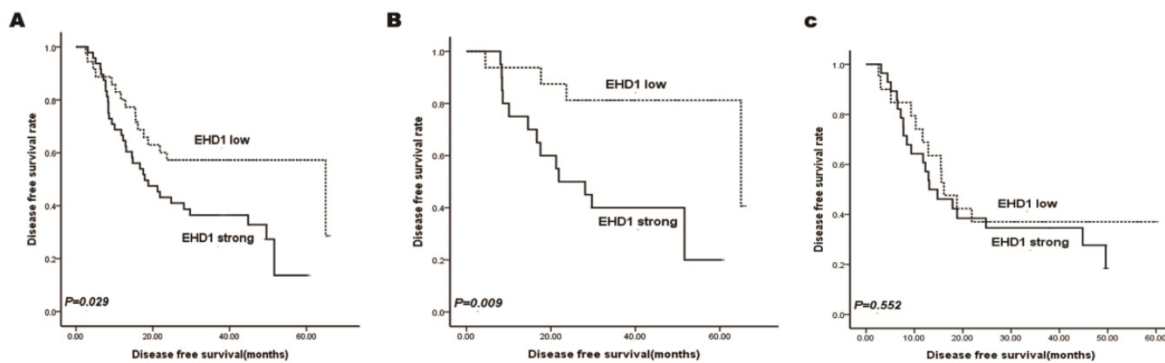


Fig. 2 Kaplan–Meier analysis for disease free survival (DFS) based on EHD1 expression status in SCLC patients. (A–C) Kaplan–Meier analysis for DFS based on EHD1 expression status in (A) all patients (B) stage I SCLC patients (C) stage II and IIIA SCLC patients.

Discussion

The results of this study revealed that the expression of EHD1 predicted DFS in a cohort of 85 SCLC patients who received a surgical resection and platinum-based adjuvant chemotherapy, which is a similar result to that found in our previous study reporting the prognostic value of EHD1 expression in NSCLC [26, 27]. Platinum-based treatment is a crucial part of the standard regimen for SCLC and NSCLC patients. Recent research showed that high mRNA levels of EHD1 were associated with cisplatin resistance in HeLa cells [28]. High levels of EHD1 expression are also associated with poor response to treatment in cutaneous T cell lymphoma [29]. These results suggested that EHD1 might mediate cisplatin resistance through regulating endocytosis.

Our study reveals that EHD1 overexpression was markedly correlated with tumor size. It suggests that the EHD1 expression level might provide information that correlates with the ability of tumor cell proliferation. Other studies observed the involvement of misregulated and mutated EHD1 in cancer pro-

gression [19, 20], invasion and metastasis [25]. EHD1 is primarily involved in *tubular recycling endosome* (TRE) membrane vesiculation [30], acting as a gatekeeper to promote the recycling of a variety of receptors from the endocytic recycling compartment (ERC) to the plasma membrane [19, 31–36]. For example, EHD1 protein is involved in endocytosis and trafficking of various membrane proteins including major histocompatibility complex (MHC) class proteins [33], insulin-like growth factor 1 (IGF1) [37, 38] and secretion of glucose transporter 4 (GLTU-4) [36, 39]. Therefore, it is possible that the loss of EHD1 activity could give rise to tumor development through the aberrant regulation of MHC class molecules, which participate in antigen presentation and destruction of abnormal cells [36]. Furthermore, some studies reported that IGF1 is associated with diverse types of cancer, such as pancreatic cancer [40], leukemia [41], hepatocarcinoma (HCC) [42] and breast cancer [43], and Zheng X et al. reported that GLUT4 protein is a common disruptor of carbohydrate metabolism [44], which is linked to elevated cancer risk. Additionally, EHD1 and its interaction partner molecule interacting

with CasL-like protein 1 (MICAL-L1) are required for the activation and transport of Src, which plays a primary role in regulating cell adhesion and migration [45], and the misregulation of Src kinase activity in cancer is related to metastasis and poor survival [46]. As previously mentioned, β 1 integrins stimulate signaling pathways affecting cell motility and migration as well as tumor cell invasiveness [21], adhesion and spreading [22, 23]. EHD1 plays a significant role in regulating β 1 integrin transport and is further involved in integrin-mediated downstream functions [20]. One study indicated that aberrant regulation of EHD1 could possibly lead to tumor development, as was shown in metastatic colon cancer, non-Hodgkin lymphoma (NHL) and partially in rhabdomyosarcoma (RMS) [24].

Summarily, EHD1 expression may affect the sensitivity of tumor cells to cisplatin-containing treatment and recurrence or metastasis through the regulation of the endocytic recycling process.

Notwithstanding, EHD1 expression status did not correlate with overall survival (OS). SCLC is an aggressive malignancy with extremely high recurrence and metastasis, though the mechanism by which it progresses is not yet clear. Recent experimental observations identified many signaling molecules, such as CEA, MGr1-Ag and receptor tyrosine kinases (RTKs), involved in its regulation [47-49]. We purport that EHD1 is an effective prognostic marker for SCLC patients, particularly for those in the early stages of disease; however, as SCLC progresses and other molecular mechanisms take over, the effect of EHD1 is reduced. Therefore, further research is required to identify the molecular mechanisms underlying EHD1 protein overexpression and its role in SCLC. A larger sample size is also needed to verify the results of this study. In conclusion, this study presents evidence that EHD1 may predict the prognosis of early stage SCLC patients receiving cisplatin treatment.

Abbreviations

EHD1: Eps15 Homology Domain 1, IHC: immunohistochemistry, FFPE: formalin-fixed paraffin-embedded, SCLC: small cell lung cancer, DFS: disease-free survival.

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Competing Interests

The authors have declared that no competing interest exists.

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