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Clinical significance of HAX-1 expression in laryngeal carcinoma

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ABSTRACT

Objective: HS1-associated protein X-1 (HAX-1) is a multifunctional protein that has been highlighted as an important marker in many types of cancers. However, little is known about the role of HAX-1 in laryngeal carcinoma. The purpose of the present study is to explore HAX-1 expression status and its associations with clinicopathologic features and survival in a well-defined cohort of laryngeal carcinoma.
Methods: We examined the expression of HAX-1 at protein and mRNA levels in laryngeal carcinoma tissues and adjacent non-tumor tissues by immunohistochemistry, Western blotting and two-step quantitative real-time PCR analysis, respectively.
Results: We observed that HAX-1 was significantly elevated in laryngeal carcinoma. The relationship between the levels of HAX-1 expression and clinicopathologic characteristics was then analyzed. Overexpression of HAX-1 was significantly correlated with T classification, lymph node metastasis, clinical stage, and pathology. Survival curves were plotted using the Kaplan–Meier method and compared using the log-rank test. We find that patients with overexpression of HAX-1 had shorter overall survival rates. Finally, the significance of various survival variables was analyzed using multivariate Cox proportional hazards model. We found that overexpression of HAX-1 was an independent prognostic factor for patients with laryngeal carcinoma.
Conclusion: Our findings hinted that overexpression of HAX-1 was a potentially unfavorable factor in the progression and prognosis of laryngeal carcinoma.

1. Introduction

Laryngeal cancer represents the second most common malignancies of the head and neck worldwide [1]. Most patients affected by laryngeal cancer are in the locoregionally advanced stage of disease at the time of diagnosis. Given the fundamental role of the larynx that plays in communication, breathing, and swallowing, patients with laryngeal carcinoma can develop dysphonia, dyspnea and dysphagia. Despite multiple and advanced therapeutic interventions of the early stages of the disease, there has been a substantial proportion of patients with localized or locally advanced disease who will eventually relapse and die [2]. Therefore, a better understanding of the molecular mechanisms underlying the initiation and progression of laryngeal carcinoma and a new strategy for the treatment of laryngeal cancer have great importance in improving outcomes for this disease.

HS-1-associated protein X-1 (HAX-1) has previously been shown to associate with HS-1, a protein specifically expressed in cells of the hematopoietic lineage, and is thought to be involved in signal transduction in B cells and apoptosis. Although HAX-1 has been detected in various cellular compartments such as endoplasmic reticulum and nuclear membrane, it is predominantly mitochondrial [3]. HAX-1 was implicated in a perplexing range of important biological functions including the regulation of apoptosis or programmed cell death, cell motility, endocytosis and calcium homeostasis, and involvement in multiple signaling pathways and cellular processes through interacting with a number of cellular and viral proteins such as polycystin-2, cortactin and Epstein–Barr virus nuclear antigen leader protein (EBNA-LP) [4–11]. Analysis of gene expression profiles revealed that the amounts of the HAX-1 gene were significantly elevated in a broad variety of cancers, including esophageal squamous cell carcinoma (ESCC), colorectal cancer (CRC), oral squamous cell

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carcinoma, lung cancer, lymphoma, melanoma, breast cancer and hepatoma [6,12–19]. More recently, it was determined that the level of increase in HAX-1 gene expression correlated with the size and grade of the tumor, with higher amounts of HAX-1 detected as the disease progressed, at least for breast cancer [16]. However, the expression of HAX-1 protein in laryngeal carcinoma tissues and a link between HAX-1 expression and laryngeal carcinoma have remained unclear until now.

To explore the expression of HAX-1 in laryngeal carcinoma tissues and adjacent noncancerous tissues and to study the relationship between this expression, we used Western blot, real-time RT-PCR and immunohistochemical method. We found that overexpression of HAX-1 in laryngeal carcinoma was an unfavorable prognostic factor for patient's progression and survival.

2. Materials and methods

2.1. Tissue specimens

Laryngeal carcinoma and adjacent non-tumor tissues used for immunohistochemistry were collected from 98 laryngeal carcinoma patients who underwent surgical excision of laryngeal squamous cell carcinoma with neck dissection in the Department of Otolaryngology-Head and Neck Surgery ENT Affiliated Hospital of Nantong University between January 2004 and December 2013. Informed consent was obtained from all patients. The clinicopathological characteristics of 98 laryngeal carcinoma patients are given in Table 1.

92 of the 98 patients were men and 6 were women; their median age was 69 years (range 46–87 years). 60 (61.2%) of the 98 patients had a glottic localization, 31 (31.6%) had a supraglottic localization and 7 (7.1%) of the 98 patients had a subglottic localization. The pathology grade was well/moderate in 60 cases, as well as poor/undifferentiation in 38 cases. The clinical classification of the lymph nodes metastasis was N0 in 53 and N+ in 45 patients. The T status was T1–T2 in 60 and T3–T4 in 38 patients. The TNM clinical stage was I–II in 36 cases and III–IV in 62 cases. 18 patients underwent a total laryngectomy, 72 had a partial laryngectomy, and 8 had a carbon dioxide (CO_2) laser surgery. 75 patients had unilateral neck dissection and 17 had bilateral neck dissection. The followed up time was 80 months, with a range of 12–80 months and the median clinical follow-up was 65 months.

Table 1
Expression of HAX-1 in 98 human laryngeal carcinoma tissues.

Clinicopathological parameters	No. case	HAX-1 expression, *n* (%)		*p*
		Low	High	
Gender				
Male	92	39	53	0.400
Female	6	4	2	
Age, year				
<60	32	19	13	0.500
≥60	66	24	42	
Smoking				
No	32	16	16	0.515
Yes	66	27	39	
T status				
T1–T2	60	39	21	<0.01*
T3–T4	38	4	34	
Lymph node metastasis				
No	53	33	20	<0.01*
Yes	45	10	35	
TNM clinical stage				
I–II	36	30	6	<0.01*
III–IV	62	13	49	
Pathology grade				
Well/moderate	60	32	28	0.022*
Poor/undifferentiation	38	11	27	
Tumor location				
Glottic	60	27	33	0.699
Supraglottic	31	14	17	0.688
Subglottic	7	2	5	0.488

* Statistical analyses were performed by the Pearson χ^2 test. $p < 0.05$ was considered significant.

Twenty pairs of fresh laryngeal carcinoma and adjacent non-tumor tissues used for quantitative PCR and Western blot were collected from the Affiliated Hospital of Nantong University, China, and snap frozen in liquid nitrogen until use. The patients had not received any therapy before admission.

2.2. Immunohistochemical staining

Immunohistochemistry for HAX-1 expression in patients' tissues was performed using standard methods. The tissue sections were deparaffinized using a graded ethanol series, and endogenous peroxidase activity was blocked by soaking in 0.3% hydrogen peroxide for 15 min. The sections were then processed in 10 mmol/L citrate buffer (pH 6.0) and heated at 120 °C for 5 min to retrieve the antigen. After being rinsed in phosphate buffered saline (PBS, pH 7.2), 10% goat serum was applied for 1 h at room temperature to block nonspecific reactions. The sections were then incubated overnight at 48 °C with anti-HAX-1 mouse polyclonal antibody (diluted 1:300; Santa Cruz Biotechnology, CA, USA). Negative control slides were also processed in parallel using a non-specific immunoglobulin IgG (Sigma Chemical Co., St. Louis, MO) at the same concentration as the primary antibody. Sections were then washed with PBS for 5 min and treated with horseradish peroxidase-conjugated goat anti-mouse antibody (Dako Cytomation, USA) for 15 min. All slides were processed using the peroxidase antiperoxidase method (Dako, Hamburg, Germany). After being rinsed in PBS, the peroxidase reaction was visualized by incubating the sections with diaminobenzidine tetrahydrochloride in 0.05 mol/L Tris buffer (pH 7.6) containing 0.03% H2O2. After being rinsed in water, the sections were counterstained with hematoxylin, dehydrated, and coverslipped.

The immunohistochemistry scoring results were evaluated independently by the three independent pathologists without knowing the patient's clinic pathological outcomes. The intensity of immunostaining in each tumor section was assessed as strong (3), moderate (2), weak (1), or negative (0); semiquantitatively using the following scale: 0% of cells (0), 1–10% (1), 11–50% (2), and >50% (3) of cells. A final immunoreactivity score (IRS) was obtained for each case by multiplying the percentage and the intensity score. Protein expression levels were further analyzed by classifying IRS values as low (based on an IRS value lower than 4.5 which was the median of IRS values) and as high (based on an IRS value greater than 4.5 which was the median of IRS values) [13]. Ethics approval to perform this study was obtained from the Human Research Ethics Committee of the Affiliated Hospital of Nantong University, Jiangsu Province, China.

2.3. Western blot analysis

Western blotting analysis was carried out with the proteins collected from the adjacent non-tumor tissues and the cancer tissues, and total proteins were extracted with homogenization buffer containing 1 M Tris–HCl pH 7.5, 1% Triton X-100, 1% NP-40 (Nonidet p-40), 10% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 0.5 M EDTA, 10 mg/ml leupeptin, 10 m g/ml aprotinin, and 1 mM PMSF, then centrifuged at 10,000 × *g* for 30 min to collect the supernatant. The concentration of the protein was measured by the BCA protein assay kit (PIERCE, Rockford, IL, USA).

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