

## Original Article

# Differential expression of homeobox D11 (HOXD11) in esophageal squamous carcinoma and its clinical significance

Han Liu, Ying Liu, Xiufeng Cao\*, Suqing Li\*

*Department of Surgical Oncology, Nanjing First Hospital, Nanjing Medical University, Nanjing, China. \*Equal contributors.*

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**Abstract:** China has a high incidence of esophageal carcinoma (EC), with EC cases accounting for more than 50% of the cases worldwide. Most of these patients have esophageal squamous carcinoma (ESC), which has a very low 5-year survival rate. We used microarrays to screen for differentially expressed mRNAs in ESC to identify new molecular markers closely related to ESC development. A human mRNA microarray was used and identified homeobox D11 (HOXD11) to be differentially expressed. Real-time quantitative polymerase chain reaction (qRT-PCR) and immunohistochemical staining were performed to verify the expression levels of HOXD11 mRNA and protein in ESC and paraneoplastic tissues (PNT). HOXD11 mRNA was significantly upregulated in 78 ESC cases ( $P < 0.001$ ). Immunohistochemistry showed that the expression of HOXD11 protein in 37 ESC cases was significantly higher than that in PNT ( $P < 0.001$ ), and the expression in ESC-positive lymph nodes was significantly higher than that in ESC-negative lymph nodes ( $P < 0.001$ ). The expression levels of HOXD11 mRNA and protein in human ESC were significantly higher than those in PNT, while low expression was observed in negative lymph nodes, and the expression in positive lymph nodes was significantly higher than that in negative lymph nodes.

**Keywords:** HOXD11, esophageal squamous carcinoma, esophageal carcinoma, qRT-PCR, immunohistochemistry

## Introduction

Esophageal carcinoma (EC) is one of common malignancies worldwide, ranking eighth in the global incidence of malignant tumors [1] and sixth in the mortality rate [2]. The pathological types of EC show obvious regional differences, with adenocarcinoma more common in Europe and America and squamous carcinoma more common in Asia [2-5]. China has a high incidence of EC, accounting for more than 50% of the global incidence of EC and a mortality rate of 17.38/100,000, ranking fourth among all malignant tumors [6].

The onset of EC is insidious [7] and highly invasive; most patients are in an advanced stage when diagnosed. Early surgery cannot remove the lesions, and therefore, the five-year survival rate is  $< 20\%$  [8], out of which 75% of patients die within 1 year of diagnosis [9]. Although significant progress has been made in the clinical diagnosis and treatment of EC in recent years,

there has been no significant decrease in mortality [7, 10]. The lymph node micrometastasis of EC in early stages is an important reason for its poor prognosis [11-13]; there is also no effective marker for early diagnosis. In order to systematically study the mRNA expression profiles of esophageal squamous carcinoma (ESC) and paraneoplastic tissues (PNT), we used a human Agilent mRNA expression profile microarray to detect the differential expression of mRNA in 6 pairs of ESC and PNT samples to screen for mRNA that is differentially expressed (up-/down-regulated by more than 2-fold). We found that homeobox D11 (HOXD11) was significantly differentially expressed in ESC and PNT. HOXD11 is a protein-coding gene derived from the HOX family; after extensive literature review, we found no report regarding the correlations between ESC and HOXD11. In this study, real-time quantitative polymerase chain reaction (qRT-PCR) was used to detect the expression level of HOXD11 mRNA in ESC tissues. We found that the expression of HOXD11 mRNA in

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**Table 1.** Clinicopathological data of 78 ESC cases for qRT-PCR detection

Items	Count
Male/Female	48/30
Age (Year)	63.46±11.92
Smoking history	19
Drinking History	11
Differentiation degree (high differentiation/moderate differentiation/poor differentiation)	5/63/10
T staging (T1/T2/T3)	15/40/23
N staging (N0/N1/N2)	60/19/8
M staging (M0/M1)	78/0

**Table 2.** Clinicopathological data of 37 patients with immunohistochemical analysis

Items	Count
Male/Female	29/8
Age (Year)	61.14±11.32
Differentiation degree (high differentiation/moderate differentiation/poor differentiation)	4/26/7
T staging (T1/T2/T3)	3/11/23
N staging (N0/N1/N2)	23/8/6
M staging (M0/M1)	37/0

ESC tissues was significantly higher than that in PNT, suggesting that HOXD11 is involved in ESC development and may be an important target for preventing and treating ESC. We further performed an immunohistochemical assay and verified that the HOXD11 protein was highly positively expressed in ESC nuclei and positive metastatic lymph nodes, whereas nearly no expression was observed in PNT and negative lymph nodes. The expression of HOXD11 was significantly tissue-specific, suggesting that it is a regulatory molecule involved in the occurrence and development of ESC and may play an important role in the occurrence, development, and metastasis of EC.

### Materials and methods

#### Collection of tissue samples

All clinical specimens used to study the expression of the HOXD11 gene were detected by qRT-PCR and immunohistochemistry, and the study was approved by the ethics review committee of the First Hospital of Nanjing Affiliated to Nanjing Medical University. Written informed

consent was obtained from all patients before sampling. All samples used in this study were obtained from inpatients who had undergone surgery between January 2006 and December 2012, among which 78 paired tissue samples of ESC and PNT (> 5 cm away from the proximal edge of ESC) and 37 immunohistochemical specimens were provided by the First Hospital of Nanjing Affiliated to Nanjing Medical University and Huaian First People's Hospital, Jiangsu; 6 pairs of fresh frozen specimens for microarray analysis (including ESC and PNT) were provided by the Affiliated Nanjing Hospital of Nanjing Medical University. None of the patients had been treated with chemotherapy, radiation, or other cancer-targeting treatment. All tissue samples were surgically separated and immediately placed in liquid nitrogen, and all cases were preliminarily examined and reviewed by two clinical pathologists.

PCR samples were provided by the First Hospital of Nanjing Affiliated to Nanjing Medical University and Huaian First People's Hospital, Jiangsu. Clinicopathological data are shown in **Table 1**.

Immunohistochemical specimens were provided by the First Hospital of Nanjing Affiliated to Nanjing Medical University and Huaian First People's Hospital, Jiangsu. Clinicopathological data are shown in **Table 2**.

Microarray samples were provided by the Department of Surgical Oncology, the First Hospital of Nanjing Affiliated to Nanjing Medical University and the clinicopathological data are shown in **Table 3**.

To prepare the microarray, total RNA was extracted from the samples according to the standard operations of Trizol reagent, which was then completed by Beijing Biochip Co., Ltd. (Beijing, China).

#### Real-time qPCR

To extract total RNA from frozen tissues, the tissue specimens were removed from liquid nitrogen, ground into powder, and 1 mL ice-cold Trizol reagent was added. Samples were placed in a 10-mL centrifuge tube and incubated at room temperature for 5-10 min. We added 200  $\mu$ L of chloroform into the tube, shook the tube vigorously for 15 s, incubated the sample at room temperature for 3 min, and then centri-

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**Table 3.** Clinicopathological data of 6 microarray samples for ESC mRNA detection

No	Gender	Age	Smoking history	Drinking history	Tissue type	Differentiation degree	T staging	N staging	M staging
1	M	54	N/A	100 g/d for 20 years	ESC	Moderate	3	2	0
2	M	54	N/A	N/A	ESC	Moderate	3	1	0
3	F	67	N/A	N/A	ESC	Moderate	3	0	0
4	M	72	N/A	N/A	ESC	Moderate	3	2	0
5	M	75	10 cigarettes/for 30 years	N/A	ESC	Moderate	3	0	0
6	F	65	N/A	N/A	ESC	Moderate	3	0	0

fuged the sample at 4°C and 12,000 rpm for 15 min. The upper phase was removed into a clean Eppendorf tube and 0.5 mL of isopropanol was added. The sample was mixed, incubated at room temperature for 10 min, and centrifuged at 4°C and 12,000 rpm for 10 min. After removing the supernatant, we added 75% of ethanol, flicked the tube wall, centrifuged the tube at 4°C and 7500 rpm for 15 min, and discarded the supernatant. The sample was dried at room temperature for 10 min, after which 20 µL of RNase-free water was added, and the RNA was fully dissolved in a 55°C water bath for 10 min. Samples were stored at -70°C.

### RNA purity

The integrity of the total RNA was examined by formaldehyde-denatured agarose gel electrophoresis using 4.5 µL of total RNA (approximately 20-30 µg). Formaldehyde gel electrophoresis buffer was added and incubated at 65°C for 15 min. Next, we conducted 5-min ice-bath incubation and then added 1 µL of ethidium bromide (1 µg/µL) and 2 µL of loading buffer. We loaded the sample and performed electrophoresis at 5 V for approximately 2 hours. After electrophoresis, the gels were placed under a UV lamp to observe the integrity of total RNA.

### Removal of contaminating DNA

The 1.2% formaldehyde-denatured agarose gel electrophoresis was used to evaluate the integrity of total RNA and remove contaminating DNA. A UV spectrophotometer was used to detect RNA content (A260 value) and purity (A260/A280 ratio), and the RNA sample was stored at -70°C until use. The PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time, Xingzhi Biological Technology Co. Ltd., Guangdong, China) and reverse transcription kit (TaKaRa Co., Shiga, Japan) were then used

for reverse transcription of cDNA products from the total extracted RNA using a two-step method, which was then inactivated in accordance with the kit instructions and stored at -20°C until use.

### Primer design and synthesis

PRIMER5.0 software was used to design HOXD11-specific primers, through searching dbEST and nr databases (the nonredundant set of GenBank, EMBL, DDBJ database sequences), the nucleotide sequences used as primers had no specificity and no DNA polymorphism. The primers were synthesized by Beijing Bioko Biotechnology Co. and had the following sequences: HOXD11 (F): TCTCCGAGTCCTCGTGGGGA; HOXD11 (R): GCAAACACCAGCGCCTTCTA.

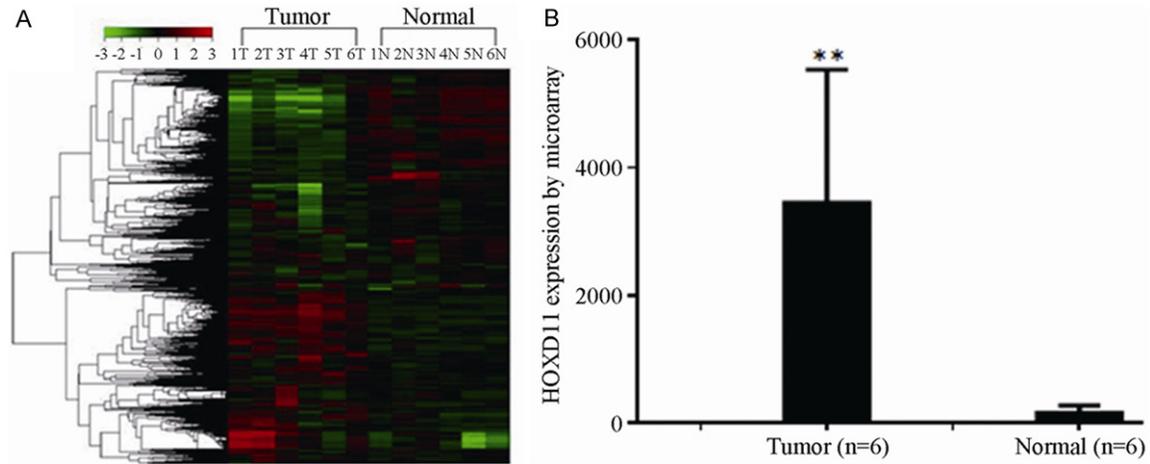
### Detecting expression of HOXD11 mRNA by qRT-PCR

A 7500 quantitative PCR instrument (Applied Biosystems, Foster City, CA, USA) was used together with a TAKARA SYBR Premix Ex Taq™ II (TliRNaseH Plus) fluorescence assay kit. Reverse transcription cDNA products were used as templates for quantitative PCR amplification based on the kit instructions. The 20-µL of reaction system included 10 µL of SYBR Green PCR Master Mix (Shanghai Genecore Biotechnology Co. Ltd., Shanghai, China), 0.5 µL of 10 µM upstream and downstream primers, 1 µL of cDNA, 7.6 µL of ddH<sub>2</sub>O, and 0.4 µL of ROC Reference Dye, which was mixed in the dark.

The conditions for mRNA and GAPDH amplification were as follows: denaturation at 95°C for 30 s, followed by 95°C for 5 s, and 60°C for 30-34 s, for a total of 40 cycles.

To analyze the qRT-PCR results, we calculated the relative target gene expression:  $= 2^{-\Delta\Delta CT} / 2^{-\Delta\Delta CT}$ , where  $\Delta CT = CtC - CtA/CtD - CtB$  and

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**Figure 1.** Upregulation of HOXD11 in ESC by microarray. A. Heatmap of expression profiles of mRNA-microarray expression profiling data, the 2,630 genes that showed significant expression changes (1,152 upregulated and 1,478 downregulated). B. Upregulation of HOXD11 was detected in 6 primary ESC by mRNA-microarray. Normal, nontumor tissue; Tumor, tumor tissue; \*\* $P < 0.01$ ; Mann-Whitney test.

$\Delta\Delta CT = (CtC - CtA) - (CtD - CtB)$  (in which CtA was the Ct value of the internal control in cancer tissue, CtB was the Ct value of the internal control in paired normal tissue, CtC was the Ct value of the target gene in cancerous tissue, and CtD was the Ct value of the target gene in normal tissue).

### Immunohistochemistry

The 4- $\mu$ m paraffin sections were washed with phosphate-buffered saline (PBS), and then incubated at room temperature with 3% hydrogen peroxide for 30 min, followed by antigen retrieval using the boiling method. Blocking serum working solution was added in a drop-wise manner and incubated at room temperature for 30 min. An appropriate amount of diluted HOXD11 rabbit polyclonal antibody (sc-154, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added and cultured at 4°C overnight. Biotin-labeled horseradish peroxidase-labeled goat anti-mouse/rabbit secondary antibody was added (Beijing ZSGB-Bio Origene Co., Ltd.), and incubated at 4°C for 30 min. The staining liquids were mixed in accordance with the ratio of 1 mL double-distilled water/one drop reagent A, then added one drop of reagent B and mixed again. Staining was observed under a microscope. When staining was fully completed, we added PBS to terminate the reactions, followed by hematoxylin re-staining for 15-30 s. PBS rather than primary antibody was used as a negative control.

### Analysis of results

All pathological sections were scanned using an Aperio pathology scanning system and analyzed using ImageScope software. Different regions were selected to analyze the expression of HOXD11.

### Statistical analysis

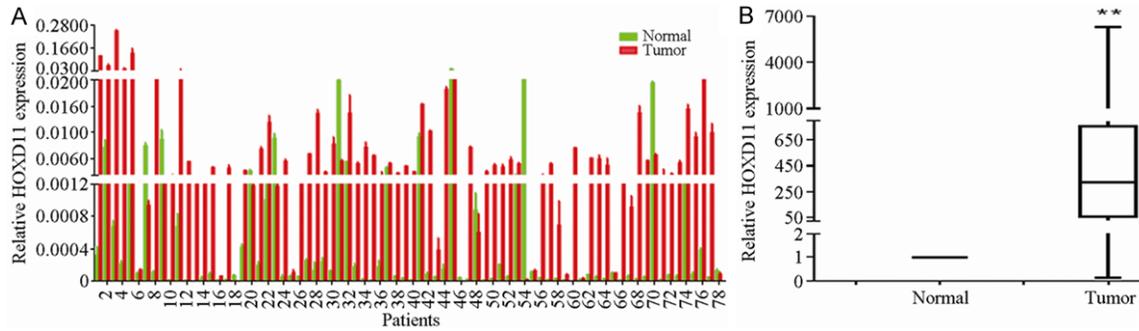
All data were analyzed using SPSS20.0 statistical software (SPSS, Inc., Chicago, IL, USA). Experimental data were expressed as the mean  $\pm$  standard deviation ( $\bar{x} \pm s$ ). qRT-PCR data were analyzed using the Normal test, and the Mann-Whitney test was used based on the data types for analysis, with  $P < 0.05$  or  $P < 0.01$  considered to indicate a statistically significant difference; these differences are marked with \* or \*\* in the figures, respectively.

## Results

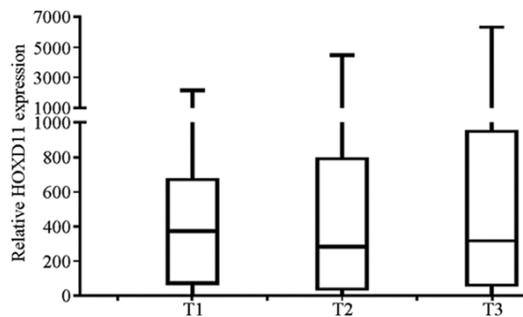
### Microarray screening

New-generation microarray technology was used to detect the mRNA expression profiles in ESC and PNT. The results showed that 1152 mRNAs were upregulated (greater than 2-fold), while 1478 mRNAs were down-regulated. HOXD11 exhibited significantly differential expression between SC and PNT. Compared with PNT, HOXD11 was upregulated by 15.3-fold in ESC. The results are shown in **Figure 1**.

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**Figure 2.** Upregulation of HOXD11 in ESC by qRT-PCR. A. The relative expression levels of HOXD11 in 78 ESC and paired nontumor tissue by qRT-PCR ( $2^{-\Delta Ct}$ ). Normal, nontumor tissue; Tumor, tumor tissue. B. The expression levels of HOXD11 in 78 ESC and paired nontumor tissue by qRT-PCR ( $2^{-\Delta\Delta Ct}$ ). Normal, nontumor tissue; Tumor, tumor tissue; \*\* $P < 0.01$ , Mann-Whitney test.



**Figure 3.** The expression level of HOXD11 and its clinical significance. Correlation between the expression level of HOXD11 and TNM staging of the 78 ESC cases. No data were statistically different.

### QRT-PCR

The expression levels of HOXD11 mRNA in ESC and PNT of 78 ESC cases are shown in **Figure 2A**. The results showed that compared with PNT, the expression of HOXD11 mRNA in ESC was significantly increased. The qRT-PCR data were analyzed using SPSS20.0 statistical software; the Normal test revealed a non-normal distribution of the data, so the Mann-Whitney test was used for analysis. The expression of HOXD11 in 78 ESC cases was significantly higher than in PNT ( $P < 0.01$ ) (**Figure 2B**).

### Correlation analysis

The analysis of the different invasive ranges of these 78 ESC cases (T1-T3) showed that the expression level of HOXD11 was gradually increased from T1-T3. Because of the limited case numbers, statistical analysis revealed no significant difference (**Figure 3**).

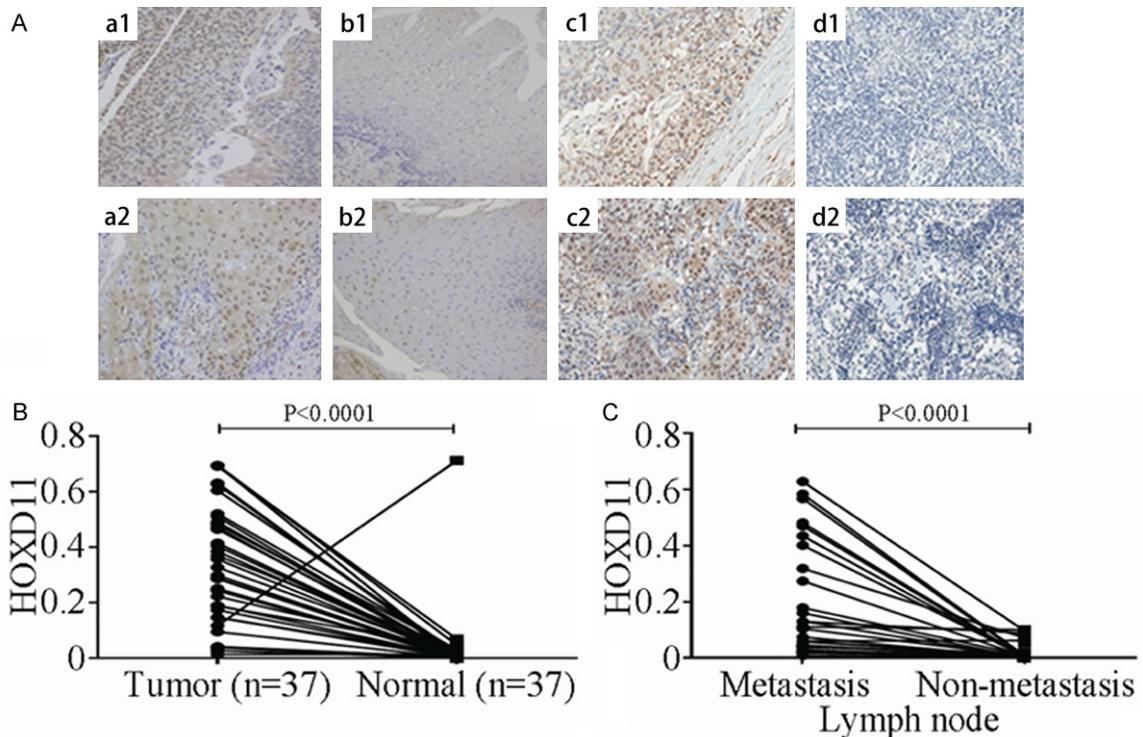
### Immunohistochemistry

Immunohistochemical analysis was used to detect the ESC and PNT samples of 37 ESC cases, as well as partial positive and negative metastatic lymph nodes. Based on the degrees of immunohistochemical-positive expression, the positive expression rate was defined as high expression ( $\geq 50\%$ ), low expression ( $< 50\%$ ), and non-expression (0%). Mapping according to the expression values of each phase clearly revealed that the expression of HOXD11 was tissue-specific, and the expression of HOXD11 in ESC was significantly higher than that in PNT ( $P < 0.001$ ). Further analysis showed that HOXD11 was very lowly expressed in negative lymph nodes, while the expression in ESC-positive lymph node tissues was significantly higher ( $P < 0.001$ ), as shown in **Figure 4**.

### Discussion

ESC is the most common disease of EC in Chinese and exhibits insidious onset. The degree of malignancy of ESC is high, and most patients are already been in the middle and late stage when diagnosed. In recent years, a growing number of studies have found that abnormal expression of gene families is closely associated with a variety of malignancies [14]. The human and vertebrate HOX gene family includes 39 genes; each HOX gene contains a 180-base pair DNA nucleotide coding sequence, which may encode for highly conserved homoeodomains containing 60 amino acids. HOX genes are the main regulatory genes involved in the development of the central nervous system, skeletal system, gastrointestinal system, geni-

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**Figure 4.** Clinical significance of HOXD11 in human ESC. A. Two representative images of IHC staining with an anti-HOXD11 antibody in 37 metastatic ESC patients, from left to right: tumor tissue (a1, a2); normal tissue (b1, b2); positive metastatic lymph nodes (c1, c2); negative metastatic lymph nodes (d1, d2), 200x magnification. B. Bar chart of average staining intensity of HOXD11 in tumor/normal tissue of 37 metastatic ESC patients ( $P < 0.0001$ , Mann-Whitney test). C. Bar chart of average staining intensity of HOXD11 in positive/negative metastatic lymph nodes of 37 metastatic ESC patients ( $P < 0.0001$ , Mann-Whitney test).

tourinary system, and limbs [15], among which HOXD is located at 2q31 [16]. As positive and negative regulatory genes involved in cell proliferation and differentiation, HOX genes are not only responsible for regulating the proliferation, differentiation, and migration of embryonic cells, but also play regulatory roles in a variety of biological behaviors of histiocytic cells in the mature body [17-20]. Recent studies have found that abnormal expression of HOX genes is closely related to the development of a variety of tumors [21-24], and changes of in the expression patterns of these genes may be a key mechanism promoting or inhibiting tumorigenesis. HOX genes also participate in the occurrence and development many human malignancies [25, 26]. There have been no reports regarding the expression of HOXD11 in ESC.

In this study, we used the human Agilent mRNA microarray to detect differential mRNA expression in 6 pairs of ESC and PNT sample groups, screened for differentially expressed mRNAs (up- or down-regulated by more than 2-fold),

and found that HOXD11 exhibited a significant expression difference in ESC and PNT. Compared with PNT, HOXD11 was upregulated by 15.3-fold in ESC. Additionally, qRT-PCR was used to detect the expression levels of HOXD11 mRNA in ESC and PNT of 78 ESC cases. HOXD11 mRNA was found to be significantly highly expressed in ESC compared to in PNT ( $P < 0.01$ ), indicating that HOXD11 is involved in the occurrence and development of ESC.

Immunohistochemical staining results showed that the HOXD11 protein was mainly expressed in the nuclei and partially expressed in the cytoplasm. Among the 37 paired tissue samples, all ESC specimens showed high positive expression of HOXD11, which was significantly higher than in PNT. Additionally, HOXD11 was highly expressed in positive metastatic lymph nodes, but only very lowly expressed in negative lymph nodes; the difference was statistically significant ( $P < 0.001$ ). These results clearly demonstrate that the expression of HOXD11 was tissue-specific, suggesting that HOXD11 can be

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used as a regulatory molecule involved in the occurrence and development of ESC, and may play an important role in the occurrence, development, and metastasis of EC.

The differential expression of HOXD11 in ESC and PNT showed that HOXD11 was highly expressed in cancer tissues and positive metastatic lymph nodes. With the increased depth of tumor invasion, the expression level of HOXD11 was also increased from T1-T3, suggesting that the expression level of HOXD11 is associated with the invasion depth of the tumor. However, we did not find a significant correlation between HOXD11 expression and T staging of ESC, which may be related to the small number of tissue samples examined. In this study, 78 ESC patients showed no distant lymph node metastasis, which may be related to the loss of surgical indications or sampling difficulties in most patients with distant metastasis. While studying the effects of the differential expression of HOXD11 in ESC and PNT on N-staging of ESC patients, the total case numbers detected were fewer, and therefore, statistical analysis was not performed.

In summary, we found that the expression of HOXD11 mRNA in human ESC was higher than that in PNT. The expression of HOXD11 protein in human ESC was also significantly higher than that in PNT, and was only very lowly expressed in negative lymph nodes, but the expression in positive lymph nodes was significantly higher than that in negative lymph nodes.

We found that HOXD11 was highly expressed in ESC and metastatic lymph nodes and that the expression level of HOXD11 was increased with an increasing invasion depth of the tumor. This finding may provide new targets for studies of molecular and signaling pathway mechanisms of ESC. This study also had some limitations; the sample sizes for qRT-PCR and immunohistochemistry were small, and statistical analysis for N staging of ESC could not be performed. Additionally, the knockdown and degradation of HOXD11 by siRNA was not evaluated, and studies examining the impacts of HOXD11 expression on cell cycle and apoptosis rate of ESC were also not performed.

### Disclosure of conflict of interest

None.

**Address correspondence to:** Drs. Xiufeng Cao and Suqing Li, Department of Oncological Surgery, The First Hospital of Nanjing Affiliated to Nanjing Medical University, Nanjing 210006, China. Tel: +86 25 87721050; Fax: +86 25 52269924; E-mail: xiufeng-caocn@163.com (XFC); hanliud@126.com (SQL)

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