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## **Disordered Expression of HOX Genes in Human Non-Small Cell Lung Cancer**

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Running Title: Disorder of HOX code in lung cancer

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## ABSTRACT

We hypothesized that the disordered tissue architecture in cancer results from the steps that the cells execute the program designed during ontogeny in a spatiotemporally inappropriate manner. HOX genes are known as master regulators of embryonic morphogenesis, and encode transcription factors which regulate the transcription of the downstream genes to realize the program of body plan. In this study, we quantified the expression levels of 39 HOX genes in 41 human non-small cell lung cancer (non-SCLC) and non-cancerous lung tissues by a comprehensive analysis system based on the real-time RT-PCR method. We found that the expression levels of HOXA1, A5, A10 and C6 in squamous cell carcinoma tissues (and HOXA5 and A10 in adenocarcinoma tissues) were significantly higher than those in the non-cancerous tissues. Comparison of HOX gene expressions between adenocarcinoma and squamous cell carcinoma tissues showed higher expressions of HOXA1, D9, D10 and D11 in squamous cell carcinoma tissues than in adenocarcinoma tissues. Immunohistochemical analysis revealed that HOXA5 and A10 proteins were localized in the cytoplasm of tumor cells in both adenocarcinoma and squamous cell carcinoma tissues. These results suggest that the disordered patterns of HOX gene expressions were involved in not only the development of non-SCLC but also histological diversity such as adenocarcinoma and

squamous cell carcinoma.

## **INTRODUCTION**

It can be hypothesized that the disordered tissue architecture in cancer results from the steps that the cells execute the program designed during ontogeny in a spatiotemporally inappropriate manner. In other words, the cancer cells may partly follow the ontogenic program to display the transformed phenotype and dynamic movement to surrounding or distant areas. Homeobox genes are a superfamily of the genes encoding transcription factors, which regulate the embryonic morphogenesis in animals (1). The clustered group of homeobox genes in human are called HOX genes. The genes contain a 180-bp DNA sequence (homeobox), which encodes a highly conserved 60-amino-acid homeodomain. The HOX proteins function as transcription factors through their homeodomain, which is responsible for recognition and binding of sequence-specific DNA motifs (2, 3). In human genome, 39 HOX genes are clustered in a similar arrangement of 13 paralog groups in four different chromosomal regions, HOXA, B, C, and D (4). During embryonic morphogenesis, HOX gene expressions are regulated in a precise spatiotemporal manner: the 3'-end genes of the clusters are expressed earlier and in more anterior domain than the genes localized in more 5'

positions (5).

Recently, there is a growing interest in investigating relationship of inappropriate expressions of HOX genes with carcinogenesis or malignant progression. Some HOX genes exhibit different expression levels in a variety of human cancers including kidney, colon, bladder, melanoma and prostate cancers, compared to normal tissues from which they were derived (6-10). For example, HOXB5 and B9 are expressed in normal kidney but not in renal cancer whereas the expression of HOXC11 is observed in human renal cancer but not in normal kidney (6). In human prostate cancer, overexpression of HOXC8 correlates with loss of differentiation phenotype (10). Increased expressions of HOXC4, C5, C6 and C11 are likely to be involved in the development of human bladder transitional cell carcinomas (8). We also found that the expression levels of *HOXA11*, *A13*, *B9*, *D12* and *D13* were higher in cutaneous melanoma than in nevus pigmentosus (9).

There are few reports on HOX gene expressions in human lung cancer. The expression of some HOX genes in human small-cell lung cancers xenografted into nude mice was different from that in normal lung (11). Analysis of HOX gene expressions by using degenerate reverse transcription-PCR showed that HOXA9, A10 and B9 were frequently up-regulated in human lung cancer cell lines and lung tumor tissues (12).

Lechner et al. reported that some HOX genes of which expression was restricted to pulmonary embryogenesis were re-expressed in lung cancer cells (13). To better understand the roles of HOX genes in lung cancer, we investigated the HOX gene expressions in 41 human non-small cell lung cancer tissues by a comprehensive analysis system to quantify the expressions of 39 human *HOX* genes based on the real-time reverse transcription-PCR method. And we further examined the relationship between HOX gene expression and clinicopathologic parameters.

## **MATERIALS AND METHODS**

**Patients and samples.** A total of 41 primary lung cancer tissues (28 adenocarcinoma and 13 squamous cell carcinoma tissues) and 15 non-cancerous tissues were obtained from surgical specimens resected from patients who underwent radical surgery without any preoperative chemotherapy or radiotherapy at the Department of Surgical Oncology, Hokkaido University Hospital and 33 affiliated hospitals in Hokkaido, Japan, between January 2001 and September 2004. Only those patients who agreed with the aim and contents of this study and who provided their written informed consent were subjected to the study. Tumor and normal tissue samples of about 5 mm-size were immediately cut from the lung resected by a standard surgical procedure, snap frozen in liquid

nitrogen, and stored at  $-80^{\circ}\text{C}$  until use. Part of each tumor sample piece was cut and examined for histopathological analysis, and the sample pieces were used for RNA extraction. All procedures in this portion of the study were approved by the Ethics Committee of Hokkaido University and the independent internal ethics committees of the affiliated hospitals.

Histological subclassification and staging of the tumors were done by reviewing the specimens taken for pathological diagnosis, according to the TNM classification. The tumor status of each case was categorized based on the pTNM pathological classification of the International Union Against Cancer (14). Pertinent major clinicopathological parameters are shown in Table 1.

**RNA extraction and cDNA preparation.** Total RNA was extracted from powdery frozen tissues of each clinical specimen which had been crushed in liquid nitrogen with a CRYO-PRESS compressor (Microtec Niton, Chiba, Japan) with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. For exclusion of contaminated genomic DNA, 50 microg of total RNA was incubated for 30 min at  $37^{\circ}\text{C}$  in 50 microL of reaction mixture containing 40 mM Tris-HCl (pH 7.2), 10 mM NaCl, 6 mM  $\text{MgCl}_2$ , 2 mM dithiothreitol, 0.04 U/microL PQ1 DNase (Promega, Madison, WI), and 0.4 U/microL RNase inhibitor. Reverse transcription reaction for

real-time PCR was performed by the same method as described in our previous report (15).

**Quantitative real-time PCR.** Quantitative RT-PCR assays were carried out by using ABI PRISM 7900HT (Applied Biosystems) with SYBR-green fluorescence under the same condition as described in our previous report (15). The primer sets for amplification of 39 HOX genes and beta-actin gene were listed in our previous report (15).

**Immunohistochemistry.** Surgical specimens were fixed in a 10% formalin solution and embedded by routine methods in paraffin for sectioning at a thickness of 4 microm. Immunohistochemical reactions were carried out by the streptavidin-biotin-peroxidase methods. Sections were deparaffinized in xylene, and rehydrated through a graded series of ethanol. The specimens were boiled in target retrieval solution at 10x concentration (Dako Cytomation, Glostrup, Denmark; diluted at 1:10) in a pressure cooker for 8 min. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide methanol for 10 min. The specimens were then washed with phosphate-buffered saline (PBS, pH7.2), placed in a serum-free protein blocker (DAKO corporation, Carpinteria, CA) for 5 min, and incubated overnight at 4 °C with the primary antibodies, anti-HOXA5 goat immunoglobulin polyclonal antibody and anti-

HOXA10 goat immunoglobulin polyclonal antibody (Santa Cruz Biotechnology, CA; diluted at 1:200). After washing in PBS, Biotinylated Link Universal (Dako Cytomation) was applied, and the specimens were incubated at room temperature for 20 min. After washing in PBS, the specimens were incubated in streptavidin conjugated with peroxidase solution (Nichirei, Tokyo, Japan) at room temperature for 15 min. After washing in PBS, immunohistochemical reactions were developed in freshly prepared 3, 3'-diaminobenzidine tetrahydrochloride (Nichirei). The sections were counterstained with hematoxylin and coverslipped in a systemic mounting medium. As a negative control, we omitted the primary antibody.

**Statistical analysis.** The relationship between the expression of each HOX and each clinicopathological parameter was determined by the Mann-Whitney U test, the Kruskal-Wallis rank test or the Spearman rank correlation test. The statistical software package applied was Statview 5.0 for Macintosh (SAS Institute, Cary, NC). A p value less than 0.01 was considered statistically significant.

## **RESULTS**

**Expressions of HOX genes in non-cancerous tissues.** Figure 1 shows the expression levels of 39 HOX genes in 15 non-cancerous lung tissues which were obtained from the

surgically resected tissues of lung cancer patients. The non-cancerous tissues showed relatively high expressions of HOXB2, B3, B4, B5 and B6 which were located at 3'-side of cluster B, and HOXA3 (median values of relative expression ratio were more than 0.0025) whereas they showed relatively low or no expression of Abd-B family of HOX genes (paralog 9 to 13) which were located at 5'-side of each cluster. Generally, there was a tendency that the expression levels varied relatively in a wide range within each sample.

**Differences in HOX gene expression between lung cancer and non-cancerous tissues.** We compared the expression levels of each HOX gene between lung cancer tissues and non-cancerous tissues. Four of 39 HOX genes, HOXA1, A5, A10 and C6, showed significant differences in the expression levels between cancer and non-cancerous tissues ( $p < 0.01$ , Mann-Whitney U-test) (Figure 2). The expressions levels of all the 4 HOX genes in squamous cell carcinoma tissues were higher than those in non-cancerous tissues. In adenocarcinoma tissues, the expression levels of HOXA5 and A10 were high compared to non-cancerous tissues. We also found significant differences in the expression levels between squamous cell carcinoma tissues and adenocarcinoma tissues: HOXA1, D9, D10 and D11 showed higher expressions in squamous cell carcinoma than in adenocarcinoma tissues (Figure 3).

**HOX gene expression and clinicopathologic parameters.** We next examined whether there was a relationship between HOX gene expression patterns and clinicopathologic parameters. Although we did not find significant difference in any HOX gene expression between cancer tissues with metastasis to lymph node and those without it, there was a tendency that the expression levels of HOXB7 in adenocarcinoma tissues with lymph node metastasis were low compared to those without it ( $p = 0.0295$ , Mann-Whitney U-test). There was no HOX gene which characterized the disease stage (IB, IIB and IIIA) or histological types of differentiation. We also examined the correlation between HOX gene expressions and levels of tumor markers. As shown in Figure 4, there was a slight correlation between CEA levels and HOXD3 or HOXD4 expression levels (HOXD3 vs CEA:  $r = 0.389$  with  $p = 0.0166$ ,  $n = 39$ ; HOXD4 vs CEA:  $r = 0.407$  with  $p = 0.0122$ ,  $n = 39$ ). There was no HOX gene of which expression correlated with the levels of other tumor markers such as SCC, NSE, CYFRA21-1 and ProGRP or smoking.

**Immunohistochemical examination for HOXA5 and A10 in non-SCLC specimens.**

We subjected non-SCLC tissues and their adjacent non-cancerous tissues of 8 specimens (4 adenocarcinoma and 4 squamous cell carcinoma) to immunohistochemical staining to detect the protein expression of HOXA5 and A10 of which expressions were

higher at mRNA levels in non-SCLC than non-cancerous tissues. Figure 5 shows representative examples of immunohistochemical staining for HOXA5 and A10. Immunoreactivity of HOXA5 and A10 was observed in cytoplasm of lung cancer cells and bronchial epithelial cells but neither in alveolar epithelial cells nor in interstitial tissues of lung cancer. The immunoreactivity of both HOX proteins was detected in squamous metaplasia tissues involved in squamous cell carcinoma. Intensity of immunoreactivity of both HOX proteins was consistent with the expression levels of mRNA for the HOX genes in the 8 samples examined (data not shown).

## **DISCUSSION**

In this study, we tried to elucidate whether inappropriate expressions of HOX genes were involved in the development and progression of non-small cell lung cancer (non-SCLC). Our comprehensive analysis of 39 HOX genes in 41 non-SCLC and 15 non-cancerous tissues revealed the expression patterns of HOX genes characteristic of adenocarcinoma or squamous cell carcinoma. Compared to non-cancerous tissues, adenocarcinoma tissues showed enhanced expressions of HOXA5 and A10, and squamous cell carcinoma tissues showed enhanced expressions of HOXA1 and C6 in addition to the two HOX genes. It is likely that the increased expressions of HOXA5

and/or A10 are positively associated with the development of non-SCLC. The HOXA5 gene product is a potent transactivator of the p53 promoter (16). The HOXA10 gene product activates p21 transcription and results in cell cycle arrest and differentiation in myelomonocytic cells (17). These reports suggest anti-oncogenic function of both HOXA5 and A10 proteins, which is contradictory to our assumption of the roles of the two HOX genes in the development of non-SCLC. However, our immunohistological analysis suggested to us the possibility of interesting interpretation: Unexpectedly, both HOX proteins existed in the cytoplasm but not in the nuclei of cancer cells, indicating that they do not function as transcription factors to regulate downstream target genes such as p53 and p21. Recently, HOX proteins are known to have other functions than DNA binding-transcription factors. For example, HOXA13 and D13 interact with Smad protein to modify Smad transcriptional activation (18). Homeodomain of HOX proteins bind to CREB binding protein (CBP) and block its acetyltransferase activity (19). These indicate that HOX proteins modulate the transcriptional activity through interaction with other proteins in a non-DNA binding manner. Therefore, we need to consider a possibility that HOXA5 and A10 proteins interfere with the function of anti-oncogenic molecules in lung cancer cells.

It is known that HOXA1 and C6, which were upregulated in squamous cell

carcinoma, affect the cell growth and death. Forced expression of HOXA1 in human breast cancer cells results in increased cell growth activity (20). Loss of HOXC6 expression by transfection with siRNA induces apoptosis in human prostate cancer cell lines (21). Therefore, the increased expressions of HOXA1 and C6 may play a promoting role in the primary growth of lung squamous cell carcinoma tissues.

Between adenocarcinoma and squamous cell carcinoma, 4 HOX genes presented different expressions. The expression levels of HOXA1, D9, D10 and D11 were significantly higher in squamous cell carcinoma than in adenocarcinoma. The expression levels of HOXD12 and D13 also tended to be high in squamous cell carcinoma ( $p = 0.05$  and  $0.01$ , respectively, Mann-Whitney U-test, data not shown). We examined the relationship between the expression of these HOX genes, and SCC and CYFRA21-1 which were tumor markers of lung squamous cell carcinoma. There was no relationship between them. As the expressions of HOXA1 and HOXD9-11 genes were also upregulated in oral squamous cell carcinoma (our unpublished data), these HOX genes are likely involved in maintaining the squamous cell carcinoma-related phenotypes other than SCC and CYFRA21-1.

We found a positive correlation between CEA levels and HOXD3 or D4 expression levels although their correlation coefficients were not so high. There are many

common phenomena between cancer and embryonic morphogenesis, one of which is the production of oncofetal proteins. This evidence provides us the possibility to consider HOX genes as candidates which regulate the production of oncofetal proteins.

In conclusion, the results presented here suggest that the disordered patterns of HOX gene expressions were involved not only in the development of non-SCLC but also in the histologically aberrant diversity such as adenocarcinoma and squamous cell carcinoma.

#### **ACKNOWLEDGMENTS**

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apoptosis in prostate cancer cells. *Oncogene* 24: 188-198, 2005.

## FIGURE LEGENDS

### Figure 1

Expression profiling of 39 HOX genes in non-cancerous tissues. The relative levels of HOX mRNA were determined by quantitative RT-PCR in 15 of the non-cancerous tissues. The graphs of HOX cluster A, B, C and D are lined from top to bottom. HOX gene paralogs 1 to 13 are lined from left to right of X-axis in each graph. The distribution of the relative expression ratios (HOX /beta-actin) was summarized by using box-whisker graphs. The central box in each plot shows the interquartile (25th to 75th percentile) range. The line in the box shows the median. The whiskers (vertical bars) were drawn to the 90th and 10th percentiles. Extreme values greater than the 90th percentile and less than the 10th percentile were plotted individually.

### Figure 2

Different expression levels of HOX genes between non-SCLC and non-cancerous tissues. The distribution of the relative expression ratios (HOX/beta-actin) was summarized by using box-whisker plots in the same manner as in Figure 1. Shaded, hatched and open boxes represent adenocarcinoma, squamous cell carcinoma and non-cancerous tissues, respectively. P values were determined by the Mann-Whitney U-

test.

### Figure 3

Different expression levels of HOX genes between adenocarcinoma and squamous cell carcinoma tissues. The distribution of the relative expression ratios (HOX/beta-actin) was summarized by using box-whisker plots in the same manner as in Figure 1. Shaded and hatched boxes represent adenocarcinoma and squamous cell carcinoma, respectively. P values were determined by the Mann-Whitney U-test.

### Figure 4

Relationships between the levels of CEA and HOXD3 or D4 in non-SCLC. (A) Correlation plots for HOXD3 (x-axis) vs. CEA (y-axis). The Spearman correlation coefficient was  $r = 0.389$  with  $p = 0.0166$  ( $n = 39$ ). B, Correlation plots for HOXD4 (x-axis) vs. CEA (y-axis). The Spearman correlation coefficient was  $r = 0.407$  with  $p = 0.0122$  ( $n = 39$ ). Closed and open circles represent adenocarcinoma and squamous cell carcinoma samples, respectively.

### Figure 5

Immunohistochemical staining of HOXA5 and A10. Immunoreactivity of HOXA5 and A10 was observed in tumor cells but not in interstitial tissues (A, HOXA5; C, HOXA10, original magnification x40). Immunoreactivity of both HOXA5 and A10 was observed in the cytoplasm but not the nuclei of the tumor cells (B, HOXA5; D, HOXA10, original magnification x400).

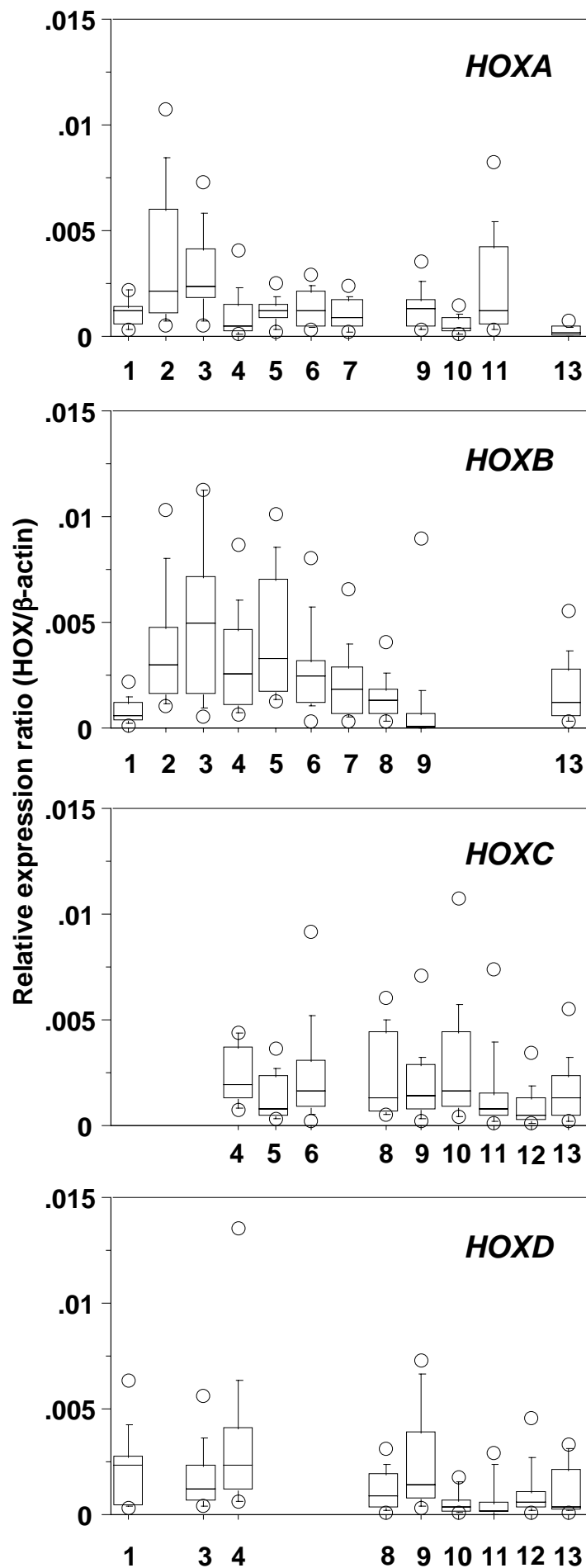


Figure 1-Abe et al.

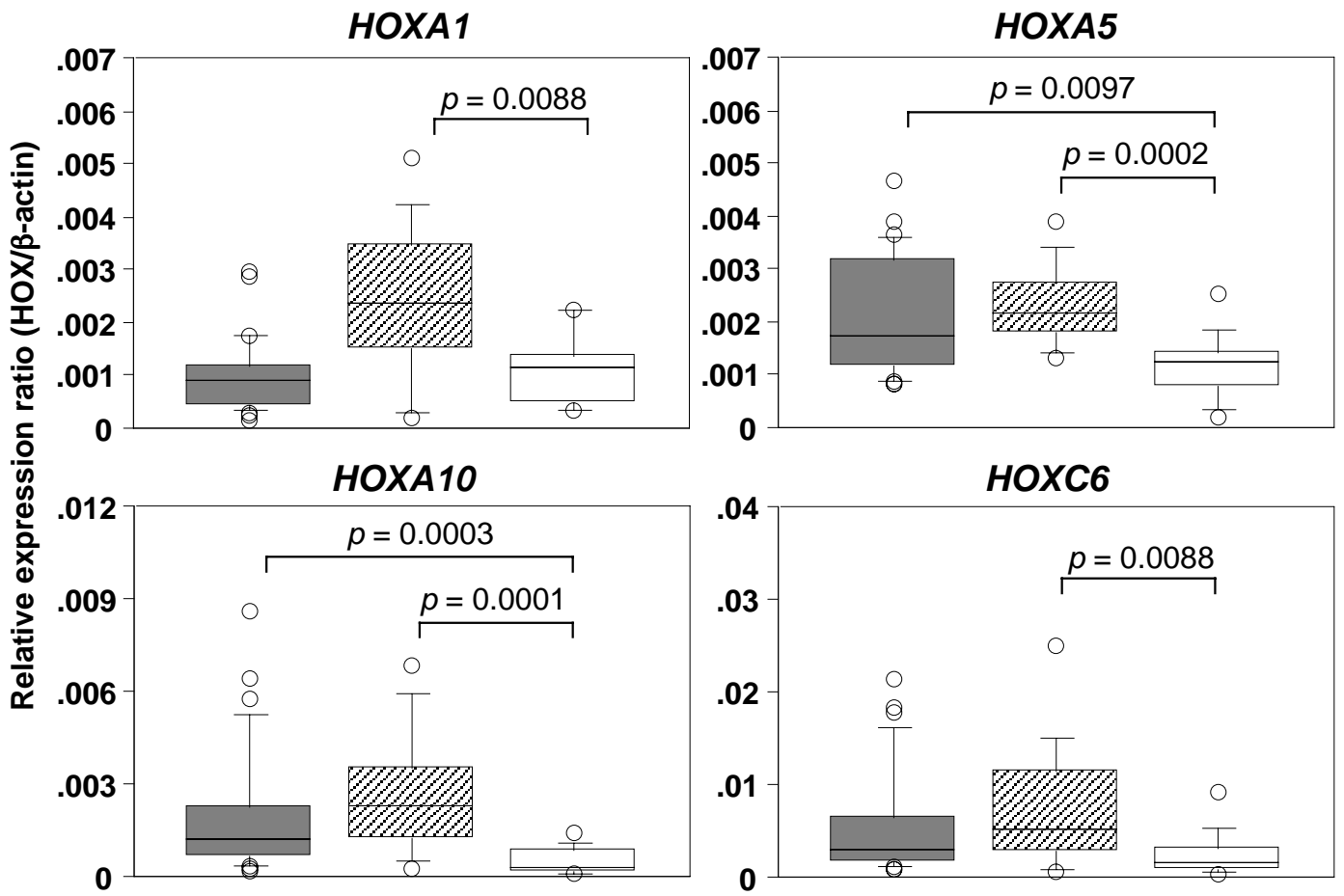


Figure 2 Abe et al.

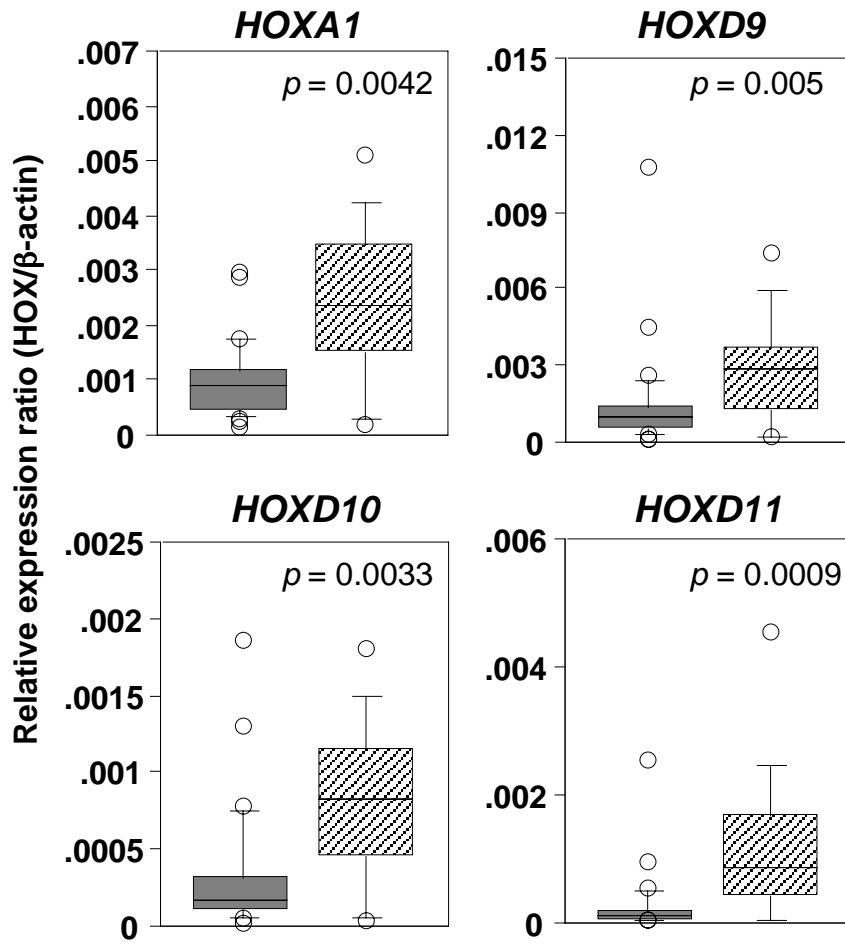


Figure 3 Abe et al.

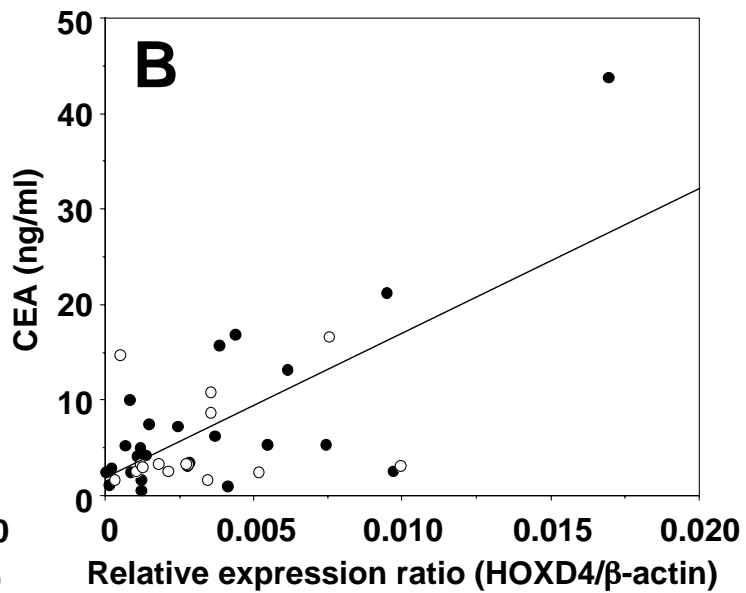
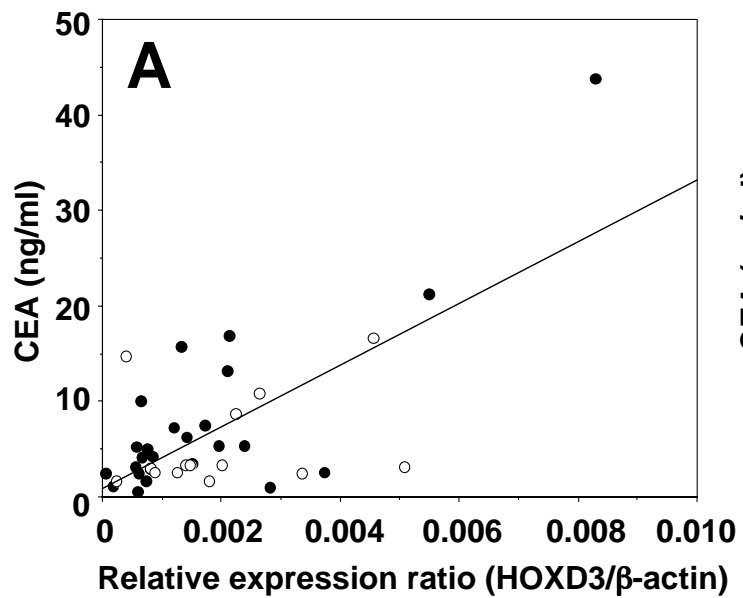


Figure 4 Abe et al.

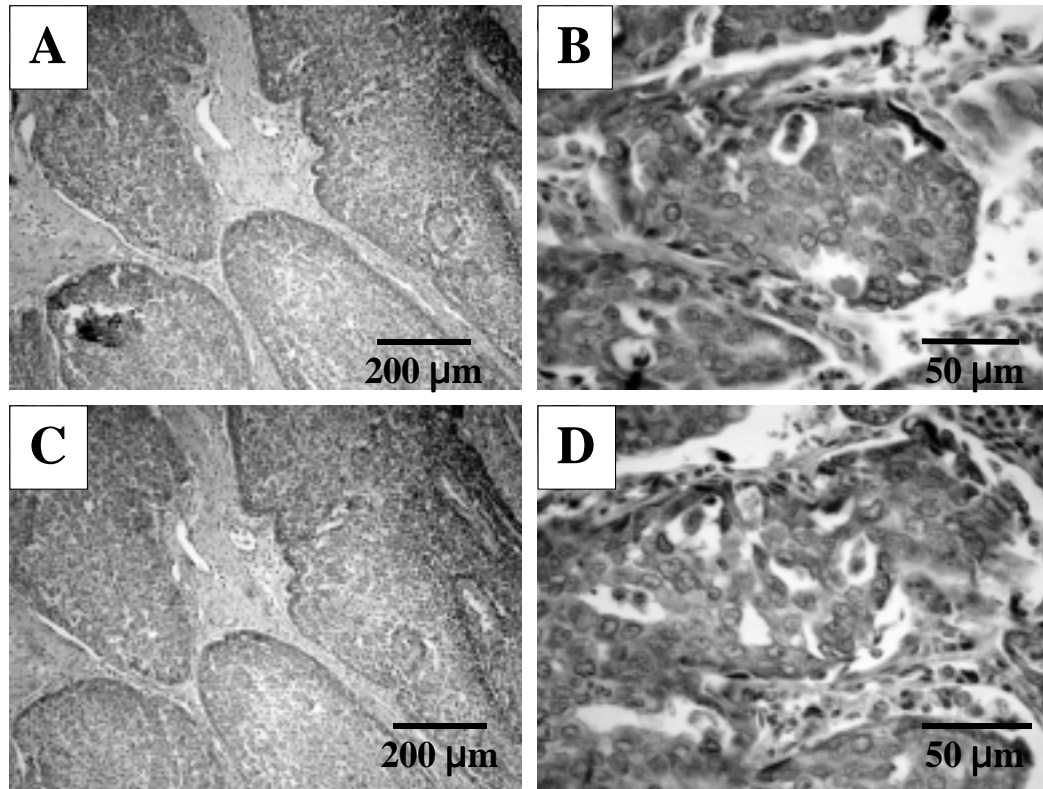


Figure 5 Abe et al.

Table I Clinicopathologic parameters

Parameters	Adenocarcinoma (n = 28)	Sqaumous cell carcinoma (n = 13)
Age, median (range)	68 (39 - 82)	72 (47 - 79)
Gender		
male	16	12
female	12	1
Differentiation		
well	8	1
moderately	11	10
poorly	7	2
pTNM		
pT2N0M0	20	7
pT2N1M0	5	3
pT2N2M0	3	3
pStage		
IB	20	7
IIB	5	3
IIIA	3	3
Smoking		
+	14	10
-	12	2
Brinkman's index		
≥ 600	5	6
< 600	21	6
Tumor marker, median (range)		
CEA (ng/ml)	5.1 (0.6 - 48.0)	3.2 (1.7 - 16.6)
SCC (ng/ml)	0.5 (0 - 1.5)	1.4 (0.7 - 2.3)
NSE (ng/ml)	4.6 (0 - 9.7)	8.2 (0 - 11.9)
CYFRA21-1 (ng/ml)	0.9 (0 - 7.0)	5.2 (0 - 53.0)
ProGRP (pg/ml)	8.5 (0 - 28.5)	17.4 (0 - 46.5)

Tumor status at the operation was according to the TNM classification (UICC, 6th edition).