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Aberrant expression of HOX genes in human invasive breast carcinoma

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Abstract. HOX genes are known not only as master genes that control the morphogenesis, but also as regulator genes that maintain tissue or organ specificity in the adult body. We hypothesized that dysregulated expression of HOX genes was associated with tumor development and malignant progression such as invasion and metastasis. In this study, we analyzed the expression patterns of 39 HOX genes in human invasive ductal breast cancer tissues and normal tissues by the real-time RT-PCR method. We found 11 HOX genes (HOXA1, A2, A3, A5, A9, C11, D3, D4, D8, D9 and D10) expression levels of which were significantly different between cancerous and normal tissues. All 10 genes except HOXC11 were expressed at lower levels in cancerous tissues than normal tissues. Comparing expression levels of each HOX gene among the different types of cancer tissues, the expression level of HOXB7 was lower in lymph node metastasis-positive cancer tissues than negative cancer tissues; those of HOXD12 and D13 were higher in progesterone receptor-positive cancer tissues than negative cancer tissues; and the expression level of HOXC5 was lower in cancerous tissues with mutated-type p53 than in normal and cancerous tissues with wild-type p53. These results suggest that the aberrant expression of HOX genes is related to the development of breast cancer and malignant behavior of cancer cells.

Introduction

The disordered tissue architecture, invasion and metastasis in cancer can be considered as that resulting when tumor cells recognize abnormal positional information. HOX genes are master regulators of morphogenesis and give cells the positional information to go onto regional specification (1). 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 the 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, the HOX genes determine positional identity along the anterior-posterior and secondary axes in animals. Within each cluster, HOX genes located at the 3'-extremity are activated first, then in the anterior embryonic domains, whereas 5'-located genes are transcribed subsequently and in more caudal areas, which is the property called colinearity rule (5).

HOX genes are also expressed in some normal adult organs in characteristic patterns besides the spatio-temporally controlled expression during embryogenesis. Our previous study using a quantitative real-time RT-PCR method uncovered that 5' HOX genes were expressed in the caudal parts of organs in the body, and that a higher number of HOX genes were expressed in more caudal regions (6). It is also well known that the expression of HOX genes, such as HOXA5 and A10, play an important role in differentiation and maturation of hematopoietic cells (7,8). This evidence suggests that HOX genes work to maintain the tissue-specific architecture and function in adult tissue and organs.

Comparative analysis of HOX gene expression between cancerous and normal tissues showed the misexpression of a particular HOX gene in certain types of carcinomas. HOXB5 and B9 are expressed in normal kidney but not in renal cancer, whereas the expression of HOXC11 is observed in human renal but not in normal kidney cancer (9). In human prostate cancer, overexpression of HOXC8 correlates with the loss of a differentiated phenotype (10). Increased expression of HOXC4, C5, C6 and C11 are likely to be involved in the development of human bladder transitional cell carcinomas

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Table I. Clinical specimens.

Case number	Age[a]	Histology	TNM stage	Maximum diameter of tumor (cm)	Lymph node metastasis	Intra-ductal spread	PgR[b]	ER[b]	Menopause	CEA[c] (ng/ml)	CA15-3[c] (U/ml)	p53 status[d]
Brl7	63	Solid[e]	IIA	2.7	P[f]	P[f]	N[f]	N[f]	Po[g]	NT	NT	M[h]
Brl8	71	Solid	IIA	3.2	N[f]	N[f]	N	N	Po	NT	NT	M
Br49	50	Pap[e]	IV	7.0	P	NI	N	N	Pr[g]	NT	NT	W[h]
Br51	50	Scir[e]	IIA	1.8	P	NI	P[f]	P[f]	Po	1.3	17.3	W
Br54	68	Solid	UIB	17.0	N	NI	N	N	Po	1.5	9.8	W
Br58	67	Solid	IIA	3.2	N	P	N	N	Po	8.7	11.4	M
Br61	49	Solid	IIA	3.0	N	P	N	P	Po	1.0	NT	W
Br69	57	Scir	I	2.0	N	N	N	P	Po	2.0	16.0	W
Br79	75	Solid	I	1.1	N	P	N	P	Po	2.5	NT	W
Br81	61	Scir	IIB	2.5	P	P	N	N	Po	1.9	36.0	W
Br82	63	Scir	IV	4.0	P	P	N	N	Po	1.7	17.0	W
Br84	48	Pap	IIB	4.0	P	N	P	N	Pr	0.7	31.0	W
Br87	61	Scir	IIA	4.7	N	N	N	N	Po	3.2	47.0	M
Br96	49	Scir	IIIB	5.3	P	P	P	P	Po	1.4	41.2	M
Br99	68	Solid	IIA	2.2	N	N	N	N	Po	NT	14.0	M
Brl05	31	Scir	IIB	5.5	P	P	P	P	Pr	0.6	13.0	W
Brl08	58	Solid	IIB	2.8	N	P	N	P	Po	1.2	22.0	W
Brl21	NI	Pap	IIB	3.5	N	P	N	N	Pr	0.7	14.0	M
BrN118	55											W
BrN119	60											W
BrN164	51											W
BrN171	41											W
BrN174	53											W
BrN236	64											W

[a]Age at operation. [b]The expression levels of progesterone or estrogen receptors in tumor tissues were measured by an enzyme-linked immunosorbent assay (ELISA). [c]CEA and CA15-3 in sera of the patients were measured by ELISA. [d]p53 status was evaluated by yeast functional assay described in Materials and methods. [e]Solid, Pap and Scir means solid tubular, papillotubular and scirrhous carcinoma, respectively. [f]Po, positive; N, negative. [g]Po, post-menopausal; Pr, pre-menopausal. [h]M, mutated-type p53; W, wild-type p53. NT, not tested; NI, no information.

(11). Experimental biological approaches showed that overexpression of some HOX genes converted tumor cells into more malignant ones. Forced expression of HOXA1 in human breast cancer cells resulted in increased cell growth activity (12). Our previous studies showed that human lung cancer A549 cells transfected with the HOXD3 gene acquired more metastatic, invasive, and motile phenotypes (13,14). We also observed that the transduction of HOXD3-antisense expression vectors into human melanoma cells dramatically diminished their invasive and motile activities (15). Taken together, inappropriate expression of a particular HOX gene is likely to be involved in tumor development and malignant progression such as invasion and metastasis, and the HOX gene(s) responsible for oncogenesis and/or tumor progression differs according to the organs or tissues from which the tumor cells have been derived.

In this study, we analyzed the expression profiles of 39 HOX genes in 18 cases of invasive breast cancer and 6 cases of non-cancerous breast tissue, and further examined their relation to clinicopathological features.

Materials and methods

Clinical samples. We collected primary cancer tissues from a total of 18 patients with histologically-verified invasive ductal breast carcinoma who underwent surgery from January 2001 to September 2003 at Hokkaido University Hospital and 15 affiliated hospitals in the Hokkaido prefecture, Japan. Only those that agreed to the aim and contents of this study and provided their written informed consent were included. One to five bulk tumor tissue samples of approximately 5 mm in size were immediately cut from the esophagus resected by a standard surgical procedure, snap-frozen in liquid nitrogen, and stored at -80°C until use. 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.

RNA extraction. Each frozen tissue was crushed into powder using liquid nitrogen and a CRYO-Press compressor (Microtec Nition, Chiba, Japan). Total RNA was extracted from

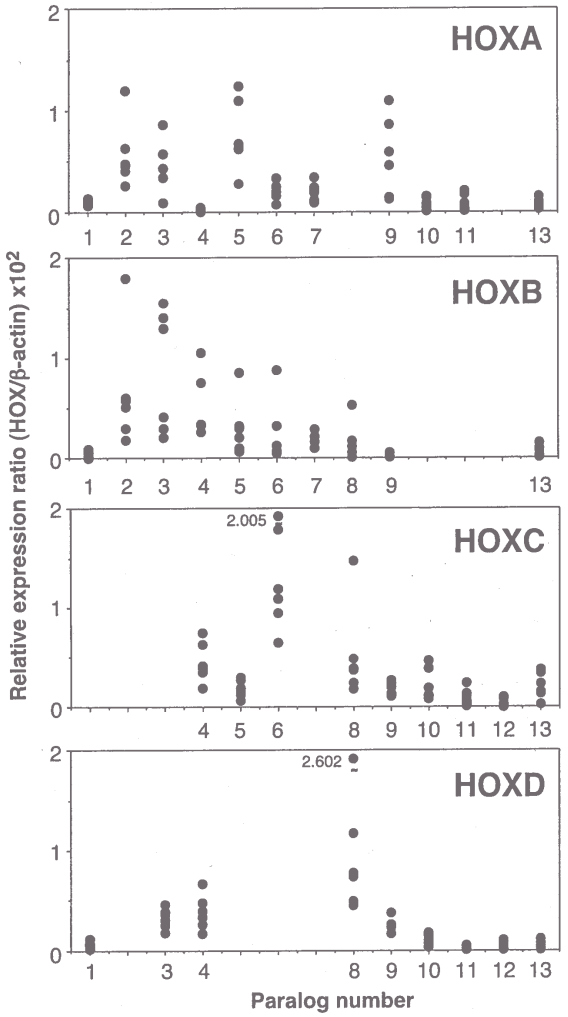

Figure 1. Expression levels of 39 HOX genes in 6 non-cancerous (normal) breast tissues. Each circle represents an individual case. The value of relative expression ratio is shown on the left side of the circle in cases when the relative expression ratio was >2.

monolayer cultures of each cell line and crushed tissues by using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Quantitative real-time reverse-transcription-PCR (RT-PCR). For real-time PCR, 1 μg of total RNA was subjected to cDNA synthesis in 100 μl of reaction mixture containing Taq Man RT buffer (Applied Biosystems, Foster City, CA), 5.5 mM $MgCl_2$, 500 μM dNTP, 2.5 μM random hexamers, 0.4 U/μl RNAse inhibitor, and 1.25 U/μl MultiScribe™ reverse transcriptase. The reverse transcription reaction was performed sequentially for 10 min at 25˚C, for 30 min at 48˚C and for 5 min at 95˚C. Quantitative PCR assays were carried out by using ABI Prism 7900HT (Applied Biosystems) with SYBR-green fluorescence. Real-time PCR amplification was performed in 20 μl of reaction mixture containing 2 μl of cDNA sample and 10 μl of QuantiTect™ SYBR Green PCR Master Mix (Qiagen, Valencia, CA), with specific primer sets described in our previous report (6). PCR was carried out by starting with a 15-min hot start at 95˚C followed by a denaturation step at 94˚C for 15 sec, an annealing step at 60˚C for 30 sec and an extension step at 72˚C for 1 min for 40 cycles. Dissociation curve analysis (95˚C for 15 sec, 60˚C for 15 sec, and 95˚C for 15 sec) was performed at the end of 40 cycles to verify the PCR product identity. Data were analyzed by using Sequence Detector Systems version 2.0 software (Applied Biosystems). Quantification was done by using the standard curve method as described previously (6). Finally, we compared relative gene expression levels as the ratio of the target *HOX* gene to the internal reference gene (*β-actin*) expression based on the initial copy number calibrated along the standard curve.

Yeast p53 functional assay. For yeast p53 functional assay, 3 μg of total RNA was subjected to cDNA synthesis in 20 μl of reaction mixture containing 200U of Moloney murine leukemia virus reverse transcriptase (Gibco BRL), 0.5 mM $MgCl_2$, 0.5 mM dNTP, 25 pmol p53-specific primer RT-1, (5'-CGGGAGGTAGAC-3'), and 7.5 mM dithiothreitol (16,17). The reverse transcription reaction was performed at 37˚C for 1 h. Yeast p53 functional assay and subsequent sequence analysis were performed to examine p53 status as described.

Statistical analysis and cluster analysis. The relationship between each HOX gene expression and each clinicopathological parameter was evaluated by the Mann-Whitney U test. The statistical software package applied was Statview 5.0 for Macintosh (SAS Institute, Cary, NC). A p-value <0.01 was considered statistically significant.

Clinicopathological parameters. Histological subclassification and staging of the tumors were done by reviewing the specimens used for pathological diagnosis, according to the TNM classification (18). The tumor status of each case was categorized based on the TNM classification (UICC, 6th edition) for pT, pN, and pM stages. Pertinent major clinicopathological parameters are shown in Table I.

Results

Expression of HOX genes in invasive ductal breast cancer tissues and non-cancerous tissues. The 6 non-cancerous tissues used were confirmed histologically to contain normal mammary glands in >50% of the section evaluated. The non-cancerous tissues expressed all HOX genes belonging to paralog 2 to 9 except HOXA4 (Fig. 1). The expression of HOX genes belonging to paralog 10 to 13 (except HOXC10 and C13) were nil or extremely low in the non-cancerous tissues.

Between invasive ductal breast cancer tissues and non-cancerous tissues, we found significant differences in the expression levels of HOX genes containing HOXA1, A2, A3, A5, A9, C11, D3, D4, D8, D9 and D10 ($p<0.01$, Mann-Whitney U test; Fig. 2). The expression level of HOXC11 was higher in the cancerous tissues than non-cancerous tissues whereas those of other HOX genes were lower in the cancerous tissues than non-cancerous tissues.

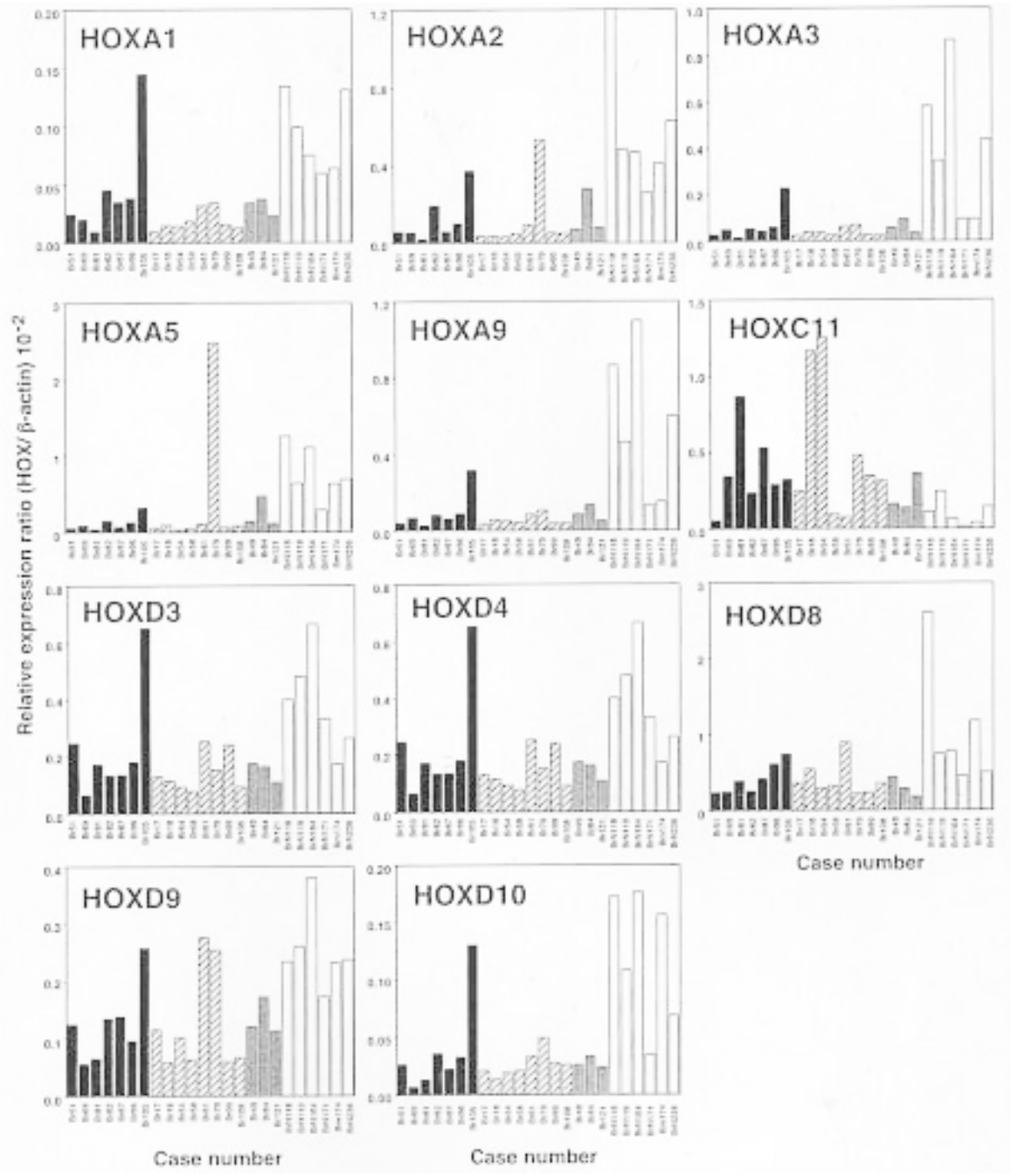

Figure 2. HOX genes in which expression levels were significantly different between breast cancer tissues and non-cancerous (normal) breast tissues. Solid tubular (closed bars), Papillotubular (hatched bars) and scirrhous (shaded bars) carcinoma tissues, and non-cancerous (normal) (open bars) tissues.

Histological type and HOX gene expression. Invasive ductal breast carcinomas (n=18) were histopathologically classified into 3 groups: 8 papillotubular carcinomas, 7 solid tubular carcinomas and 3 scirrhous carcinomas. We compared the HOX gene expression patterns among the 3 different histological types of carcinomas. It was found that HOXA1 was expressed at a higher level in the solid tubular type than papillotubular or scirrhous type of carcinomas (p=0.0039, Mann-Whitney U test; Fig. 2).

Lymph node metastasis and HOX gene expression. To find the particular HOX genes involved in progressive phenotypes, we compared the expression levels of each HOX gene between breast carcinoma with lymph node metastasis (8 cases) and

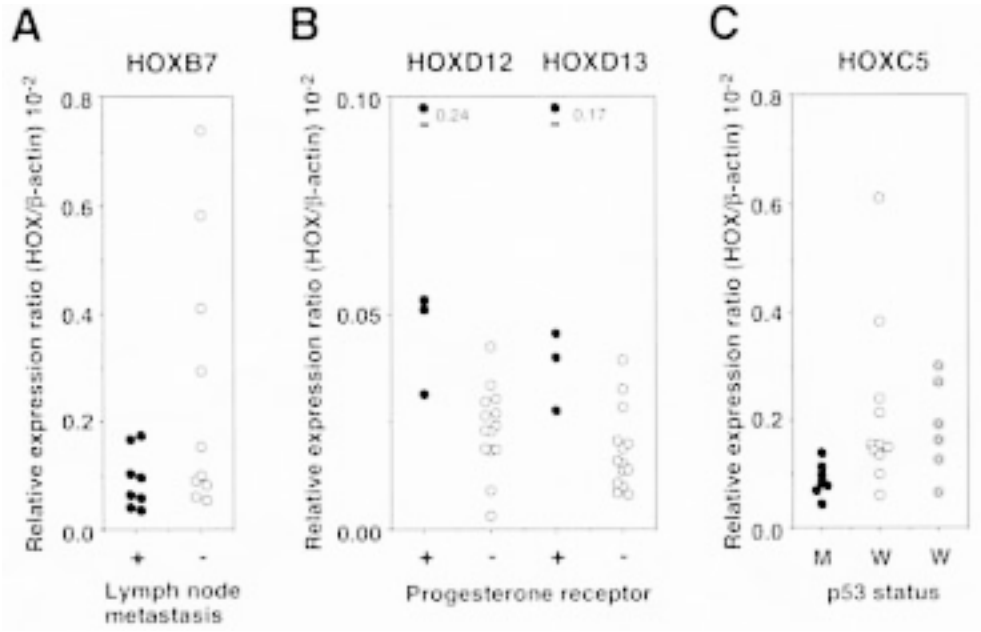

Figure 3. HOX genes in which expression levels were significantly different in clinicopathological parameters such as the presence or absence of lymph node metastasis, progesterone receptors or mutation of TP53. (A) The expression levels of HOXB7 in breast cancer tissues with or without lymph node metastasis ($p<0.01$, Mann-Whitney U test). (B) The expression levels of HOXD12 and D13 in breast cancer tissues with or without progesterone receptors ($p<0.01$, Mann-Whitney U test). (C) The expression levels of HOXC5 in non-cancerous (normal) breast tissues (grey circle) and cancer tissues with mutated-type TP53 (●) or wild-type TP53 (○). M and W mean mutated-type and wild-type TP53, respectively.

the one without (10 cases). The expression level of HOXB7 in lymph node metastasis-positive cancers was significantly low compared to that in lymph node metastasis-negative cancers ($p=0.0067$, Mann-Whitney U test; Fig. 3). No particular HOX gene characterized tumor size or intraductal spread (data not shown).

Hormonal conditions and HOX gene expression. We examined the relationship between expression patterns of HOX genes and the expression of hormone receptors, such as the progesterone receptor (PgR) and estrogen receptor (ER). PgR-positive cancer tissues (4 cases) showed higher expression levels of HOXD12 and D13 genes than PgR-negative ones (14 cases) ($p=0.0058$ and 0.0079, respectively, Mann-Whitney U-test; Fig. 3) whereas there were no HOX genes with different expression levels between ER-positive and -negative cancer tissues (data not shown). No HOX genes were found to be correlated with a menopausal condition (pre- or post-menopause) or age (data not shown).

Tumor markers in sera and HOX gene expression. We examined the serum levels of CEA and CA15-3, which are known tumor markers of breast cancer. No correlation was found between the levels of CEA or CA15-3 and expression levels of any HOX gene (data not shown).

Mutation of tumor suppressor TP53 gene and HOX gene expression. To examine the influence of TP53 status on the expression of HOX genes, we first determined the TP53 status by yeast p53 functional assay. The mutated-type TP53 was found in 7 of 18 cancer tissues (Table I). Between cancer tissues with the mutated-type and wild-type TP53 gene, the expression level of HOXC5 was significantly lower in the former than the latter or normal tissues ($p=0.011$, Mann-Whitney U test; Fig. 3).

Discussion

We tried to demonstrate the hypothesis that misexpression of the HOX gene, a master-regulating gene of morphogenesis, is associated with development and malignant progression of breast cancer. First, we compared the HOX gene expression patterns between cancerous and non-cancerous tissues to clarify that HOX genes are related to the development of breast cancer. In non-cancerous tissues, it was found that the expression levels of HOX genes belonging to paralog 2 to 9 were high. These HOX genes are known to be expressed in cervical (paralog 1 to 4) and thoracic parts (paralog 5 to 8) during embryogenesis (5). The HOX gene expression patterns of non-cancerous breast tissues are similar to those of adult lung and thymus, which neighbor the breast on the body axis (6). Thus, in the adult body, some organs including the breast may maintain the HOX gene expression patterns in a manner of spatial colinearity as observed during embryogenesis.

To find the HOX gene(s) associated with development of breast cancer, we compared the HOX gene expression patterns between invasive ductal cancer and non-cancerous tissues. It was revealed that the expression levels of HOX genes belonging to cluster A (HOXA1, A2, A3, A5 and A9) and D (HOXD3, D4, D8, D9 and D10) were significantly lower in the cancerous tissues than non-cancerous tissues, whereas HOXC11 expression level was higher in cancerous tissues than non-cancerous ones. Neighboring HOX genes on the same cluster showed similar expression patterns, for

example A1/A2/A3, D3/D4 and D8/D9/D10. This suggests that these HOX genes may be controlled by common upstream regulatory components in adult breast tissues and that a dysfunction of the regulatory components occurs in breast cancer.

It is speculated that the altered expression of these 11 HOX genes may change the expression of their downstream target genes and promote the development of breast cancer or breast cancer-related cell behavior. It is difficult to explain the biological roles of each HOX gene in carcinogenesis or cell behavior of breast cancer, however we could speculate on the present results and investigations reported by others. For example, the transduction of HOXA5 into human breast cancer cells activated the p53 tumor suppressor gene promoter (19). Expression of HOXA5 in breast cancer cells expressing wild-type p53 led to apoptosis while those lacking the p53 gene did not (19,20). Further, the HOXA5 promoter region was methylated in 80% of p53-negative breast cancer specimens (19). Such evidence indicated that the compromised HOXA5 function could limit p53 expression in breast cancer. In the present study, we observed that HOXA5 expression levels in cancer tissues were lower than those in non-cancerous tissues of which p53 status was wild-type. Therefore, the decreased expression of p53 through the down-regulation of HOXA5 is thought to be one of the factors involved in breast carcinogenesis.

It was demonstrated by CAT reporter and gel shift assay that HOXD9 transactivates the chicken E-cadherin promoter (21). The decreased expression of HOXD9 may induce the down-regulation of E-cadherin to promote carcinogenesis or invasion of breast cancer cells since E-cadherin is known to be a tumor and/or invasion suppressor (22-24).

We also found decreased expression of HOXA1 and B7 in solid tubular type of carcinomas and carcinomas with lymph node metastasis, respectively. Experimental biological approaches revealed that HOXA1 had an oncogenic function in human breast cancer cells, and HOXB7 may play an important role in malignant progression through the production of an angiogenic factor, bFGF (12,25,26). We have no evidence to interpret the contradiction between these reports and our results.

Nonetheless, it is noteworthy that expression levels of HOXC5 in breast cancer tissues with mutated-type p53 were significantly lower compared to those in cancer tissues with wild-type p53. Hence, the possibilities are: 1) wild-type p53 is necessary to transcribe HOXC5 gene; and 2) mutated-type p53 functions as a repressor for HOXC5 gene. HOXC5 gene is composed of 2 exons and 1 intron. Although we searched the binding sequences for p53 from Ξ3 kbp upstream of the transcription starting site to Ξ700 bp downstream of exon 2 and whole intron 1, we could not find any binding sites (data not shown). Therefore, it seems that p53 regulates the HOXC5 expression through an indirect pathway. Another possibility is that a cell growth advantage due to a dysfunction of p53 may be involved in HOXC5 expression, as some reports indicated the potential link of HOX gene expression with cell cycle progression (27-29). It is of interest that p53 plays a role in the expression network of HOX genes including HOXA5 and C5. We intend to study the roles of both HOX genes by conducting further cell biological experiments.

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