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Aberrant expressions of HOX genes in colorectal and hepatocellular carcinomas

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Abstract. HOX genes are known as master regulator genes which give cells positional information in embryogenesis. In this study, we compared the expression patterns of 39 HOX genes among human colorectal carcinomas from the right large intestine (cecum, ascending and transverse colon), those from the left large intestine (discending and sigmoid colon, and rectum) and hepatocellular carcinoma. The expression levels of each HOX gene were quantified by analysis based on the real-time RT-PCR. The expression patterns of HOX genes in colorectal and hepatocellular carcinoma tissues differed from those in their normal or non-cancerous tissues. Between the tumor tissues in the right-side large intestine and those in the left-side, different HOX genes were expressed in association with cancer. Further, the expression levels of HOXD8 in liver-metastatic tissues of colorectal carcinomas were as low as in non-cancerous liver tissues, and were significantly lower than those in the primary tissues. These results suggest that dysregulated expressions of HOX genes play an important role in carcinogenesis and malignant progression of colorectal and hepatocellular carcinomas.

Introduction

Hox genes are one of the members of homeobox genes that function as developmental regulatory genes (1). The genes have a common sequence element of 180 bp, the homeobox, which encodes a highly conserved 60-amino-acid homeodomain. The homeodomain binds to DNA motif in a sequence-specific manner to function as a transcription factor (2). In mammals, 39 Hox genes, present in four gene clusters termed HoxA, HoxB, HoxC and HoxD, are found on separate chromosomes (3). During embryonic development, the Hox genes are expressed spatio-temporally in a highly coordinated manner and establish regional identities along the antero-posterior body axis (4). It has recently become apparent that the expression patterns of Hox genes are organ- or tissue-characteristic not only in the embryo but also in the adult, suggesting that Hox genes work as regulators of differentiation or maturation in organs and tissues of post-natal body as well as embryo (5,6). Further, there is accumulating evidence indicating that expressions of particular HOX genes are dysregulated in certain types of carcinomas. For example, our previous reports showed differences in expression patterns of 39 HOX genes between cancerous tissues and non-cancerous tissues of lung, breast, oral and esophageal cancers and melanoma (7-11). Other groups also reported abnormal expressions of HOX genes in cancers of kidney, colon, prostate, and uterus (12-15). Cell biological approach demonstrated that misexpression of some HOX genes altered malignancy of human tumor cells in culture. Enforced expression of HOXA1 in human breast cancer cells resulted in enhancement of cell proliferation (16). Knockdown of HOXC10 expression by shRNA decreased invasiveness of cervical cancer cells (17). Forced expression of HOXB13 in renal cell carcinoma cells suppressed colony formation and induced apoptotic features (18) whereas the transduction of antisense-HOXB13 into ovarian cancer cells with a high expression of HOXB13 reduced their invasiveness (19). We also reported that overexpression of HOXD3 enhanced invasive and metastatic ability of lung cancer cells through the activation of integrin αvß3 and TGF-ß pathways (20-22). Thus the accumulating evidence suggests that HOX genes are involved in oncogenesis and malignant progression.

It is well known that one of the functions of HOX genes is to give cells positional information during embryogenesis (1,4). Therefore, it is intriguing to determine whether the expression patterns of HOX genes represent the spatial position where tumor cells exist. In this study, we compared the expression patterns of HOX genes among tumor tissues

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Table I. Clinicopathological features of the colorectal adenocarcinoma and hepatocellular carcinoma specimens.

	Characteristic	No.
Colorectal carcinoma		
Primary site	Gender	
	Male	27
	Female	13
	Age, median (range)	66 (48-89)
	Site	
	Cecum	3
	Ascending	5
	Transverse	5
	Descending	3
	Sigmoid	10
	Rectum	14
	Differentiation	
	Well	16
	Moderate	20
	Poor	4
	Depth of invasion	
	pT1	2
	pT2	5
	pT3	28
	pT4	5
	Lymph node metastasis	
	Positive	24
	Negative	16
	Hepatic metastasis	
	Positive	21
	Negative	19
	Venous/lymphatic invasion	
	Positive	26
	Negative	14
	CEA, median (range)	9 (0.2-5000)
	≤2.5 ng/ml	9
	>2.5 ng/ml	22
	CA19-9, median (range)	6.2 (0-15000)
	≤37 U/ml	18
	>37 U/ml	10
Hepatic metastatic site	Site where derived from	
	Ascending	5
	Sigmoid	3
	Rectum	3
Hepatocellular carcinoma	Gender	
	Male	20
	Female	5
	Age, median (range)	65 (43-79)
	Differentiation	
	Well	7
	Moderate	8
	Poor	6
	Unknown	4
	Pathologic stage	
	I	2
	II	14
	III	6
	IVA	1
	Unknown	2
	Vp, Vv, Va and/or B	
	Positive	5
	Negative	20
	Viral status	
	HBV	7
	HCV	10
	Non-B - non-C	7
	Unknown	1
	AFP, median (range)	9.3 (1.9-50000)
	<20 ng/ml	15
	≥20 ng/ml	9
	Unknown	1
	PIVKA II, median (range)	54 (0-36800)
	<28 mIAU	9
	≥28 mIAU	12
	Unknown	4

Vp, invasion of portal vein; Vv, invasion of hepatic vein; Va, invasion of hepatic artery; B, invasion of bile duct. The tumor status was assessed according to the TNM classification (UICC, 6th edition).

grown in different organs: primary sites and hepatic metastatic sites of colorectal carcinomas, and primary sites of hepatocellular carcinomas. The cecum, the ascending colon and the proximal two thirds of the transverse colon derive from the midgut whereas the distal third of the transverse colon, the descending colon, the sigmoid colon and the upper anal canal derive from the hindgut (23). Therefore, we also compared the HOX gene expression patterns in colorectal carcinomas, by dividing colorectum into two parts: proximal (right-sided) large intestine (cecum, ascending and transverse colon) and distal (left-sided) one (descending, sigmoid colon and rectum) since large intestine derives from two different embryologic origins.

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Patients and samples. We collected primary cancer tissues from a total of 40 patients with histologically verified colorectal adenocarcinoma, metastatic liver lesions from 11 patients with colorectal adenocarcinoma, and primary tissues from a total of 25 patients with hepatocellular carcinoma, who had undergone surgery from 2000 to 2002 at the Hokkaido University Hospital and its affiliated hospitals (total 33 institutions) in Hokkaido prefecture, Japan. From 8 of the 11 patients, metastatic liver lesions of colorectal carcinoma were obtained as well as the primary tumors. Only those who agreed to the aim and contents of this study and who provided their written informed consent were included. One to five bulk tumor tissue samples of about 5 mm size were immediately cut from the colorectum and/or liver resected by a standard surgical procedure. Twenty normal mucosa tissues, which were at least 5 cm away from the tumor edge, were obtained by sharply dissecting the mucosa leaving the muscularis propria. Nineteen non-cancerous tissues were also obtained from the livers. All the tissues were 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. Clinicopathological features of specimens examined are shown in Table I.

RNA extraction and cDNA preparation. Each frozen tissue sample was powdered in liquid nitrogen by using a Cryopress (CP-100W, Microtech Nition Co., Chiba, Japan). Total RNA was extracted by using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Prior to reverse transcription, total RNA was treated with 1 unit/μl DNase I and 10 units/μl RNase inhibitor (Toyobo, Osaka, Japan) at 4˚C for 15 min. For real-time PCR, 1 μg of total RNA was subjected to cDNA synthesis in 100 μl of reaction mixture containing TaqMan RT buffer (Applied Biosystems, Foster City, CA), 5.5 mM $MgCl_2$, 2 mM dNTP, 2.5 mM random hexamers, 0.4 U/μl RNAase inhibitor, 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 real-time reverse-transcription-PCR (RT-PCR). Quantitative RT-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, 10 μl of QuantiTect SYBR Green PCR Master Mix (Qiagen, Valencia, CA), with specific primer sets which had been described in our previous report (5), except HOXA10 and HOXD4 (HOXA10: sense, 5'-CTTCCGAGAGCAGCAAAGCC-3'; anrisense, 5'-CTTCTTCCGACCACTCTTTGCC-3'; HOXD4: sense, 5'-ACCCCTGGATGAAGAAGGTGC-3'; antisense, 5'-ACGGCGCCTTGTCAGATAC-3').

PCR was carried out 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. Data were analyzed by using Sequence Detector Systems version 2.0 software (Applied Biosystems). Quantification was done by using the standard curve method as previously described (5). Finally we presented relative gene expression levels as the ratio of the target HOX gene to the internal reference gene (ß-actin) expression based on the initial copy number calibrated along the standard curve.

Statistical analysis. The relationship between each HOX gene expression and each clinicopathological parameter was calculated 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.

Results

Expressions of HOX genes in normal colorectal mucosa tissues and non-cancerous tissues around hepatocellular carcinomas. Expression levels of 39 HOX genes in 20 normal colorectal mucosa tissues and 19 non-cancerous liver tissues are shown in Fig. 1. Colorectal mucosa tissues showed relatively high expressions of HOXA9, A11, A13, B5, B6, B7, B13 compared to other HOX genes. There were differences in expression levels between the right and the left-side large intestine: the expression levels of HOXB13 and D13 in the left were significantly higher than those in the right whereas the expression levels of HOXB6 and B9 in the right were significantly higher than those in the left. In non-cancerous liver tissues, the expressions of HOXA, HOXC and HOXD were silent or extremely low, and only HOXB2, B3 and B4 were expressed at relatively high levels.

Aberrant expressions of HOX genes in colorectal and hepatocellular carcinomas. We next compared the expression patterns of 39 HOX genes between colorectal carcinoma and normal mucosa tissues (Fig. 2). Ten HOX genes were expressed differently in carcinoma versus normal tissues of the left-sided large intestine: the expression levels of HOXA9, B3, B8 and B9 were significantly high, whereas those of HOXB2, B13, D1, D3, D4, D8 and D12 were significantly low, in carcinoma tissues. In the right-side large intestine, the expressions of only 2 HOX genes were different between carcinoma and normal tissues: the expression level of HOXA9 was significantly high whereas that of HOXD1 was significantly low in carcinoma tissues. In hepatocellular carcinoma, the expression levels of 28 HOX genes (HOXA3, A5, A6, A7, A9, A10, A11, A13, B1, B6, B7, B8, B9, B13, C5, C6, C8, C9, C10, C11, C12, C13, D1, D3, D4, D8, D9 and D10) were significantly higher in cancer tissues than in non-cancerous tissues whereas no HOX genes decreased their expressions in hepatocellular carcinoma tissues (Fig. 3).

Liver metastasis-related HOX genes in colorectal carcinoma. To explore HOX genes related to hepatic metastasis of colorectal carcinoma, we first compared the expression patterns of HOX genes between the primary colorectal carcinoma tissues with hepatic metastasis and those without hepatic metastasis. As shown in Fig. 4A and B, the expression levels of HOXA3 and D11 were significantly higher in primary tissues with hepatic metastasis than in those without metastasis

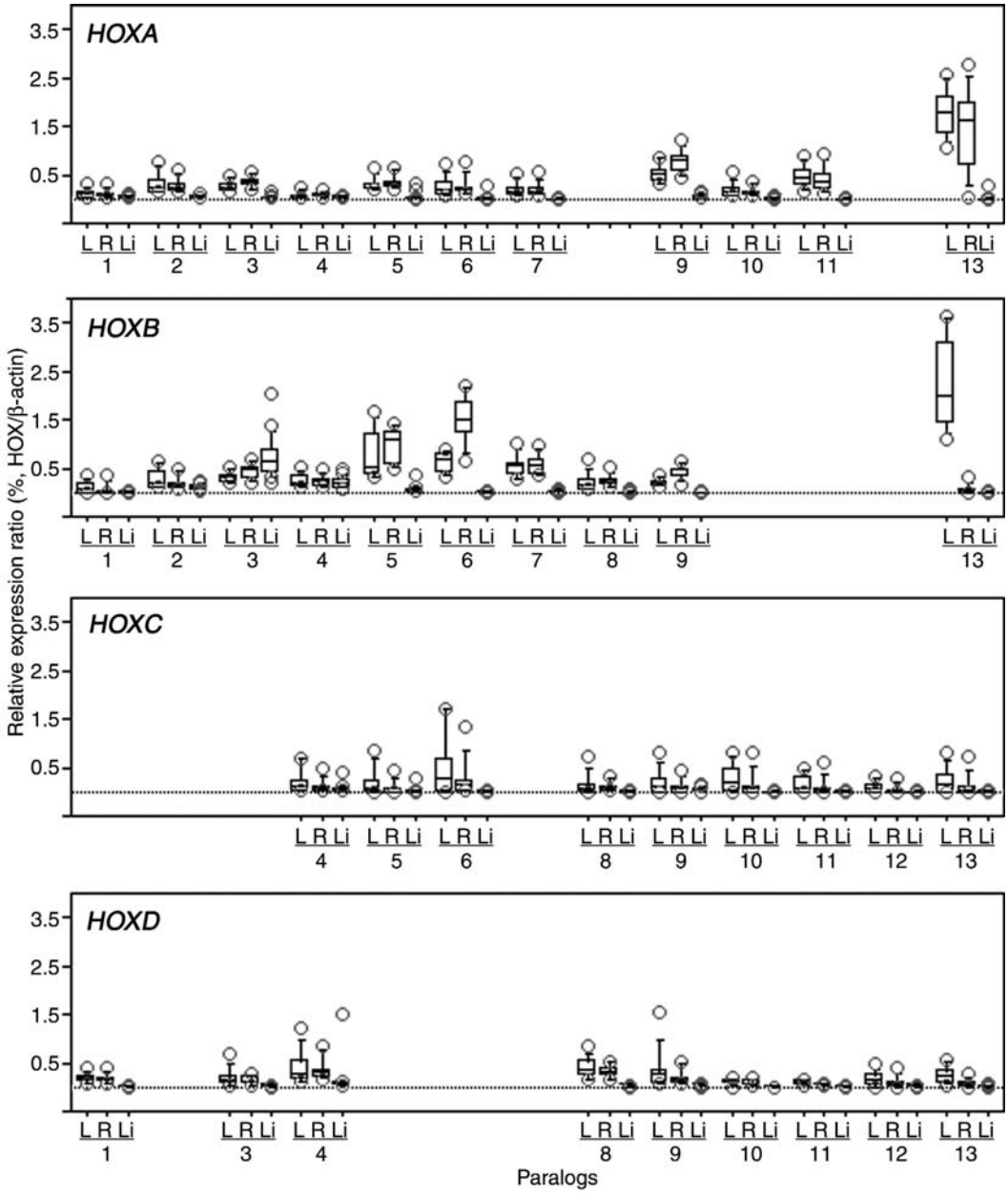

Figure 1. Expression profiling of 39 HOX genes in normal (non-cancerous) colorectum mucosa and liver. The relative levels of HOX mRNA were determined by quantitative RT-PCR in 10 proximal colon, 10 distal colon or rectum and 19 liver samples. The graphs of HOX cluster A, B, C and D are lined from top to bottom. HOX gene paralogs 1-13 are lined from left to right of X-axis in each graph. The distribution of the relative expression ratio (HOX/ß-actin) is summarized by using boxplots. The central box in each plot shows the interquartile (25th-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. L, mucosa of left large intestine (cecum, ascending and transverse colon); R, mucosa of right large intestine (descending, sigmoid colon and rectum); Li, liver tissues. In comparison between the left-side and the right-side large intestine, there were significant differences in expression levels of HOXB6, B9, B13 and D13 (p=0.0009, 0.0032, 0.0002 and 0.0025 respectively). P-values were determined by Mann-Whitney U test.

among the carcinomas arisen in the right, but not in the left-side large intestine. We further analysed expression levels of HOX genes in the primary colorectal cancers and the 11 liver metastases that were obtained from a total of 36 patients who had liver metastasis at the time of surgery. The expression levels of HOXD8 in liver metastatic foci were significantly lower than those in primary tissues regardless whether they had developed in the right or the left-side large intestine, or whether they had liver metastasis or not (Fig. 4C). Additionally, in the eight cases in which both the primary tissues and the metastatic tissues were available, HOXD8 expression levels in the metastatic foci were low compared to those in the matched primary tissues except for one case (Fig. 4D).

Invasion-related HOX genes in hepatocellular carcinoma. We examined relationships between the expression level of HOX genes and clinicopathological parameters such as stage, differentiation, invasion and production of tumor markers (AFP and PIVKA II) in hepatocellular carcinoma. It was found that the expression levels of HOXA5 were significantly lower in HCC tissues with invasion of artery, vein and/or bile duct compared to those without invasion (Fig. 4E).

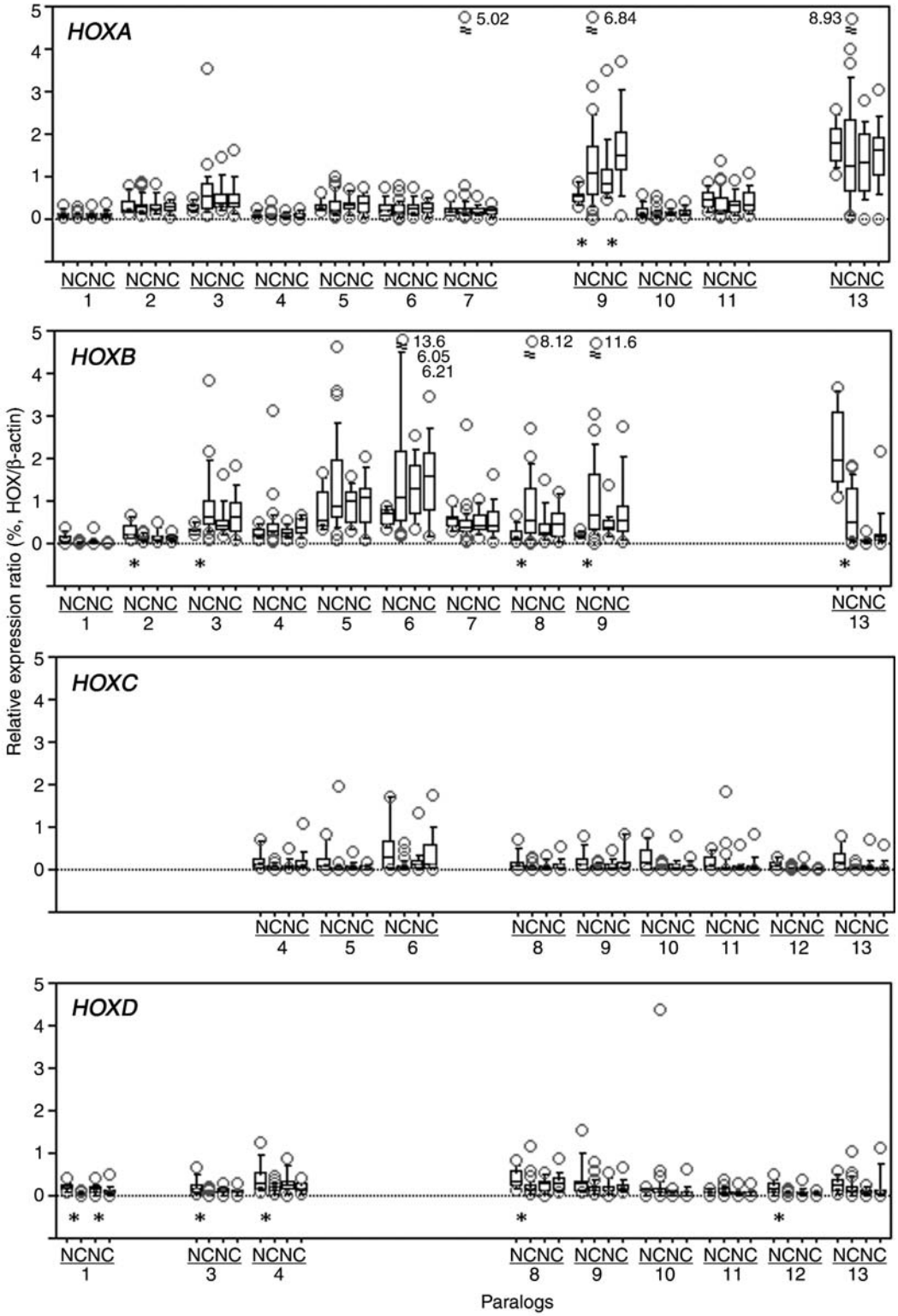

Figure 2. Comparison of HOX gene expressions between normal (non-cancerous) mucosa and colorectal cancer tissues. The distribution of the relative expression ratio (HOX/ß-actin) is summarized by using boxplots in the same manner as in Fig. 1. N, normal mucosa tissues; C, cancer tissues. N and C in the left above the paralog numbers represent the tissues from the left-side large intestine whereas those in the right represent the tissues from the right-side large intestine. *Statistically significant difference compared to N and C ($p<0.01$ by Mann-Whitney U test).

Discussion

In the present study we analysed the expression patterns of 39 HOX genes in normal colon mucosa, colorectal carcinoma and hepatocellular carcinoma tissues. Analysis of normal (non-cancerous) tissues showed organ-characteristic HOX expression patterns. The prominent high expression of HOXA13 was specific to mucosa of large intestine. Our analysis of HOX gene expressions by the same method showed no expression of HOX paralog 13 genes in mucosas of digestive tracts such as oral cavity, esophagus or stomach (10,11; unpublished data). It was also noted that the mucosa

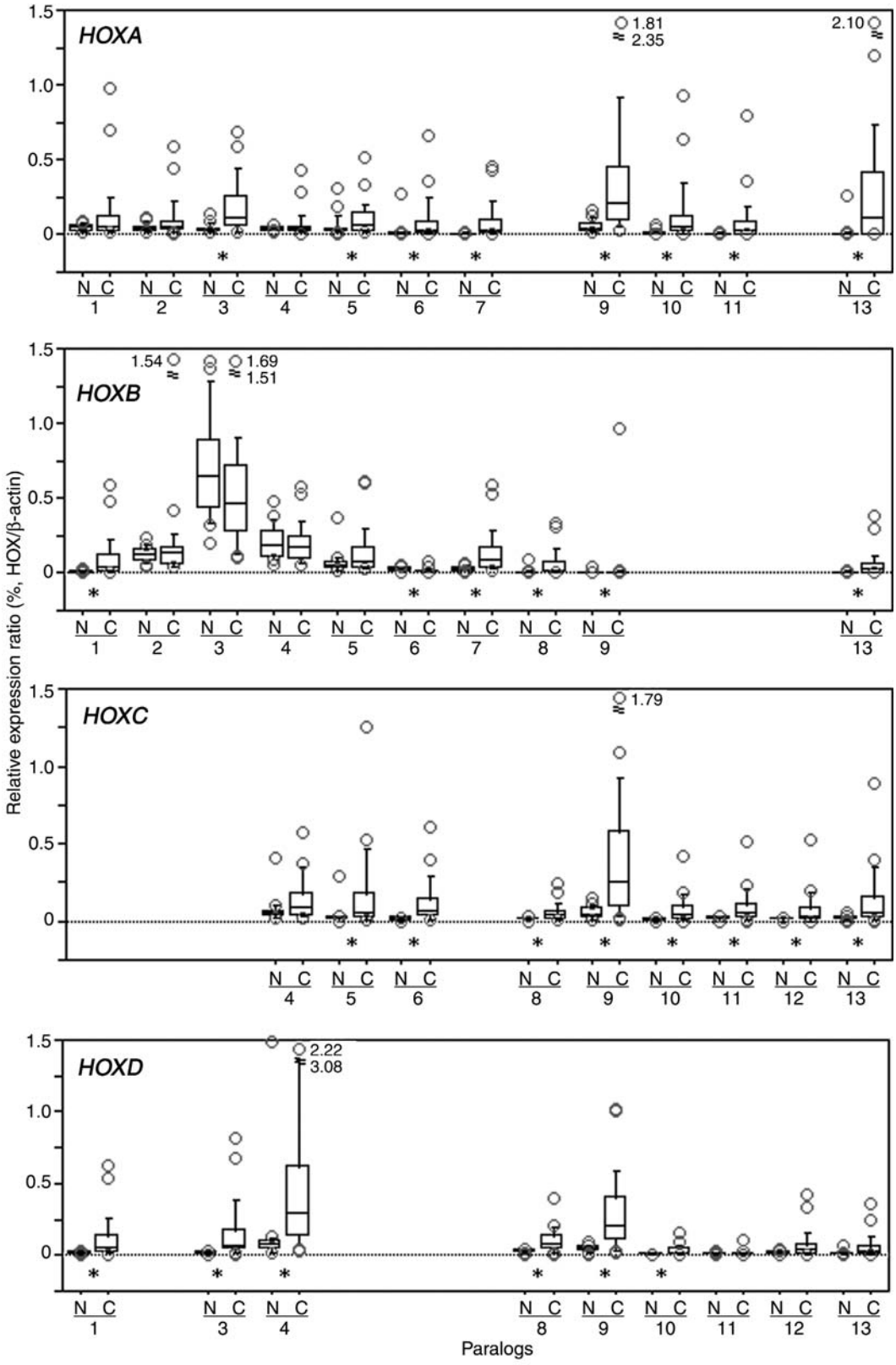

Figure 3. Comparison of HOX gene expressions between normal (non-cancerous) and hepatocellular carcinoma tissues. The distribution of the relative expression ratio (HOX/ß-actin) is summarized by using boxplots in the same manner as in Fig. 1. N, normal (non-cancerous) liver tissues; C, cancer tissues. *Statistically significant difference compared to N and C ($p<0.01$ by Mann-Whitney U test).

of the left-side large intestine but not that of the right-side expressed high levels of HOXB13 as well as HOXA13. It is well known that the order of the genes within a cluster corresponds to the segments of order along anterior-posterior axis during embryogenesis: e.g., the 5'-end of the HOX cluster such as paralog 13 genes is expressed most posteriorly (4). Glebov *et al* took pinch biopsies of normal colonic mucosa from the ascending and descending colon and compared gene

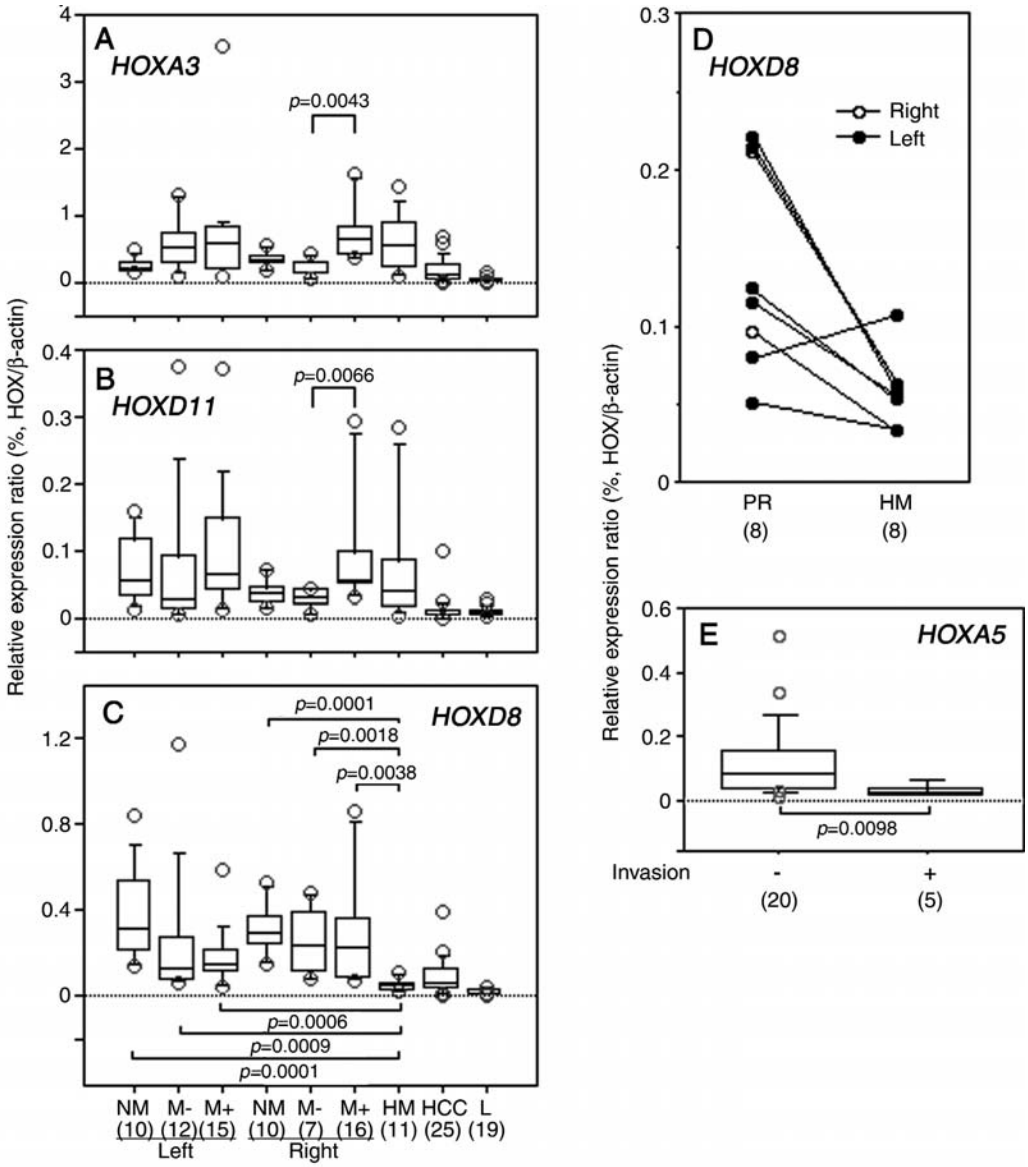

Figure 4. HOX genes related to invasion and metastasis of colorectal and hepatocellular carcinoma (HCC). Comparison of expression levels of HOXA3 (A), HOXD11 (B) and HOXD8 (C) among primary tissues of colorectal carcinoma and hepatic metastatic tissues, and primary HCC tissues. NM, normal (non-cancerous) mucosa tissues of colorectum. M^-, primary tissues of colorectal carcinoma without hepatic metastasis. M^+, primary tissues of colorectal carcinoma with hepatic metastasis. Left, tissues from descending and sigmoid colon, and rectum. Right, tissues from cecum, ascending and transverse colon. HM, hepatic metastatic tissues. HCC, hepatocellular carcinoma tissues. L, normal (non-cancerous) liver tissues. Comparison of expression levels of HOXD8 between hepatic metastatic tissues and the matched primary tissues (D). PR, primary tissues. HM, hepatic metastatic tissues. Comparison of expression levels of HOXA5 between HCC tissues with invasion of hepatic artery, portal vein, hepatic vein and/or bile duct and those without invasion (E). The distribution of the relative expression ratio (HOX/ß-actin) is summarized by using boxplots in the same manner as in Fig. 1 (A, B, C and E). Numerals in the parentheses are sample numbers examined. P-values were evaluated by Mann-Whitney U test.

expressions between them by cDNA microarray analysis (24). They demonstrated that the expression levels of HOXB13 and HOXD13 in descending colon were higher than those in ascending one whereas the expression level of HOXB6 in descending colon was lower than that in ascending one (24). These expression patterns of HOX genes in adult large intestine resemble those in the embryonic stage (25-27). Taken together, it is suggested that HOX genes play a role in maintaining the architecture and/or function of large intestine in not only embryonic stage but also post-natal stage.

One of the most noteworthy points of our results is that the number of HOX genes of which expressions were altered in cancerous tissues was markedly numerous in the left-side large intestine compared to the right-side one. In the latter, only two HOX genes changed their expression levels in cancerous tissues: increased expression of HOXA9 and decreased of HOXD1. On the other hand, in the left-side large intestine, total 11 HOX genes including these two genes altered their expressions in the cancerous tissues. Namely, the increased expression of HOXA9 and the decreased expression of HOXD1 occurring in both sides of the large intestine suggest that such an alteration of gene expressions may be involved in carcinogenesis and/or malignant progression of colorectal cancer, regardless of their developing site. It is well known that genetic and epigenetic status of cancers developed in the right-side large intestine differ from those of

cancers from the left-side large intestine. For example, frequencies of loss of heterozygosity (LOH) and mutation of TP53 are high in cancers from the left-side large intestine whereas those of microsatellite instability (MSI) and CpG islands methylator phenotype (CIMP) are high in cancers from the right-side large intestine (28-33). Is there any relationship between such a difference in genetic and epigenetic characteristics dependent on sites (right- or left-side) where tumors arise and the difference in tumor-associated changes of HOX gene expression? LOH at the chromosomal regions in 5q, 17p and 18q is often detected in tumors arisen in the left-side large intestine (28). In our study, although the expression levels of 7 HOX genes decreased in the left-side tunors, none of the HOX genes was localized at these chromosomal regions, suggesting that LOH was not responsible for decreased expressions of some HOX genes. It is unlikely that mutant TP53 causes alteration of HOX gene expressions. There are so far no reports indicating that p53 regulates the transcription of HOX genes although there are reports on transcriptional regulation of TP53 by HOXA5 (34). It does not seem probable that the high frequency of MSI and CIMP is directly linked to cancer-associated HOX code in the right-side large intestine because there was few HOX genes of which expressions were changed in cancer, and the expressions of HOXA genes, especially HOXA4-6 which are known to be hypermethylated in myeloid and lymphoid malignancy, were low even in normal mucosa tissues. Thus we speculate that there was no direct linkage between such genetic and epigenetic characteristics and the alteration of HOX code in a site-specific manner. Meanwhile, the left-sided tumors are known to produce more amounts of growth factors such as EGF, TGF-α, insulin-like growth factor (IGF)-I and IGF-II than the right-sided tumors (35,36). The growth factors in tumor microenvironment may affect HOX gene expressions; or HOX genes abnormally expressed in tumors may promote the production of such growth factors. Although so far there is no evidence indicating either that EGF, TGF-α and IGFs directly regulate HOX gene expression or that these growth factor genes are direct target for any HOX genes. Interestingly, there is a report indicating that HoxA9 induces IGF-I receptor expression in B-cell leukemia cells (37). As shown in this study, the expression of HOXA9 increased in tumors from both sites whereas IGFs production was high in the left, but not the right-sided tumors, resulting in that IGFs gave only the left-side tumor cells advantage for expressing malignant properties such as growth and survival.

The present study revealed that the expressions of most of HOX genes were silent in adult non-cancerous liver. The results were similar to those of our previous study in which we analysed HOX expression patterns of mRNA from normal adult liver (5).

The expression levels of 28 HOX genes were altered in hepatocellular carcinomas (HCCs). All of them increased their expression in HCCs, and, except for HOXA3 and B1, they are adjacent on the same cluster. This expression pattern resembles those in oral squamous carcinoma or esophageal squamous carcinoma observed in our previous studies (10,11). Such expression patterns suggest the possibility that these adjacent HOX genes are cis-acitvated and controlled by common upstream regulatory components, and that epigenetically regulatory components may be dysregulated in cancers such as HCC, or oral and esophageal squamous cell cancers. The expression of HOXA5 in HCCs with invasion of vessels and/or bile ducts was markedly low compared to that in HCCs without invasion. As the expression level of HOXA5 was higher in HCC tissues than in non-cancerous tissues, it is thought that HOXA5 plays a positive role in development of HCCs, but not in their invasion. There are some reports indicating that decreased expression of HOXA5 correlated with malignant phenotypes. For example, inactivation of HOXA5 is associated with poor prognosis in myeloid and lymphoid malignancy (38). Loss of HOXA5 expression is also observed in breast cancer (34,39). HOXA5 is known to transactivate the tumor suppressor TP53 gene, and hMLH1 gene which encodes DNA mismatch repair protein (34,40). Thus, decreased expression of HOXA5 may induce mutation-prone status in tumor cells, resulting in emergence of more malignant tumor cells.

The expression pattern of HOXD8 was one of the most significant findings in this study. The HOXD8 was the only HOX gene of which expression level was different between the primary tissues and hepatic metastatic tissues in colorectal cancers. The expression level of HOXD8 in the primary tissues of left-sided large intestine tumors was lower than that in normal mucosa of the same-side large intestine whereas there was no significant difference of HOXD8 expression between tumor tissues and normal mucosa tissues in the right large intestine. There was no difference in the expression levels of HOXD8 between the primary tumors with hepatic metastasis and those without hepatic metastasis either in the right- or in left-side tumor tissues. However, the expression levels of HOXD8 in hepatic metastatic tissues were significantly lower than those in primary tissues. From these findings, HOXD8 is thought to function as a metastasis suppressor. If HOX genes give the cells positional information in adult, loss of HOXD8 may give the colorectal cancer cells misinformation to recognize liver as an orthotopic organ. The alteration of positional information may give an advantage to the tumor cells to adapt themselves to the micro-environment of the liver. In this sense, decreased expression of HOXD8 in hepatic metastatic tissues fascinates us to understand especially from the viewpoint of organ-specific metastasis. We need to clarify the expressions of HOXD8 in metastatic tissues other than liver and to identify the genes transcriptionally regulated by HOXD8 in order to demonstrate its function as a suppressor to hepatic metastasis.

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