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OVER-EXPRESSION OF *HOX-8*, THE HUMAN HOMOLOGUE OF THE MOUSE *Hox-8* HOMEOBOX GENE, IN HUMAN TUMORS

Makoto Suzuki, Masamitsu Tanaka, Toshio Iwase, Yasuhisa Naito,
Haruhiko Sugimura, and Isamu Kino*

First Department of Pathology, Hamamatsu University School of Medicine,
3600 Handa-cho, Hamamatsu 431-31, Japan

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SUMMARY: A human ovarian yolk sac tumor cDNA library was screened for homeobox genes with an oligonucleotide probe under low stringent condition. Three homeobox genes were isolated, two of which were identified as HHO.c1 and HB24. The third was highly homologous with the mouse *Hox-8* gene and was designated as *HOX-8*. Studies on RNAs from 25 human tumor tissues and cell lines showed that the profile of *HOX-8* expression was different from those of HHO.c1 and HB24. The expression of *HOX-8* was not detected in hematopoietic tumor cells, in which HHO.c1 and HB24 were highly expressed. *HOX-8* was expressed at higher levels in a variety of tumors of epithelial origin than in their corresponding normal tissues more frequently than HHO.c1 and HB24. All three homeobox genes were highly expressed in a yolk sac tumor, an immature tumor of gonadal origin. These results suggest that *HOX-8* plays a more important role in human tumors of epithelial origin than those of hematopoietic origin.

Homeobox genes, which encode DNA binding proteins that recognize specific sequences and modulate transcriptional activity, are expressed during embryogenesis with positional specificity (1, 2). Recently, altered expressions of some homeobox genes were observed in certain types of human neoplasms. For example, persistent *in vitro* over-expression of *Hox2.4* results in transformation of mouse fibroblasts (3) and expression of *Hox11* is associated with alteration of chromosome translocation of t(10;14) in human T-cell leukemia (4). The expression of class I homeobox genes in hematopoietic tumors has been studied extensively (5,6,7,8). Abnormalities in the expressions of some homeobox genes have also been observed in kidney cancers (9). But little is known about the expressions of homeobox genes in tumors of epithelial origin.

During investigations of homeobox genes in a human ovarian yolk sac tumor, a rare malignant tumor involving juvenile gonads, we isolated three human homeobox genes including *HOX-8*, a putative human homologue of *Hox-8* (10). We compared the expression levels of these genes in various human tumor tissues and cell lines, and found that *HOX-8* was expressed at high level in tumors of epithelial origin, but not in the corresponding normal tissues or in hematopoietic tumors.

*To whom correspondence should be addressed.

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MATERIALS AND METHODS

Tissues and Cell Lines. Samples of human tumors and normal tissues were obtained from surgically resected specimens. The tumors studied were two colon cancers, three gastric cancers, one renal cell carcinoma, two thyroid cancers, six ovarian cancers, one lymphoma of the stomach, one thymoma, one yolk sac tumor and one testicular seminoma. Cell lines established from malignant human hematopoietic cells and choriocarcinoma were gifts from the Japanese Cell Resource Bank. All the samples were immediately frozen in liquid nitrogen and stored at -80 °C.

RNA Preparation. Total RNAs were extracted from various human tissues and cell lines by guanidinium/cesium chloride ultracentrifugation, and poly(A)$^+$ RNAs were obtained by oligo(dT) cellulose column chromatography (11).

cDNA Library Construction. First and second strand cDNAs were synthesized with a cDNA synthesis system (Amersham) and an oligo(dT) primer from 5 μg of the yolk sac tumor mRNA. The cDNA was filled in with T4 DNA polymerase and ligated to the adaptor containing *Eco*RI site. The size fractionated cDNA was ligated to *Eco*RI digested λgt10 under the conditions recommended by the supplier (Stratagene). Ligated DNA was then packaged into phage particles with Gigapack II Gold Packaging Extract (Stratagene).

Screening of a cDNA Library. A 30-bp synthesized oligonucleotide, the complementary sequence of the most conserved portion in the homeodomain of *ftz* gene, 5'-CTTCATGCGTCGGTTTTGTAACCAGATCTT-3', was end-labeled (MEGALABEL, Takara) with [γ-^{32}P]ATP and used as a hybridization probe. Filters prepared from the cDNA library were hybridized under low stringent conditions with 5× SSPE (1×SSPE is 150 mM NaCl, 10 mM $NaH_2PO_4 \cdot H_2O$, 1 mM Na_2EDTA) at 37°C and washed in 5× SSPE at 45°C.

DNA Sequencing. The cDNA clones isolated from the library were subcloned into pUC18 plasmid vector and sequenced by the double stranded dideoxy-chain termination protocol, using T7 DNA polymerase (Sequenase; U.S. Biochemical). The sequences were determined using universal primers and synthetic oligonucleotide primers complementary to each cDNA. In some cDNAs, overlapping subclones were generated by the stepwise deletion method using a Kilo Sequence Deletion kit (Takara) and sequenced with Sequenase. Sequence homology in the DNA Data Base of Japan was searched using the FASTA program (12).

Northern Blot Hybridization. Total RNAs and poly(A)$^+$ RNA from various tissues and cell lines were separated in denaturing agarose gels and transferred to nitrocellulose membranes. As hybridization probes, isolated cDNAs were labeled by [^{32}P] with random primer (Takara). Hybridization was performed followed by washing under high stringent conditions and autoradiography. Hybridization and washing conditions were as described by Sambrook *et al.* (11).

RESULTS AND DISCUSSION

Isolation and nucleotide sequence analysis of cDNA clones.

A human yolk sac tumor cDNA library was constructed, and 50,000 phages were screened using duplicate filters from each plate with 5,000 cDNA plaques. After two rounds of screening with end-labeled oligonucleotide probe, 16 candidate clones were isolated. Finally, three independent clones were selected based on the results of restriction mapping and cross-hybridization.

These three isolated cDNA clones were characterized by partial sequencing. Homology search of each sequence of 200-400 nucleotides (nt) disclosed that two of the three cDNAs were identical with nucleotides 207-891 and 1081-1890 of the human homeobox genes HHO.c1 (13) and HB24 (14), respectively. The third clone was highly homologous with the mouse homeobox gene *Hox-8*, implying that this cDNA is a putative human homologue of the mouse *Hox-8*. Complete sequencing of this cDNA showed that it was 1,804 nt in length and was the counterpart of the 3' region of mouse *Hox-8*. Our cDNA shared 95% of 172 amino acids in the coding sequence, and it further encompassed 1285 nt

of the 3' non-coding region to the poly(A)$^+$ tail (DDBJ accession number, D14970). In the homeodomain, the deduced amino acid sequence of our clone was completely identical with that of *Hox-8*. Therefore, we concluded that our isolated cDNA is the human counterpart of mouse *Hox-8* (*HOX-8*), though it lacked about 300 nt of the 5' end expected by sequence alignment with *Hox-8*.

Expression of *HOX-8* and other homeobox genes in various human tumors.

The expression levels of isolated homeobox genes in various human cancer tissues and cell lines were examined with Northern blotting. As a probe for *HOX-8*, we used a *Pst*I digested 1.5kbp fragment of the 3' region of the cDNA, which did not cross hybridize with the other homeobox containing cDNAs that we isolated. The 0.7 kb insert of HHO.c1 cDNA and the 0.8 kb insert of HB24 cDNA were also used as hybridization probes.

First, we examined the expressions of these genes in human leukemia cell lines, in which lineage specific expressions of class I homeobox genes have been reported (6,7). The 1.6 kb and 1.4 kb transcripts of HHO.c1 were highly expressed in the erythroid cell lines HEL and K562, while the 2.3 kb transcript of HB24 was expressed in the myelomonocytic cell lines KG1, U937 and HL60. In contrast, no expression of *HOX-8* was observed except for a weak band of cross-hybridization to 28S rRNA (Fig. 1).

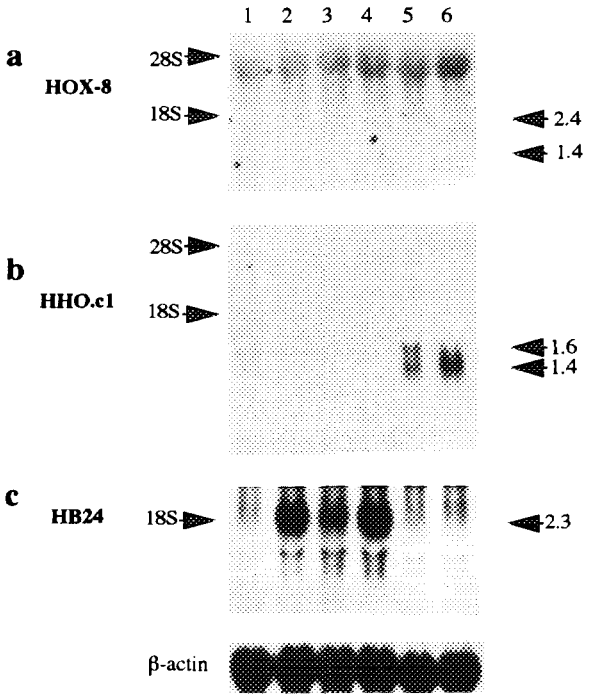

Fig.1. Expressions of *HOX-8*, HHO.c1 and HB24 in the human hematopoietic cell lines. Lane : 1, MOLT-4 (acute lymphoblastic leukemia); 2, KG-1 (acute myelogenous leukemia); 3, U-937 (histiocytic lymphoma); 4, HL-60 (promyelocytic leukemia); 5, K-562 (erythroleukemia); 6, HEL (erythroleukemia). Samples of 15 μg of total RNA were hybridized with [^{32}P] labeled cDNA probes of (a) *HOX-8*, (b) HHO.c1 and (c) HB24. Hybridization of the same filters with β-actin probe is shown as a control. Transcript sizes are indicated in kb.

Among various human tumors of epithelial origin, *HOX-8* mRNA was highly expressed as two major transcripts of 2.4 kb and 1.4 kb in adenocarcinoma of the colon with or without cross-hybridization to 28S rRNA. Its expressions in other types of epithelial tumors, including gastric cancers, renal cell carcinoma and ovarian cancers of several histological types, were weak, and its expression was not detected in the various corresponding normal tissues. On the other hand, expression of HHO.c1 mRNA was observed in tumors and/or corresponding normal tissues of colon, stomach, kidney and ovary. HB24 was only weakly expressed in all the epithelial tumors examined and in the corresponding normal tissues (Fig. 2).

Among the tumors of immature gonadal origin examined, *HOX-8*, HHO.c1 and HB24 were all highly expressed in the ovarian yolk sac tumor from which we isolated the cDNAs of these three genes. *HOX-8* mRNA was also abundant in testicular seminoma and a choriocarcinoma cell line (BeWo). HHO.c1 mRNA was highly expressed in the seminoma but not in the choriocarcinoma, while weak expression of HB24 mRNA was detected in testicular seminoma (Fig. 3). A broad band of cross-hybridization to 28S rRNA was also observed previously when HHO.c1 was hybridized to total RNAs (13). Ribosomal cross-

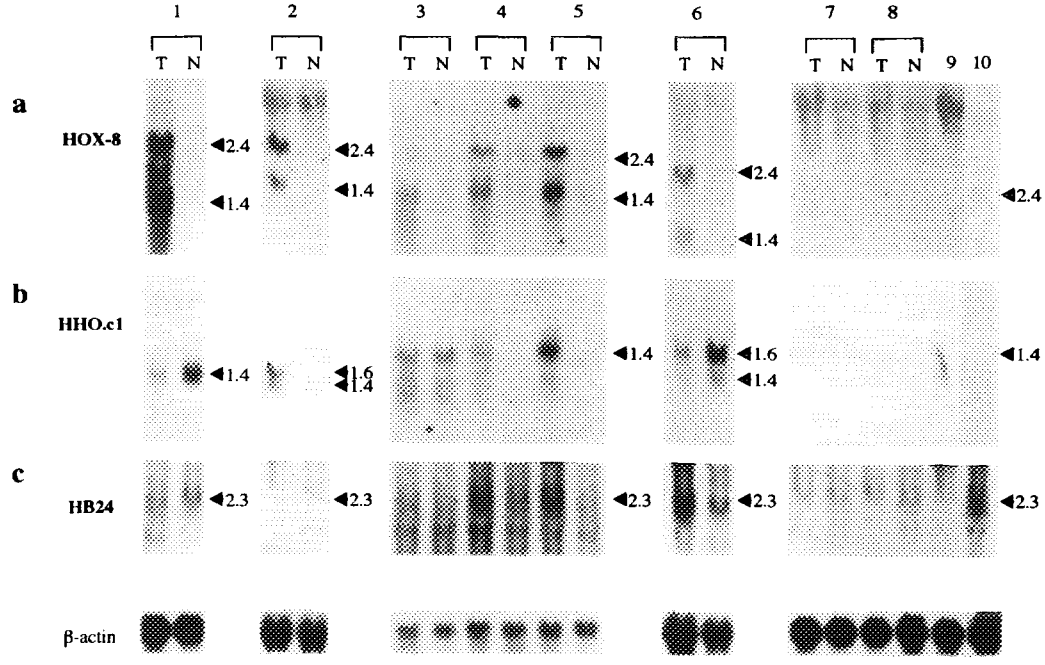

Fig.2. Expressions of *HOX-8*, HHO.c1 and HB24 in various human tumors. Samples of 15 μg of total RNA from colon cancer (cases 1,2), stomach cancer (cases 3,4,5), kidney cancer (case 6), thyroid cancer (cases 7,8) lymphoma of the stomach (case 9) and thymoma (case 10) were hybridized with [^{32}P] labeled cDNA probes of (a) *HOX-8*, (b) HHO.c1 and (c) HB24. Numbers above lanes indicate case numbers, and the letters T and N indicate tumor tissue and the corresponding normal tissue, respectively, except in cases 9 and 10. Hybridization of the same filters with β-actin probe is shown as a control. Transcript sizes are indicated in kb.

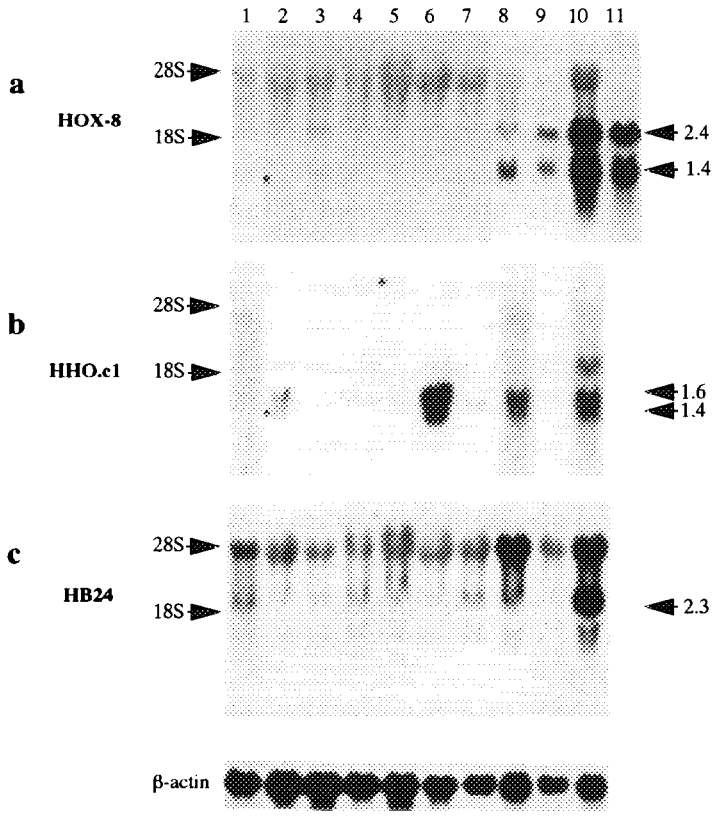

Fig.3. Expressions of *HOX-8*, HHO.c1 and HB24 in human ovarian tumors and immature gonadal tumors. Samples of 15 μg of total RNA were extracted from normal ovary (lane 1), poorly differentiated adenocarcinoma (lanes 2 and 3), serous cystadenocarcinoma (lanes 4-6), mucinous cystadenocarcinoma (lane 7), testicular seminoma (lane 8), the choriocarcinoma cell line BeWo (lane 9) and a yolk sac tumor (lane 10). Three micrograms of poly(A)$^+$ RNA was obtained from the same specimen as for lane 10 (lane 11). Northern blot hybridizations were performed on total RNAs, except for the blot with *HOX-8* probe shown on the right, with [^{32}P] labeled cDNA probes of (a) *HOX-8*, (b) HHO.c1 and (c) HB24. Hybridization of the same filters with β-actin probe is shown as a control. Transcript sizes are indicated in kb.

hybridization was excluded by an experiment with poly(A)$^+$ RNA of the yolk sac tumor (Fig. 3, lane 11).

During embryogenesis, homeobox containing genes are expressed with positional and temporal specificity (2). The peptide sequence of homeobox gene products contains a helix-turn-helix motif which is the DNA binding domain to control gene expression as a transcription factor (15). Furthermore, immunohistochemical analyses have shown that the levels of homeobox proteins are increased in human cancers (16).

In this study, we isolated three homeobox genes from a human yolk sac tumor of the ovary: one was a human homologue of mouse *Hox-8*, and the others were HHO.c1 and HB24. *Hox-8*, a mouse homeobox gene that is related to the *Drosophila msh* homeobox

gene, is reported to be expressed in the surface ectoderm and in the optic vesicle during mouse embryonic development (10), and also in developing chicken limb buds (17). There is no report concerning the expression level of *Hox-8* in neoplasms. We found that *HOX-8* mRNA is highly expressed in immature tumors, including a yolk sac tumor, seminoma and choriocarcinoma, and that its expression is higher in various tumors of epithelial origin than in the corresponding normal tissues. Although *HOX-8* was expressed at high levels in tumors of epithelial origin, it was not expressed in any hematopoietic lines so far examined.

HHO.c1 shares 87% homology with *Hox-2.2* at the nucleotide level, and is a member of the class I homeobox genes (18). The class I homeobox genes were reported to be expressed with lineage-specificity in hematopoietic cell lines (7,8). Recently, altered expressions of some class I homeobox genes have been observed in human kidney cancer (9). We found that HHO.c1 was expressed in several tumors of epithelial origin, but that its expression levels in tumors and the corresponding normal tissues differed in different cases. HB24 has been isolated as a gene distantly related to class I homeobox genes (14). HB24 mRNA is expressed predominantly in mature hematopoietic cells, and is highly expressed in acute myelogenous leukemia (19). The expression of HB24 in non-hematopoietic tumors has not been reported. In this work we observed its expression at high level only in an ovarian yolk sac tumor.

Yolk sac tumor, also named endodermal sinus tumor, resembles yolk sac epithelium histologically, grows down into the extraembryonic mesenchyme and has highly malignant biological characteristics (20). The over-expressions of different classes of homeobox genes in a yolk sac tumor supports the idea that this tumor is in an early stage of differentiation. Our find that *HOX-8* is preferentially expressed in tumors of epithelial origin suggests that it is more important in these tumors than in hematopoietic tumors.

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