A Homeobox Protein, Prox1, Is Involved in the Differentiation, Proliferation, and Prognosis in Hepatocellular Carcinoma

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Abstract

Purpose: It has been shown that a lymphatic differentiation master gene, proxl, also plays an essential role in fetal hepatocyte migration. Its expression is detected in embryonic hepatoblasts and in adult hepatocytes. Hepatoma cells are similar to embryonic hepatoblasts to a certain extent because they both proliferate and invade the surrounding tissue. To address the possibility that Prox1 may be involved in the tumorigenesis of hepatocellular carcinoma (HCC), human clinical samples were analyzed.

Experimental Design: To screen proxl as a potential tumor suppressor gene, its expression was analyzed in HCC cell lines and in human HCC tissues. Its growth-confering abilities were assessed by transiently overexpressing Prox1 in HCC cell lines and by knocking down its expression by RNA interference.

Results: We found that there was a significant correlation between Prox1 expression and the differentiation scores of the tumors. Subsequently, we also showed that low expression of Prox1 in tumors was closely associated with a poor prognosis. The specific knockdown of Prox1 by RNA interference strongly accelerated in vitro cell growth, whereas the overexpression of Prox1 greatly suppressed the growth.

Conclusions: Our results suggest that Prox1 is involved in the differentiation and progression of HCC, and thus it may be a candidate for the development of novel diagnostic and therapeutic strategies for HCC.

Primary hepatocellular carcinoma (HCC) is one of the most common solid tumors in many countries of the world, especially in Asia and Africa, representing the third cause of mortality among deaths from cancer (1). Chronic infections with hepatitis B virus or hepatitis C virus and alcoholic cirrhosis are responsible for the majority of HCC cases. Other risk factors include prolonged dietary exposure to aflatoxin (2), primary hemochromatosis, and cirrhosis associated with genetic liver diseases (3); however, the principal risk factor varies among countries. Although much is known about both the cellular changes that lead to HCC and the histologic findings suggesting that HCC needs a multistep process in expressed genes, the molecular pathogenesis of HCC is not well understood. In addition, a great deal of effort has been devoted to establishing a prognostic model for HCC by using clinical information and pathologic classification to provide information at diagnosis on both survival and treatment options. Nevertheless, many issues still remain unresolved (e.g., a trustworthy staging system to separate patients with HCC into homogeneous groups with respect to prognosis does not exist; ref. 4).

Homeobox proteins are known to play essential roles in the determination of cell fate and the development of the body plan. The roles of homeobox proteins have been documented individually, although these total physiologic roles remain unclear. Several homeobox genes are the targets of chromosomal translocations in malignancies and are thought to be potential oncogenes. Deregulation of such a homeobox gene may give rise to tumorigenesis in target organ. The homeobox gene proxl is related to the Drosophila prospero gene, which mediates cell fate decisions of neuroblasts (5). Proxl is the master gene of lymphangiogenesis but it is also expressed in the developing central nervous system, lens-secreting cone cells, R7 photoreceptors, and midgut (6–8). Furthermore, the expression is detected in embryonic hepatoblasts and in adult hepatocytes (9). Analysis of proxl-null mice showed its potential roles in lens fiber elongation (10), development of the lymphatic systems (11), and hepatocyte migration (12). They also showed a 70% reduction in liver size due to reduced proliferation of hepatoblasts. One of these studies suggested...
that inactivation of Prox1 caused abnormal cellular proliferation and down-regulated expression of the cell cycle inhibitors (10). Interestingly, a report showed that Prox1 controlled progenitor cell proliferation and horizontal cell genesis in mammalian retina from the analysis of the gene-targeting mice (13). In some situations, Prox1 seems to suppress cell proliferation, probably by regulating the cell cycle, and thus prox1 may have a role in tumorigenesis. In fact, mutations and aberrant DNA methylation of prox1 have been observed in hematologic malignancies (14).

These information prompted us to screen prox1 as a potential tumor suppressor gene. Hepatoma cells are similar to embryonic hepatoblasts to a certain extent because they both proliferate and invade the surrounding tissue, and Prox1 could be the driving force behind these cellular activities. Is there a relationship between HCC and Prox1? In this study, we report the down-regulation of Prox1 in HCC, and investigate its involvement in human hepatocellular carcinogenesis.

Materials and Methods

Cell lines and human tissue specimens. Hep3B, Huh7, Alexander, HepG2, and HeLa cell lines were cultured in DMEM (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum and antibiotics at 37°C in humidified air containing 5% CO2.

Cancerous and corresponding noncancerous frozen tissues and optimum cutting temperature–embedded tissues obtained by surgical resection of 52 cases of HCC were retrieved from the 1998-2003 surgical pathology files of Kyoto University Hospital (see Table 1). All samples were obtained with informed consent and their use was approved by the ethics committee of the institution.

Semiquantitative reverse transcription-PCR. Total RNAs were extracted from clinical samples of HCC and cultured cells employing TRIzol reagent (Invitrogen, San Diego, CA) according to the protocol of the manufacturer. Extracted RNA was treated with DNase I (Boehringer Mannheim, Mannheim, Germany). First strand cDNAs were synthesized with oligo dT primer and SuperScript II RNAse H Reverse Transcriptase (Invitrogen). Each single-stranded cDNA was diluted for subsequent PCR amplification. Standard PCR procedures were carried out in 25-μL volumes of PCR buffer (10× ExTaq Buffer, TaKaRa Bio, Otsu, Japan). For detecting prox1, the following primers were used: 5' CAGATGGAGAAGTACGCAC-3' and reverse 5'CATCTCAGTCATGGGATCTG-3'; set 3 (forward 5'CAGCAGCTCAGCAGGCTACTTC-3', reverse 5'TCCTCTGCTGATCAGG-3'); and set 4 (forward 5'CTCTTCCGACGTTAAGTCAACG-3', reverse 5'TCCTCTGCTGATCAGG-3'). GAPDH was used as an endogenous control using commercially available primers (Applied Biosystems). Real-time PCR was done in triplicate using a thermal cycler ABI PRISM 7700 (Perkin-Elmer Applied Biosystems, Foster City, CA) in accordance to the instructions of the manufacturer. Standard curves for templates of prox1 and GAPDH were generated by serial dilution of the PCR products. The Ct values obtained for Prox1 were normalized by the corresponding Ct values of GAPDH.

Immunohistochemistry. Cryosections of 5-μm thickness were prepared and fixed in 4% parafomaldehyde. Endogenous peroxidase was quenched with 3% H2O2 in methanol for 10 minutes, and they were blocked with TNB buffer (TSA Biotin system kit/NEL700, Perkin-Elmer) for 30 minutes. Anti-human prox1 antibody (Relia Tech GmbH, Braunschweig, Germany) was diluted 1:100 and incubated with the sections at 4°C for 16 hours. The slides were washed thrice with TNT buffer (TSA Biotin system) and incubated with biotin-conjugated antirabbit antibody (1:1,000; DakoCytomation, Glostrup, Denmark) at room temperature for 30 minutes. TSA Biotin System (Perkin-Elmer) was used to enhance staining, and peroxidase activity was envisioned with diaminobenzidine kit (DakoCytomation). A subset of the sections was counterstained with hematoxylin. All the sections were mounted in Entellan (Merck, Darmstadt, Germany).

| Variable                   | n (%) |
|----------------------------|-------|
| Sex                        |       |
| Male                       | 40 (76.9) |
| Female                     | 12 (23.1) |
| Hepatitis B virus antigen   |       |
| Positive                   | 13 (25.0) |
| Negative                   | 39 (75.0) |
| Hepatitis C virus antibody  |       |
| Positive                   | 25 (51.0) |
| Negative                   | 25 (48.1) |
| Differentiation            |       |
| Well                       | 22 (42.3) |
| Moderate                   | 21 (40.5) |
| Poor                       | 9 (17.3)  |
| Tumor status               |       |
| T1                         | 3 (5.8) |
| T2                         | 15 (28.8) |
| T3                         | 22 (42.3) |
| T4                         | 12 (23.1) |
| Nodal status               |       |
| N0                         | 49 (94.2) |
| N1                         | 3 (5.8) |
| Metastasis status          |       |
| M0                         | 46 (88.5) |
| M1                         | 6 (11.5) |
| Pathological status        |       |
| I                          | 3 (5.8) |
| II                         | 13 (25.0) |
| III                        | 18 (34.6) |
| IVa                        | 11 (21.2) |
| IVb                        | 7 (13.5) |
Survival rate. The differential expression of Prox1 was divided into two groups (high versus low) according to the value of real-time PCR. The cutoff value was set up at $C_0 = 5.5$ (Ct value). The survival rate was analyzed by the Kaplan-Meier method. SPSS software was used for the statistics.

RNA interference. Two target sequences were assigned at different locations of human prox1 mRNA, designed to match nonconserved sequences. siRNAs were 21-nucleotide-long double-stranded RNA oligos with a two-nucleotide overhang at the 3′ end: siRNA-1 (sense 5′-GGAGCGAUGUAUGGUATT-3′, antisense 5′-UAACAUAUC-CAUGCCUCCCAT-3′) and siRNA-2 (sense 5′-CCUUAUUCGGGAGUGCAATT-3′, antisense 5′-UUGCACUUCCCGAAUAAGGTG-3′). Negative control siRNA was purchased from Qiagen (Hilden, Germany).

Western blotting. Whole-cell extracts were prepared in radioimmunoprecipitation assay lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40] and protein concentration was measured with Coomassie Plus Protein Assay Kit (Pierce, Rockford, IL). Extracts (10-20 μg) were resolved on 12.5% SDS-PAGE gel. After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane, blocked with 5% nonfat milk, and probed with antibodies against Prox1 (1:200) or h-actin (1:10,000) for 1 hour. The blot was washed with TBS containing 0.05% Tween 20 and exposed to horseradish peroxidase–conjugated secondary antibodies for 1 hour, and finally detected by the enhanced chemiluminescence method (Western Lighting Chemiluminescence Reagent Plus, Perkin-Elmer).

Tet-off system. pTet-off regulator plasmid, which contains the tTA transactivator gene under the control of the cytomegalovirus promoter/enhancer, pTRE2hyg response plasmid, which contains a multicloning site immediately downstream of the Tet response element (TRE), and pTRE2hyg-Luc plasmid, which contains the gene encoding firefly luciferase cloned into the pTRE2hyg, were purchased from BD
Expression of prox1 in human HCC. We carried out RT-PCR on some hepatoma cell lines to assess the level of prox1 mRNA expression and detected the highest prox1 expression in Hep3B and lower levels in Huh7, Alexander, and HepG2 (Fig. 1A). We next examined prox1 expression in human HCC and corresponding noncancerous liver tissues by semiquantitative RT-PCR. Prox1 expression was found in cancer tissues as well as normal tissues, and the density value ratio of tumor to nontumor was evaluated. Well-differentiated tumors tended to exhibit the highest prox1 expression levels, and its expression decreased in proportion to the tumor stage, although there was no significant difference statistically (Fig. 1B).

To confirm the expression of prox1 in cancer cells, we carried out immunohistochemical staining in cancerous human liver samples (Fig. 2A and B). Immunostaining revealed high levels of Prox1 staining in the nuclei of cancerous cells in 3 of 6 HCC tissues examined, and stronger staining could be detected in hepatocytes in 4 of 12 noncancerous tissues. We could not detect Prox1 staining in a number of HCC, We could not detect Prox1 staining in a number of HCC, and stronger staining could be detected in hepatocytes in 4 of 12 noncancerous tissues. We could not detect Prox1 staining in a number of HCC, and stronger staining could be detected in hepatocytes in 4 of 12 noncancerous tissues.
proportion to the tumor stage (Fig. 3A). The mean relative expression of prox1 in 9 well-differentiated tumors was 0.043, that in 22 moderately differentiated tumors was 0.021, and that in 21 poorly differentiated tumors was 0.008 ($P = 0.045$, between well and moderate; $P = 0.010$, between moderate and poor).

**Correlation between Prox1 expression and survival rate.** The overall cancer-specific survival was defined as the period from the date of operation to the date of cancer death. We compared the overall survival rate of 52 HCC patients with the expression of prox1 using the Kaplan-Meier method. The differential expression of prox1 was divided into two groups (high and low)
according to the cutoff of the Ct values from real-time PCR (−5.50). The P value for the survival curve was determined by the log-rank test. Consequently, we found that there was a significant difference (P = 0.014) in the survival rates between high and low expressions of prox1 (Fig. 3B). The patients with higher expression (the 5-year survival rate of the 16 patients was 81.25%) had much more favorable prognosis than those with lower expression (that of the 36 patients was 52.78%). On the other hand, our data showed that there was no statistically significant difference in the disease-free survival rates between high and low expression of prox1, probably because HCC has a tendency to recur much easier (Fig. 3C).

**Genetic analyses of prox1 gene in HCC.** Molecular characterization of tumors caused by tumor suppressor genes revealed that the second mutational "hit" usually involved large portions of the particular chromosome, which could be detected by LOH analyses. Genetic events that result in LOH play a major role in the development of tumors, as they convert a heterozygous configuration of a tumor suppressor gene into homozygosity. To investigate whether any loss could be observed in the vicinity of prox1, we carried out LOH analyses in 30 HCC cases using eight polymorphic markers on chromosome 1 (Supplemental data 1). The mean heterozygosity of the microsatellite markers was 75%. The frequency of LOH for each marker in the tumors ranged from 0% to 22.2%, with an average of 8.0 ± 6.8% (mean ± SD). Although 12 tumors exhibited LOH at one or more locus, none showed loss at the two microsatellite markers F (D1S213) and G (D1S2811) spanning the prox1 region (1q32.2-32.3). Therefore, LOH may have no important effect on the suppression of Prox1 in HCC.

To then investigate whether mutations exist in the coding region of prox1, cDNAs derived from 30 human HCCs were amplified by PCR and sequenced as a population. We compared the sequences with the published human prox1 sequence but could not find any mutations in all 30 samples. Therefore, the mutation of prox1 gene is not essential for liver carcinogenesis.

**Knockdown of prox1 expression by RNA interference promotes cell proliferation in Hep3B and Huh7.** Previous data suggested that Prox1 played important roles in control of proliferation and differentiation (13). To more rigorously explore this possibility, we employed two sets of siRNA to knock down endogenous Prox1 expression in two HCC cell lines, Hep3B and Huh7, which had shown the highest expression of prox1. To confirm gene-specific knockdown, we used a negative control siRNA, which had no known homology with mamma-

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for 72 hours at 24-hour intervals, we showed that knockdown of Prox1 protein significantly promoted cell proliferation in the cells (Fig. 4F).

The effect of prox1 overexpression on the proliferation of HCC cell lines. To explore the biological function of Prox1, we next investigated the effects of overexpression of Prox1 on cell proliferation. pTRE2hyg-prox1 and pTRE2hyg-Luc plasmids were transfected into Hep3B-pTet-off cells and incubated with doxycycline. After 24 hours, the transfected cells were counted and plated onto six-well plates at $1 \times 10^5$ per well with and without doxycycline. To test whether the Tet-off system was effective, Western blot analysis was carried out on days 3 and 5. The presence of doxycycline inhibited the expression of Prox1 protein at both time points tested (Fig. 5A). Cell growth was monitored by MTT assay at days 1, 3, and 5 after the passage (Fig. 5B). The assay showed that overexpression of Prox1 suppressed cell proliferation by $>30\%$, whereas that of the control gene luciferase had no effect.

Discussion

In this study, we have shown that the level of Prox1 expression is associated with both clinical and biological aspects of HCC. Various levels of prox1 mRNAs were expressed in HCC cell lines and tumor samples, indicating that they have a diverse influence on the differentiation and prognosis of HCC. In our clinical study, a lower expression level of prox1 considerably corresponded to a poorer differentiation of HCC. Furthermore, we showed that prox1 gene expression was associated with a poor prognosis for patients with HCC. The results of these experiments reveal new insights into the specific expression patterns of Prox1 within HCC tumors and suggest novel functions for Prox1 (i.e., as a tumor suppressor gene or a factor to regulate the progression of malignant character of HCC cells).

In that case, how is prox1 gene regulated? A previous study reported that the expression of the prox1 gene was silenced in various hematologic cell lines, and bisulfate sequencing analysis revealed that DNA methylation of intron 1 might cause this silencing and other cell lines had DNA mutations in the prox1 gene (14). To address the mechanism of down-regulation of prox1 gene expression in HCC, hepatoma cell lines (Hep3B and Huh7) and HeLa cells were treated with the demethylating agent 5-azacytidine (0, 0.02, 0.5, and 1 $\mu$mol/L). However, this had no effect on restoring prox1 gene expression in the three cell lines (data not shown). Subsequently, we carried out mutation screening of prox1 mRNA and LOH analyses of prox1 in 30 HCC tumors, but we could not find any mutations or LOH in these samples. Previously, it was reported that a high frequency of LOH was not observed at the chromosome arm 1q, which is the location of prox1 gene (15). Our results are in good agreement with theirs. However, the mechanism of down-regulation of prox1 gene expression remains undisclosed. We surmise that there is an unknown mechanism that regulates the prox1 gene.

In attempting to determine the possibility of prox1 as a tumor suppressor gene, we employed the RNAi technique for knockdown of its expression and analyzed its phenotype. We found that a transient knockdown of prox1 significantly accelerated the growth of HCC cell lines in vitro, and we also showed that overexpression of prox1 resulted in suppression of cell proliferation. These results suggest that overexpression of Prox1 can strongly inhibit tumorigenesis, and our findings indicate that Prox1 is an attractive candidate for a diagnostic and therapeutic target. We have recently found that Prox1 overexpression conferred a slower growth phenotype to some cancer cell lines and enabled them to form much smaller tumors in nude mice. It remains to be determined how the reduction of Prox1 expression contributes to the differentiation of HCC and influences the survival of patients with HCC. Because of the proliferation and/or differentiation activity in HCC cells, decreased Prox1 expression may enhance the unregulated growth or recurrence of HCC cells.

In summary, we have presented evidence of Prox1 expression in malignant hepatoma cell lines and in HCC specimens. Furthermore, we have shown that decreased Prox1 levels are correlated with the progression of differentiation of HCC and predict poor prognosis for patients with HCC after surgery. Our findings indicate that prox1 may be a novel candidate gene for the development of diagnosis and therapeutic strategies for HCC.

3 Unpublished data.

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