Downregulation of alpha-fetoprotein expression by LHX4: a critical role in hepatocarcinogenesis

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Introduction

Hepatocellular carcinoma (HCC) is the sixth most common malignancy and the third most common cause of cancer-related mortality worldwide (1). Hepatitis B virus and hepatitis C virus infection are the main etiologic factors in >80% of HCC cases (2). Other risk factors include alcohol abuse, non-alcoholic steatohepatitis, primary biliary cirrhosis and hemochromatosis. HCC is not amenable to standard chemotherapy and is resistant to radiotherapy. Current curative treatments for HCC are surgical resection and liver transplantation. However, recurrence or metastasis is quite common among patients who have had a resection and the survival rate is 30–40% at 5 years post-operatively (3).

In recent years, research has focused on uncovering the molecular mechanisms that underlie HCC. Genetic alterations clearly play a major role in hepatocarcinogenesis, and abnormalities in several critical molecular pathways have been identified as contributing to HCC progression. These pathways include Wnt/β-catenin, p53, pRb and mitogen-activated protein kinase pathways (4,5). Since hepatocarcinogenesis is a complex process and HCC is still a difficult disease to treat, these issues point to the importance of the continuous identification and characterization of novel genes involved in hepatocarcinogenesis, which may enable more effective treatment of HCC.

LHX4 is a LIM-homeodomain (LIM-HD) transcription factor that belongs to the subfamily of homeobox genes. The characteristic features of LHX4 proteins are two LIM–protein–protein interaction domains followed by a DNA-binding homeodomain. The LIM domain is recognized by a number of cofactors that mediate LIM-HD function. Different types of cofactors will mediate the formation of distinct transcriptional–regulator complexes that regulate transcription in a tissue-specific manner (6). During embryonic and postnatal development, LHX4 gene is expressed in hindbrain, cerebral cortex, pituitary gland and spinal cord, suggesting that LHX4 plays a role in nervous system development (7,8). Mutations in LHX4 genes are associated with combined hormone deficiency diseases in human and animal models (9,10). Aberrant expression of the LHX4 gene by the chromosomal translocations has been observed in several types of leukemia (11,12). Moreover, the methylation of LHX4 is more pronounced in primary lung tumor samples than paired non-cancerous tissues (13).

Although the inappropriate expression of LHX4 has been found in several cancers, the functional roles of LHX4 in carcinogenesis remain unclear. In this study, we discovered that LHX4 was frequently lost in tumor tissues obtained from patients with HCC and that low expression of LHX4 was associated with tumor undifferentiation state and high AFP level in HCC. Functional studies revealed that ectopic expression of LHX4 reduced AFP expression, which leads to the suppression of HCC growth. Our results support that LHX4 functions as a potential tumor suppressor in hepatocarcinogenesis.

Materials and methods

Subjects

This study was approved by the ethics committee of the National Taiwan University Hospital and registered at ClinicalTrials.gov (a service of the US National Institutes of Health, NCT01247506). Written informed consent from each subject was obtained. Samples were obtained from a total of 75 patients who underwent curative hepatectomy for HCC between January 2002 and December 2005. Three normal liver tissues were obtained from living liver donors at the same hospital. All donors were examined to be free of liver diseases. Clinical parameters were assessed using a retrospective chart review.

Western blotting

Total proteins were extracted using RIPA buffer (50 mM Tris–HCl, 1% NP-40, 0.05% sodium deoxycholate, 150 mM NaCl and 1 mM ethylenediaminetetraacetic acid), supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany). An equal amount of protein was subjected to 10% sodium dodecyl sulfate–Tris glycine polyacrylamide gel electrophoresis gel, followed by electroblotting onto a polyvinylidene difluoride membrane. After blocking in 5% skim milk, the membrane was incubated with 1 μg/ml rabbit polyclonal LHX4 antibody (Abnova, Taipei, Taiwan), followed by the secondary antibody. The enhanced chemiluminescence detection system was used to detect the immunocomplex. Rabbit polyclonal anti-alpha-fetoprotein (AFP) antibody (Thermo, Fremont, CA), rabbit polyclonal anti-albumin antibody (Abnova), mouse monoclonal anti-β-actin antibody (Novus, Littleton, CO) and mouse monoclonal anti-flag antibody (Sigma–Aldrich, St Louis, MI) were used at a dilution of 1:2000, 1:8000, 1:5000 and 1:5000, respectively. The monoclonal anti-flag antibody was used to recognize the DDK tag of LHX4 protein.

Evaluation of LHX4 expression level

The expression of LHX4 protein was studied in HCC tumor tissues and their paired non-tumor tissues from the same patient by western blotting.

Abbreviations: AFP, alpha-fetoprotein; HCC, hepatocellular carcinoma; LIM-HD, LIM-homeodomain; PCR, polymerase chain reaction.

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Densitometric quantification of signal was analyzed with LabWorks software and the signal was normalized with β-actin. The expression levels were classified quantitatively based on the normalized signals. The average signals of the HCC tumor and paired non-tumor tissues were 0.7 and 1.4, respectively. Sixty-six patients were divided into two groups according to the LHX4 expression signal in HCC tumor tissues: ‘Low’ denotes that the signal value was lower than median (<0.2) and ‘High’ denotes that the signal value was higher than median (>0.2).

Immunohistochemistry
Paraffin-embedded specimens of 15 cases of HCC were obtained from Department of Pathology (National Taiwan University Hospital). Human Adult and Fetal Normal Tissue Array was purchased from US Biological Company (Marblehead, MA). Following deparaffinization and antigen retrieval, the slides were blocked with 3% hydrogen peroxide for 10 min. Sections were incubated with rabbit anti-LHX4 antibody (1:30; LifeSpan, Seattle, WA), rabbit anti-AFP antibody (1:200) or rabbit anti-Ki-67 antibody (1:100; Thermo) at 4°C for overnight. After washing, the sections were incubated with Histofine Stain MAX PO (Polymer detector system; Nichirei, Tokyo, Japan) at room temperature for 30 min and then developed with 3, 3-diaminobenzidine chromogen solution in 3, 3-diaminobenzidine buffer substrate. Sections were visualized with 3, 3-diaminobenzidine and counterstained with hematoxylin. Normal rabbit IgG was used as a substitute for the primary antibody as negative controls. All sections were observed and photographed with an Axioskop 2 microscope (Carl Zeiss, Oberkochen, Germany).

Cell lines and DNA transfection
The human HCC cell lines, PLC5, Hep3B, HepG2, Huh7 and normal hepatocyte CL-48 were maintained at 37°C with 5% CO2 in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum and 100 U of penicillin and 100 µg of streptomycin/ml. Cells were transfected using TurboFect reagent (Fermentas, Glen Burnie, MD) following the manufacturer’s protocol. Each transfection was brought to a total of 1 µg of DNA. Transfections were carried out in 24-well plates.

Generation of stable transfectants overexpressing LHX4
Human TrueORF<sup>TM</sup> LHX4 plasmid was purchased from OriGene Technologies (Rockville, MD). TrueORFs have a C-terminal fusion of MYC/DDK tag. The cloning expression vector is pCMV6-Entry. To establish stable transfectants that constitutively express LHX4, HepG2 cells were transfected with pCMV6-LHX4 plasmids. After culturing in complete medium containing G418 (1000 µg/ml) for 2 weeks, individual clones were isolated and determined for LHX4 expression by western blotting. A stable transfectant expressing pCMV6-Entry empty vector was used as a control.

Reverse transcription—polymerase chain reaction
Total RNA was extracted using Trizol reagent (Invitrogen) following the manufacturer’s instructions. Complementary DNA was then synthesized from one microgram total RNA using random hexamer and MMLV reverse transcriptase (Fermentas). The primer sequences are described in Supplementary Table S1, available at Carcinogenesis Online. The optimized polymerase chain reaction (PCR) cycling parameters were as follows: one cycle of denaturation at 94°C for 30 s; 25 cycles (semiquantitative) of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s, followed by one cycle of final extension at 72°C for 10 min. PCR products were analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Immunofluorescence
Cells transfected with either pCMV6-Entry empty vector or Myc/DDK-tagged LHX4 clone were plated onto glass coverslips and fixed at 4% (vol/vol) paraformaldehyde/phosphate-buffered saline 48 h after transfection. They were subsequently permeabilized in phosphate-buffered saline containing 0.5% (vol/vol) Triton X-100 for 15 min and were blocked in phosphate-buffered saline containing 5% (wt/vol) bovine serum albumin for an hour. Cells were labeled with rabbit anti-AFP antibody and mouse anti-flag antibody, followed by Alexa-488-conjugated goat anti-rabbit immunoglobulin G and Alexa-594-conjugated goat anti-mouse immunoglobulin G (Molecular Probes, Carlsbad, CA). Nuclei was stained with Hoechst 33342. Photographs were taken using a fluorescence microscope (Axiophot 2, ZEISS).

Measurements of cell proliferation
Cell proliferation of stably transfected HepG2 cells was measured using the cell proliferation reagent WST-1 (Roche, Mannheim, Germany). In brief, stable pCMV6-LHX4 transfectants and empty vector transfectants were plated in duplicates in 24-well plates at 5 × 10<sup>4</sup> per well. Fifty microliters of WST-1 was added to the medium (450 µl) at the time of harvest, according to the manufacturer’s instructions. One hour after adding WST-1, cellular viability was determined by measuring the absorbance of the converted dye at 450 nm. Cell proliferation of transient transfectant was determined using a hemocytometer. Cells were seeded (5 × 10<sup>4</sup> per well for Huh7; 7 × 10<sup>4</sup> per well for HepG2) onto 24-well plates. Twenty-four hours later, culture medium was removed and replaced with 1 ml fresh medium containing expressing plasmids and transfection reagent. Cells were detached by trypsinization and the cell count was determined at the indicated times. Cells transfected with empty vector were used as controls. Each experiment was done at least thrice in duplicate wells. The results were given as mean cell numbers ± standard deviation (SD)/well.

Colony formation assay
Colony formation assay was performed using monolayer culture. Cells (1 × 10<sup>5</sup>) were plated in a 6 cm dish and transfected with expression plasmids pCMV6-LHX4 or the empty vector pCMV6-Entry (5 µg each) using TurboFect (Fermentas). Forty-eight hours after transfection, cells were replated (1 × 10<sup>4</sup>) in a fresh 6 cm dish. After selection with G418 for 14–21 days, surviving colonies were fixed with 100% ice-cold methanol and stained by crystal violet for counting. Each treatment was performed in duplicates in three independent experiments.

Effects of AFP on the cell proliferation
Purified human AFP was obtained from AbD Serotec (Kidlington, UK). Total 2 × 10<sup>6</sup> cells per well of HepG2 stable transfectants were plated into 96-well plates and cultured for 24 h. The cultures were replaced with medium without fetal bovine serum and treated with different concentration of AFP (0, 5, 10 and 20 µg/ml) for 48 h. At the time of harvest, 20 µl of WST-1 was added to the cells cultured in 200 µl medium per well. One hour after adding WST-1, cellular viability was determined by measuring the absorbance of the converted dye at 450 nm. Relative cell viability was calculated as the absorbance of treated cells relative to that of cells before treatment. Data expressed as the mean ± SD correspond to three separate experiments performed in duplicate.

Statistical analysis
All statistical analyses were performed using the SPSS 16.0 statistical software package. Due to the non-normal distribution of protein expression, statistical evaluation was performed using non-parametric tests (Wilcoxon signed rank test). Continuous variables were presented as mean ± SD or median and range and analyzed using the Student’s t-test or Mann–Whitney U-test, respectively. The chi-square or Fisher’s exact test was performed to assess the correlation between LHX4 expression and clinicopathological characteristics. P < 0.05 was considered statistically significant.

Results
Decreased LHX4 expression in tumor tissues from HCC patients
In an attempt to explore the role of LHX4 in hepatocarcinogenesis, human primary HCC tissues were analyzed for LHX4 expression in comparison with their paired non-tumor tissues by western blotting. A representative blot was shown in Figure 1A. The expression level of LHX4 of each specimen was quantified and normalized with β-actin (Figure 1B and C). As shown in Figure 1B, LHX4 expression was downregulated >2-fold (NT/T > 2) in 62% (41/66) of HCC tissues examined. The mean of LHX4 expression in non-tumor tissues was ~2-fold of that in HCC tumor tissues (Figure 1C). The downregulation of LHX4 levels in tumor tissues was found to be statistically significant (P < 0.0001), which indicates the importance of LHX4 in human HCC.

LHX4 expression is associated with tumor differentiation and AFP levels
To clarify the clinical significance of decreased LHX4 expression in HCC, LHX4 protein expression patterns in HCC tumor tissues were compared with their clinicopathological features (Table I). Among 66 patients, low expression of LHX4 protein was significantly correlated with male dominance (P = 0.04), higher serum AFP values (P = 0.02) and poor tumor differentiation (P = 0.02). We next investigated the relationship between LHX4 expression and the differentiation grade of HCC by analyzing and comparing an independent set of HCC tumor tissues with normal liver samples. As shown in Figure 2A (upper blot), LHX4 expression in grade I tumors, well-differentiated lesions, was almost the same as that of normal liver tissues. However, LHX4 expression was abruptly downregulated in moderate-differentiated grade II tumors and was further reduced...
in poor-differentiated grade III tumors. Thus, the expression levels of LHX4 gradually decreased in accordance with histological undifferentiation. Since increased AFP levels in HCC patients have been associated with histologically undifferentiated state (14), the association of AFP with LHX4 protein level was further analyzed in these human HCC tissues. Accompanied by decreased LHX4 level, an increase of AFP protein expression was also observed (Figure 2A, middle blot). This inverse correlation for HCC tissues is consistent with the serum AFP values (Table I). Additionally, the relationship between the expressions of LHX4 and AFP in various HCC cell lines was also studied by western blotting. The data revealed that HCC cell lines exhibited significantly lower levels of LHX4 expression.

Fig. 1. LHX4 expression was downregulated in tumor tissues from HCC patients. (A) The representative data of LHX4 expression identified by western blotting in HCC tumor tissues (T) and paired non-tumor tissues (NT) from the same patient. β-Actin was used as an internal control. (B) LHX4 expression in 66 pairs of tumor and non-tumor tissues of HCC. The intensity of western blotting was retrieved using LabWorks software. Each LHX4 intensity was divided by the corresponding intensity of β-actin from the same tissue sample to adjust for the sample variation. (C) Decreased LHX4 protein expression was present in 66 pairs of HCC tissues studied. The boxes represent the interquartile range (25–75th percentiles). The horizontal line inside the box indicates the median. The difference in LHX4 expression level between T and NT groups were analyzed by Wilcoxon signed rank test. *P < 0.0001.
compared with the levels in normal liver tissue (Figure 2B). Besides, the expression level of AFP was significantly higher in cells with low LHX4 expression than in those with high expression. These results indicate that loss of LHX4 expression was often found in poor-differentiated HCC, where AFP is frequently reactivated.

Immunohistochemical analysis was conducted on paraffin-embedded HCC specimens to further verify the inverse correlation between LHX4 and AFP expression. As shown in Figure 2C (a), LHX4-positive signal was highly presented in nuclei and/or cytoplasm of surrounding non-tumor tissues, whereas this signal was reduced in HCC tumor tissues. On the contrary, AFP was highly expressed in tumor tissues but was not detectable in adjacent non-tumor tissues [Figure 2C (b)]. We also determined the expression of Ki-67, a commonly used marker for cell proliferation, together with LHX4 in serial tissue sections. As a tumor marker, Ki-67 signal was stronger in HCC tumor tissues than adjacent non-tumor tissues [Figure 2C (c)]. Primary antibody was replaced with preimmuned normal rabbit IgG to serve as negative control [Figure 2C (d)]. Collectively, decreased expression of LHX4 in tumor tissues was accompanied by a lower degree of differentiation (high AFP) and a higher extent proliferation (high Ki-67).

Table I. Relations between LHX4 protein levels and clinicopathologic features in HCC

<table>
<thead>
<tr>
<th>Variables</th>
<th>Patientsa (n)</th>
<th>LHX4 expressionb</th>
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<th>P value</th>
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<tbody>
<tr>
<td></td>
<td>All cases</td>
<td>Low, n (%)</td>
<td>High, n (%)</td>
<td></td>
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<tr>
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<td>51</td>
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<td>Female</td>
<td>15</td>
<td>4 (26.7)</td>
<td>11 (73.3)</td>
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<tr>
<td>Age (years)</td>
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<td>58.7 ± 13.1</td>
<td>62.2 ± 11.4</td>
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<td>19 (52.8)</td>
<td></td>
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<tr>
<td>Hepatitis virus C</td>
<td>18</td>
<td>12 (66.7)</td>
<td>6 (33.3)</td>
<td></td>
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<tr>
<td>Hepatitis virus B and C</td>
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<td>1 (25.0)</td>
<td>3 (75.0)</td>
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<td>Non-B, Non-C</td>
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<td>3 (37.5)</td>
<td>5 (62.5)</td>
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<td>Serum AFP (ng/ml)</td>
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<td>41 (2–87320)</td>
<td>12 (4-3001)</td>
<td>0.011f</td>
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<td>Histology grade</td>
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<td></td>
<td></td>
<td>0.021c</td>
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<td>I: well differentiated</td>
<td>13</td>
<td>2 (15.4)</td>
<td>11 (84.6)</td>
<td>0.523e</td>
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<tr>
<td>II: moderately differentiated</td>
<td>42</td>
<td>25 (59.5)</td>
<td>17 (40.5)</td>
<td></td>
</tr>
<tr>
<td>III: poorly differentiated</td>
<td>11</td>
<td>6 (54.5)</td>
<td>5 (45.5)</td>
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<tr>
<td>TNM stage</td>
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<tr>
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<td>20 (51.3)</td>
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<tr>
<td>II</td>
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<td>12 (60.0)</td>
<td>8 (40.0)</td>
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<tr>
<td>III</td>
<td>7</td>
<td>2 (28.6)</td>
<td>5 (71.4)</td>
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<td>Tumor size</td>
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<td>9 (40.9)</td>
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<tr>
<td>Present</td>
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<td>20 (50.0)</td>
<td>20 (50.0)</td>
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<td>20 (50.0)</td>
<td>0.183e</td>
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<td>4 (50.0)</td>
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<tr>
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<tr>
<td>Present</td>
<td>15</td>
<td>9 (60.0)</td>
<td>6 (40.0)</td>
<td></td>
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<tr>
<td>Cirrhosis</td>
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<tr>
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<td>23 (57.5)</td>
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<tr>
<td>Present</td>
<td>26</td>
<td>16 (61.5)</td>
<td>10 (38.5)</td>
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</table>

Age was expressed as mean ± SD. Serum AFP levels were expressed as median and range. Significant P value are bold.

- The expression level of LHX4 was quantified by western blotting as described in the Materials and Methods.
- The total number was <66 due to missing data.
- Chi-square test.
- Student’s t-test.
- Fisher’s t-test.
- Mann–Whitney U-test.

Table I. Relations between LHX4 protein levels and clinicopathologic features in HCC

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Taken together, as demonstrated in Table I and Figure 2, the expression levels of LHX4 and AFP are inversely correlated in patient-derived tumors and human HCC cell lines. We found no correlation between LHX4 and other clinicopathological features (Table I). These results suggest that the association of LHX4 with AFP expression have a specific role in relation to the tumor differentiation state in HCC.

Ectopic expression of LHX4 suppresses AFP expression in hepatoma cells

To better understand the clinical findings described previously, an in vitro system was established to dissect the molecular mechanisms. Because the endogenous levels of LHX4 were marginally expressed in HepG2 cells, we chose this cell to establish LHX4 stable cell lines. Successful expression was determined by reverse transcription–PCR and western blotting (Figure 3A and B). Among the LHX4 subclones, we selected two representative overexpression clones, namely HepG2-LHX4-7 and HepG2-LHX4-9, which expressed a moderate and high level of LHX4, respectively.

The expression pattern of AFP and albumin were first analyzed to evaluate whether ectopic expression of LHX4 restores cell
differentiation status. Reverse transcription–PCR and western blotting showed that HepG2-LHX4-9 cells expressed very low levels of AFP messenger RNA and protein when compared with the cells expressing empty vector. Besides, HepG2-LHX4-7, which moderately increased LHX4 expression, exhibited an intermediate phenotype. On the other hand, HepG2-LHX4-9 cells and respective control cells expressed similar levels of albumin messenger RNA and protein (Figure 3A and B). These data suggest that AFP downregulation in HepG2-LHX4-9 cells was gene specific, supporting current view on autonomous regulation of AFP and albumin gene expression (15).

Transient transfection was also performed to clarify that AFP repression was not due to clonal effect of LHX4 stable cells. Compared with the cells expressing empty vector, AFP expression was decreased in both Huh7 and HepG2 cells overexpressing LHX4. The decreased expression of AFP occurred in a dose-dependent manner (Figure 3C). These data suggest that AFP downregulation in HepG2-LHX4-9 cells was gene specific, supporting current view on autonomous regulation of AFP and albumin gene expression (15).

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Fig. 2. LHX4 expression inversely correlated to AFP expression in primary HCC tissues and human hepatoma cells. (A) Western blotting of LHX4 and AFP expression in HCC tissues. Grade I, Grade II and Grade III represented well, moderately and poorly differentiated tumor tissues, respectively. LHX4 protein levels decreased in parallel with HCC tumor progression and correlated negatively with AFP expression. Three normal liver samples were included as the control. Representations of three independent experiments are shown. (B) A comparison of expression pattern of LHX4 and AFP in hepatoma cells. LHX4 expression was severely repressed in hepatoma cells compared with normal liver. AFP levels were inversely correlated with LHX4 expression. Results were representative of three independent experiments. The blots were stripped and reprobed with anti-beta-actin antibody to show equal loading of total protein (A and B). (C) Immunohistochemistry of LHX4 (a), AFP (b) and Ki-67 (c) in paraffin-embedded consecutive HCC tissue sections at ×100 magnifications. Expression levels of LHX4 were higher in non-tumor (NT) tissues compared with tumor (T) tissues. The intensities of AFP and Ki-67 in tumor tissues were higher than those in non-tumor tissues. The primary antibody was replaced with normal rabbit IgG as negative control (d). These figures were representative of 15 slices.

AFP promoter analysis in silico is carried out to provide an explanation for the regulation of LHX4 in AFP expression. Using comparative sequencing analysis with the rVista computational tool (16), the putative LIM-HD-binding site is positioned −128 to −121 base pairs upstream from the transcription start site in the human AFP promoter. Therefore, we postulated that AFP is a potential target gene of LHX4.

Ectopic expression of LHX4 inhibits proliferation in hepatoma cells

In many tissues, growth arrest and differentiation are intricately linked. For instance, rapid growing cells typically differentiate less (18). We further explored whether re-expression of LHX4 would inhibit hepatocellular growth. As shown in Figure 4A, HepG2-LHX4-9

transfected with empty vector showed no staining with flag antibodies [Figure 3D (a and c)]. Taken together, these results indicate LHX4 facilitates cellular differentiation by decreasing AFP expression in hepatoma cells.
Fig. 3. LHX4 suppresses AFP expression in hepatoma cells. (A and B) Expression pattern of AFP and albumin in the cells stably transfected with empty vector and LHX4 expression vector. Enhanced expression of LHX4 downregulated AFP expression, as determined by reverse transcription–PCR (A) and western blotting (B). GAPDH and β-actin were used as loading control, respectively. (C) Huh7 (left panel) and HepG2 (right panel) cells were transiently transfected with empty vector and LHX4 expression vector in a different amount followed by western blotting for AFP. Each transfection was brought to a total of 1 μg of DNA with the replenishing empty vector. Transfections were carried out in 24-well plates. (D) Immunofluorescent images of Huh7 and HepG2 transfectants. Cells were transiently transfected with empty vector (a and c) or vector expressing LHX4 (b and d). Two days after transfection, cells were fixed with 4% paraformaldehyde and subjected to immunofluorescence staining. AFP expression (green fluorescence) was monitored using rabbit anti-AFP antibodies. LHX4 (red fluorescence) was detected using mouse anti-flag antibodies against the DDK-tag of LHX4 protein. Hoechst 33258 was used along with the secondary antibody to detect the nucleus.
Roles of LHX4 in hepatocarcinogenesis

Fig. 4. LHX4 inhibits proliferation in hepatoma cells and this inhibitory effect was reversed by replenishing AFP. (A) Cell proliferation of stably transfected HepG2 cells was assessed by WST-1 assay. HepG2-LHX4-9 cells exhibited a reduced proliferation when compared with empty vector control cells (named HepG2-Vector). (B) The effects of LHX4 on hepatoma cell growth were further confirmed by colony formation assay. Left panel showed the representative images of the colony formation in cells transfected with empty vector or LHX4 expression vector. Quantitative analysis of colony numbers was shown in the right panel. (C) Huh7 (left panel) and HepG2 (right panel) cells were transiently transfected with the empty vector or vector-expressing LHX4. One, 2 and 4 days after transfection, cells were harvested and counted using a hemocytometer. The data represent the number of cells per well. Introduction of LHX4 gene reduced the growth of Huh7 and HepG2 on day 4, at 79 and 81%, respectively, in comparison with cells expressing empty vector. (A–C) These figures were done at least thrice in duplicates and each data represent the mean and SD. One asterisk (*) indicates \( P < 0.05 \) while two asterisks (**) indicate \( P < 0.01 \), comparing with empty vector control by the Student’s t-test. (D) AFP reverses the growth-inhibitory effect of LHX4 to levels comparable with control cells. HepG2 cells stably expressing LHX4 (HepG2-LHX4-9) or empty vector (HepG2-Vector) were incubated with purified human AFP protein (0, 5, 10 and 20 mg/l) for 48 h and cell proliferation was measured by WST-1 assay as described earlier. The proliferation of treated cells was expressed as mean ± SD. As shown in Figure 4D, growth-inhibitory effect was up to 20% on the fourth day after LHX4 transfection compared with the control group (\( P < 0.05 \)).

Restoration of AFP compensates the growth-inhibitory effect of LHX4

AFP has been suggested to play an important role for hepatoma cell growth (19, 20). We hypothesized that downregulation of AFP directly mediates the growth-inhibitory effect of LHX4. To further address this issue, purified human AFP protein was added to both empty vector control and HepG2-LHX4-9 cells and cell proliferation quantified. Addition of exogenous AFP had little effect on the proliferation of empty vector control cells (Figure 4D). As previously observed, the HepG2-LHX4-9 cells exhibited a significantly decreased level of proliferation when compared with empty vector controls (\( P = 0.015 \); Figure 4D). Importantly, AFP was able to significantly restore the proliferation of HepG2-LHX4-9 cells to the same level as control cells (Figure 4D). These results indicated AFP as a downstream mediator of LHX4-driven growth inhibition.

In summary, our findings demonstrated reconstitution of LHX4 in hepatoma cells could repress AFP expression and convert hepatoma cells into a more differentiated and growth arrested phenotype.

Developmental activation of LHX4 is associated with postnatal AFP repression in liver

Many molecular pathways that control normal development are critical for carcinogenesis. To gain further insights into the mechanism of LHX4 in hepatocarcinogenesis, developmental expression pattern of LHX4 in liver was examined by Human Adult and Fetal Normal Tissue Array (US Biological). As depicted in Figure 5, LHX4 protein was nearly absent in fetal liver (Figure 5A) and normally expressed in adult liver (Figure 5B). It is known that AFP proteins are expressed during fetal development, normally absent after birth and frequently reactivated in HCC. We further analyzed AFP expression in the same batch of specimens. Consistent with previous reports, AFP was highly presented in fetal liver (Figure 5C) but was undetectable in adult liver (Figure 5D). These data showed that LHX4 was developmentally activated in liver and inversely correlated with AFP expression, suggesting that LHX4 might be involved in postnatal AFP repression in liver.

Discussion

Homeobox genes comprise a family of developmental regulators that are vital for all aspects of growth and differentiation. Aberrant regulations of homeobox gene expressions have been reported in various forms of cancers (21–24). Homeobox genes that are upregulated in cancers may be normally expressed during development and/or in undifferentiated cells, whereas the others that are downregulated in cancers may be normally expressed in adulthood and/or in differentiated tissues (25). In the present study, we offered evidence for the first time that LHX4 protein expression was significantly downregulated in HCC tumor tissues. Besides, we showed that LHX4 was normally expressed in differentiated tissues by comparing expression
levels of LHX4 protein in fetal and adult livers. This expression profile conformed to the rules described above and was consistent with other homeobox genes (26,27).

By analyzing the correlation between expression pattern of LHX4 and clinicopathological features, low expression of LHX4 correlates with undifferentiated tissue types of HCC and an inverse correlation between LHX4 expression and AFP levels was demonstrated in both serum and HCC tissues. In parallel with analysis of LHX4 dysregulation in HCC, we explored its biological activities in hepatoma cells, including Huh7 and HepG2. These cell lines offer the advantage of very low endogenous levels of LHX4, comparable with that seen in HCC, so that the effects of increased ectopic LHX4 can be readily discerned. In this system, reconstituted LHX4 expression leads to decreased AFP expression and growth inhibition. Furthermore, a potential role of LHX4 in postnatal AFP repression, which was observed in the results of tissue array, supports the possibility that LHX4 could be involved in AFP reactivation in HCC. Collectively, the findings in hepatoma cells as well as human HCC specimens indicate that the histological undifferentiation observed in low LHX4-expressing tumor tissues could be explained by the increased expression of AFP.

Previous studies have reported that LHX4 harbored both transcriptional activation and repression capacities. LHX4 was shown to have a direct stimulatory effect on the prolactin gene promoter. This interaction could be enhanced by other homeobox-containing transcription factors such as Pit-1 or be inhibited by interacting proteins such as the selective LIM-binding protein, SLB (28). In addition, SSX2, a transcriptional corepressor, could interact with LH4X and exert an inhibitory effect on the LHX4-mediated transactivation in human synovial sarcoma (29). These lines of evidence demonstrated that modulation of LHX4 activities occur through protein–protein interactions. In the present study, LHX4 overexpression in HepG2 cells decreased AFP expression at both messenger RNA and protein levels, indicating a regulatory role for LHX4 in the control of AFP transcription. In addition, in silico analysis identified a putative LHX4-binding site at the human AFP promoter. In this regard, it is reasonable to surmise that LHX4 could suppress AFP transcription through consensus sequence binding. Furthermore, the decreased proliferation in LHX4-overexpressing cells was restored by the replenishment of recombinant AFP, lending additional support that AFP is a direct and functional target for LHX4. Future molecular studies are necessary to confirm the direct interaction between LHX4 and the AFP promoter and to investigate whether other cofactors are also involved in this transcriptional repression.

Determining terminal differentiation is a feature of the LHX4 gene function that has been conserved throughout evolution (30). Studies of a pituitary cell side population containing pituitary stem cells have revealed that LHX4 is critical for differentiation into specific pituitary hormone-producing cells (31). In accordance with this function, our study demonstrated that LHX4 was developmentally activated in liver and forced re-expression of LHX4 in dedifferentiated hepatoma cell lines confer downregulation of oncofetal protein AFP expression, implying LHX4 might have a potential role in hepatic differentiation. Treating malignant tumors through the induction of cell differentiation has been an attractive concept. Differentiation therapy could force hepatoma cells to differentiate and lose their self-renewal property. However, limited benefit has been achieved in the treatment of solid tumors, including HCC (32). Our study provides a rationale for further evaluation of LHX4 differentiation therapy in HCC.

In conclusion, our findings are the first to report on tumor suppressive activities of LHX4 in HCC and to define the molecular mechanisms for LHX4 in hepatocarcinogenesis. Targeting LHX4 to downregulate AFP could have therapeutic implications in HCC.

Supplementary material
Supplementary Table S1 can be found at http://carcin.oxfordjournals.org/

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