Zinc Fingers and Homeoboxes 2 Inhibits Hepatocellular Carcinoma Cell Proliferation and Represses Expression of Cyclins A and E

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BACKGROUND & AIMS: Zinc-fingers and homeoboxes 2 (ZHX2) represses transcription of several genes associated with liver cancer. However, little is known about the role of ZHX2 in the development of hepatocellular carcinoma (HCC). We investigated the mechanisms by which ZHX2 might affect proliferation of HCC cells. **METHODS:** We overexpressed and knocked down ZHX2 in HCC cells and analyzed the effects on proliferation, colony formation, and the cell cycle. We also analyzed the effects of ZHX2 overexpression in growth of HepG2.2.15 tumor xenografts in nude mice. Chromatin immunoprecipitation and luciferase reporter assays were used to measure binding of ZHX2 target promoters. Levels of ZHX2 in HCC samples were evaluated by immunohistochemistry. **RESULTS:** ZHX2 overexpression significantly reduced proliferation of HCC cells and growth of tumor xenografts in mice; it led to G1 arrest and reduced levels of Cyclins A and E in HCC cell lines. ZHX2 bound to promoter regions of *CCNA2* (which encodes Cyclin A) and *CCNE1* (which encodes Cyclin E) and inhibited their transcription. Knockdown of Cyclin A or Cyclin E reduced the increased proliferation mediated by ZHX2 knockdown. Nuclear localization of ZHX2 was required for it to inhibit proliferation of HCC cells in culture and in mice. Nuclear localization of ZHX2 was reduced in human HCC samples, even in small tumors (diameter, <5 cm), compared with adjacent nontumor tissues. Moreover, reduced nuclear levels of ZHX2 correlated with reduced survival times of patients, high levels of tumor microvascularization, and hepatocyte proliferation. **CONCLUSIONS: ZHX2 inhibits HCC cell proliferation by preventing expression of Cyclins A and E, and reduces growth of xenograft tumors in mice. Loss of nuclear ZHX2 might be an early step in the development of HCC.**

Keywords: Mouse Model; Carcinogenesis; shRNA; CCK-8.

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third most common cause of cancer deaths worldwide.[1] Environmental factors, most notably hepatitis B virus and hepatitis C virus infection and chronic alcohol exposure, are known risk factors of HCC development.[1,2] A number of genetic loci that contribute to HCC progression also have been identified.[2,3] Numerous studies have investigated transcriptional changes in HCC, including microarray analyses of global gene expression changes. Although differences in expression of a number of liver-enriched transcription factors have been reported, consistent differences have not always been observed and it is not clear which of these are relevant to HCC progression.[4] However, several genes that are expressed abundantly in the fetal liver, silenced at birth, and reactivated in HCC have been identified,[4] including *α-fetoprotein* (*AFP*), *H19*, and *glypican 3* (*GPC3*).[5–8] A better understanding of how these genes are reactivated in HCC may elucidate transcription changes that occur during liver cancer progression.

Although the *AFP*, *H19*, and *GPC3* genes are silenced at birth in most mouse strains, these 3 genes continue to be expressed in the adult liver of BALB/cJ mice.[9] Further studies indicated that the incomplete repression of these 3 genes in BALB/cJ mice is caused by a natural mutation in the *zinc fingers and homeoboxes 2* (*ZHX2*) gene.[9,10] More recent studies indicated that ZHX2 also regulates hepatic enzymes involved in plasma lipid homeostasis, including lipoprotein lipase.[11] ZHX2 is a member of a small family that also includes ZHX1 and ZHX3.[12–14] These proteins are predicted to contain 2 zinc-fingers and 4 or 5 homeodomains, motifs that could confer protein interaction and nucleic acid binding activities. The experiment of yeast 2-hybrid indicated that ZHX proteins can form homodimers as well as heterodimers with each other and with the A subunit of nuclear factor Y (NF-YA).[13–15] Current studies have suggested that ZHX proteins are expressed ubiquitously and found primarily in the nucleus, where they function as transcriptional repressors.[14,16] We previously showed that ZHX2 reduces AFP secretion[17] and GPC3 expression (unpublished data) in human HCC cell lines. Cotransfection assays by us and others also have shown that ZHX2 can repress the promoters of *AFP* and the NF-YA–regulated genes *cdc25C* and *Hexokinase II*.[14,17,18]

Abbreviations used in this paper: AFP, α-fetoprotein; ChIP, chromatin immunoprecipitation; CHO, Chinese hamster ovary; EGFP, enhanced green fluorescent protein; GPC3, glypican 3; HA, hemagglutinin; HCC, hepatocellular carcinoma; mRNA, messenger RNA; MVD, microvascular density; NF-YA, A subunit of nuclear factor Y; NLS, nuclear localization signal; PCR, polymerase chain reaction; shRNA, short hairpin RNA; siRNA, small interfering RNA; ZHX2, zinc-fingers and homeoboxes 2.

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Several studies investigating ZHX2 and HCC have provided conflicting data. By using methylation-sensitive restriction fingerprinting, Lv[19] et al showed hypermethylation of the *ZHX2* promoter in some HCC samples and HepG2 cells that correlated with the lack of ZHX2 expression. This silencing of expression suggests that ZHX2 might function as a tumor suppressor. In contrast, using immunohistochemical analysis, Hu et al[20] reported increased ZHX2 staining in HCC samples compared with normal liver; this study also noted higher ZHX2 expression in poorly differentiated and metastasis samples. These data are consistent with ZHX2 having tumor-promoting properties.

In the present study, we investigated the role of ZHX2 in the growth of liver cell lines both in vitro and in vivo. Our data showed that ZHX2 inhibits HCC cell growth. We also showed that ZHX2 represses Cyclin A and Cyclin E expression, which might account for the growth-inhibitory properties of ZHX2. Nuclear localization of ZHX2 is critical for its inhibitory effects. These data are supported by analysis of clinical samples, which show decreased nuclear expression of ZHX2 in HCC samples compared with adjacent nontumor tissue.

Materials and Methods

Cell Lines, Plasmids, and Small Interfering RNAs

The human HCC cell lines HepG2, SMMC7721, and QSG7701, Chinese hamster ovary (CHO) cells, and human embryonic kidney 293 cells were purchased from the Shanghai Cell Collection (Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China). The HepG2.2.15 cell line was obtained from the Shandong Academy of Medical Sciences (Shandong, China). These cells were maintained as described previously.[17]

ZHX2 expression vectors pcZHX2 (full-length ZHX2 with a carboxy-terminal hemagglutinin [HA] tag cloned in pcDNA3.0) and pZHX2 (ZHX2–enhanced green fluorescent protein [EGFP] fusion protein) and short hairpin RNA (shRNA) vectors against human ZHX2 (pS1674, pS2360) were described previously.[17] Truncated forms of human ZHX2 containing homeodomain 1 and homeodomain 2 in which the nuclear localization signal (NLS) was present or absent (ZHX2[242–446] and ZHX2[242–439], respectively) were generated by polymerase chain reaction (PCR) amplification of pZHX2 using primers shown in Supplementary Table 1 and cloned into pEGFP-N1 (Invitrogen, Beijing, China). The luciferase reporter plasmids pGL3-Ap and pGL3-Ep were constructed by cloning the promoter regions of human *Cyclin A* (−505 to +361, the transcription initiation site designated as +1) and *Cyclin E* (−402 to +72), respectively, into the promoterless pGL3-basic vector (Promega, Madison, WI).[21,22] The small interfering RNAs (siRNAs) against *Cyclin A*, *Cyclin E*, and *Cyclin D1* (Supplementary Table 2) were synthesized by the Shanghai Genepharma Co (Shanghai, China).

Analysis of Cell Proliferation, Cell Cycle, and In Vivo Tumor Growth

Cell viability was measured using the Cell Counting Kit-8 (CCK-8; Beyotime, Nanjing, China) and standard colony formation assays were used to measure cell proliferation. Each experiment was repeated 3–4 times. For cell-cycle analysis, cells were collected 48 hours after transfection with indicated plasmids, stained with propidium iodide (Sigma, St Louis, MO), and assayed using a Beckman Coulter Flow Cytometer (Fullerton, CA).

Male BALB/c nude mice (4–6 weeks of age) were purchased from the Animal Research Committee of the Institute of Biology and Cell Biology (Shanghai, China) and housed in the Shandong University School of Medicine animal facility according to protocols approved by the Shandong University Animal Care Committee. HepG2.2.15 cells (1×10^7) were transplanted subcutaneously into nude mice. After reaching a diameter of 0.5 cm, tumors were injected with plasmid (20 μg/100 μL) every fourth day for a total of 3–4 injections. Tumor size was monitored every other day. Mice were killed 4 days after the final injection and the tumors were isolated and weighed. Animal experiments were repeated at least twice and 6 mice were included in each cohort. Cell proliferation in each tumor was assayed by immunoperoxidase staining with an anti–Ki-67 antibody (ab15580; Abcam, Cambridge, MA). Eight fields of roughly 1000 tumor cells for each section were scored independently by 3 pathologists.

Western Blotting

Cytoplasmic, nuclear, or whole-cell extracts were prepared and analyzed by Western blotting as previously described using anti-ZHX2 (Abcam), anti–Cyclin A (4656; Cell Signaling Technology, Danvers, MA), anti–Cyclin E (sc-25303; Santa Cruz Biotechnology, Santa Cruz, CA), anti–Cyclin D1 (ab6152; Abcam), anti-p21 (sc-6246; Santa Cruz Biotechnology), anti-p27 (ab32034; Abcam), anti-GFP (AG281; Beyotime), anti–histone H2A.X (BS5524; Bioworld Technology, Inc, St Louis Park, MN), anti–lamin A/C (BS1446; Bioworld Technology, Inc), and anti–β-actin (Sigma).[17]

Transfections, Fluorescent Staining, and Luciferase Assays

CHO and human embryonic kidney 293 cells transfected with indicated plasmids were stained with 4′,6-diamidino-2-phenylindole (Sigma) and observed for GFP and 4′,6-diamidino-2-phenylindole using fluorescence microscopy (Olympus, Tokyo, Japan). HepG2 cells were co-transfected with reporter plasmids (0.25 μg) and expression plasmids (0.75 μg) using Lipofectamine 2000 (Invitrogen, Beijing, China). Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega).[17]

Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation (ChIP) assays were performed with ZHX2-HA (pcZHX2)-transfected HepG2 cells. Briefly, transfected cells were fixed in 1% formaldehyde after 48 hours and sonicated to shear DNA to 200∼1000 bp. Supernatants obtained after centrifugation at 13,000×g for 10 minutes were used for immunoprecipitations using an anti-HA antibody (ab9110; Abcam) or control IgG. Immunoprecipitated DNA was used for PCR amplification. Total cellular DNA was used as input control.

Patient Samples and Immunohistochemical Staining

Eighty-two tumor tissues and 78 adjacent nontumor tissues were collected from patients with primary HCC who underwent surgery between October 30, 2010, and August 31, 2011, at Qilu Hospital and Shandong Provincial Hospital, Shandong University (Supplementary Table 3). Cell differentiation-