

Construction of a recombinant eukaryotic human ZHX1 gene expression plasmid and the role of ZHX1 in hepatocellular carcinoma

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Abstract. The zinc-fingers and homeoboxes protein 1 (ZHX1) consists of 873 amino acid residues, is localized in the cell nucleus and appears to act as a transcriptional repressor. Previous studies have shown that ZHX1 interacts with nuclear factor Y subunit α (NF-YA), DNA methyltransferases (DNMT) 3B and ZHX2, all of which are involved in tumorigenesis. However, the exact role of ZHX1 in tumorigenesis remains unknown. The aim of the current study was to construct a recombinant eukaryotic expression plasmid containing the human ZHX1 (hZHX1) gene and to investigate the biological activities of ZHX1 in hepatocellular carcinoma (HCC). Reverse transcription-polymerase chain reaction (RT-PCR) was used to amplify the N- and C-terminal fragments (ZHX1-N and ZHX1-C, respectively) of the hZHX1 gene. The two PCR fragments were cloned into the pEASY-T1 vector and subcloned into the pcDNA3 plasmid to generate a recombinant pcDNA3-ZHX1 plasmid. Following identification by enzyme digestion and DNA sequencing, the recombinant pcDNA3-ZHX1 plasmid was transfected into SMMC-7721 cells. The level of ZHX1 expression was detected by RT-PCR and western blot analysis.

Cell growth curve assays were used to evaluate the effect of ZHX1 on cell proliferation. Moreover, the differential expression of ZHX1 between cancer and adjacent cirrhotic liver tissue was investigated by quantitative PCR (qPCR). Enzyme digestion and DNA sequencing confirmed the successful construction of the recombinant plasmid, pcDNA3-ZHX1. qPCR and western blot analysis demonstrated that ZHX1 was efficiently expressed in SMMC-7721 cells and overexpression of ZHX1 may inhibit the proliferation of SMMC-7721 cells. In addition, reduced ZHX1 expression is widespread among cancer tissues from HCC patients. In conclusion, a recombinant eukaryotic expression plasmid, pcDNA3-ZHX1, was successfully constructed. In addition, the current results indicate that a low expression of ZHX1 may be responsible for hepatocarcinogenesis.

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common type of cancer worldwide and the third most frequent cause of cancer-related mortality (1). Chronic viral infections and alcohol exposure are the predominant risk factors of hepatocarcinogenesis (2). Hepatocarcinogenesis exhibits a multistage course, from chronic liver disease, dysplastic nodules and early through to advanced HCC, in which numerous cancer-related genes involved (3). A number of genes may be used as diagnostic or prognostic molecular markers of HCC (4). For example, glypican-3 (GPC3) is an important diagnostic marker for early HCC (5). α -fetoprotein (AFP) is a potential predictor of survival and tumor recurrence for AFP-producing HCC (6); however, it has been discontinued as a marker for HCC diagnosis (7). GPC3 and AFP may be transcriptionally regulated by zinc-fingers and homeoboxes protein 2 (ZHX2) (8-10), indicating that ZHX2 acts as an important transcriptional regulator in HCC. Our previous study demonstrated that ZHX2 inhibits the proliferation of HCC cells and the growth of xenograft tumors in mice (11).

ZHX2 is a member of the ZHX family, which also includes ZHX1 and ZHX3. The three members share a number of

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common features, including expression in various tissues and localization in the cell nucleus. These ZHX family members contain two C₂H₂ zinc-finger motifs and four to five homeodomains, appear to be transcriptional repressors, interact with the α subunit of nuclear factor-Y (NF-YA) and form homodimers or form heterodimers with other family members (12-17). In addition, ZHX1 was observed to bind to DNA methyltransferases (DNMT) 3B (18). NF-YA is a subunit of NF-Y, which was shown to be involved in tumorigenesis (19). In addition, DNMT3B and ZHX2 are important in hepatocarcinogenesis (11,20). Data from these studies indicate that ZHX1 may be involved in tumorigenesis, including in hepatocarcinogenesis.

In the current study, the correlation between ZHX1 and HCC was investigated; thus, a recombinant eukaryotic expression plasmid, pcDNA3-ZHX1, was constructed and transfected into SMMC-7721 cells. The potential biological activity of ZHX1 in HCC was also investigated.

Materials and methods

Reagents. SYBR[®]-Green Polymerase Chain Reaction (PCR) Master mix, TRIzol reagent and Lipofectamine[™] 2000 were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). ReverTra Ace quantitative PCR (qPCR) RT kit and KOD DNA polymerase were obtained from Toyobo (Shanghai, China). 2X Taq DNA polymerase, TIANgel Midi Purification kit and TIANprep Mini Plasmid kit were obtained from Tiangen Biotech Co., Ltd. (Beijing, China). DNA markers, pEASY-T₁ vector and *Escherichia coli* DH5 α were purchased from Beijing TransGen Biotech Co., Ltd. (Beijing, China). Restriction enzymes and T4 DNA ligase were from Takara Bio, Inc. (Shiga, Japan). RPMI-1640 and fetal calf serum (FCS) were obtained from Gibco-BRL (Carlsbad, CA, USA). Polyclonal rabbit anti-ZHX1 antibody was from Abcam (Cambridge, MA, USA) and the monoclonal mouse anti- β -actin antibody was from Sigma-Aldrich (St. Louis, MO, USA). Cell counting kit-8 (CCK-8) was purchased from Beyotime Institute of Biotechnology (Shanghai, China). The SMMC-7721 liver cancer cell line was obtained from the Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Science (Shanghai, China). PCR primers were synthesized by the Shanghai Biosune Biotechnology Co., Ltd., (Shanghai, China).

Patients. This study was approved by the Ethics Committee of the Provincial Hospital affiliated to Shandong University for Clinical Investigation. The study included 12 patients with HCC who underwent surgery for radical resection. Patients were excluded if they had received any other therapy prior to the surgery. Specimens of cancer liver tissue were obtained from the patients during surgery, when written consent had been obtained. Specimens of adjacent cirrhotic liver tissues were obtained as controls during the same time period. Tissues were stored at -70°C for qPCR. For each patient, the diagnosis was confirmed by a paraffin section.

Primer design and gene amplification. The full length of the human ZHX1 (hZHX1) gene coding sequence (CDS) is 2622 bp (GenBank accession no. NM_001017926.2), which is

difficult to obtain from one PCR amplification. To acquire full-length hZHX1 complementary DNA (cDNA), two pairs of primers were designed as follows: P1F 5'-CGCggatccATG GCAAGCAGGCGAAAAT-3', which contained a *Bam*HI site and P1R, 5'-GGTGGGAATGCTATTAACAGGGATTAAG-3' for the N-terminal fragment of ZHX1 (ZHX1-N); P2F, 5'-CTTCTGGATTGGCACAGGTGAT-3' and P2R, 5'-GctctagaCAAAGATTGGGCAAATTCACAG-3', which contained an *Xba*I site for the C-terminal fragment of ZHX1 (ZHX1-C). Lowercase letters indicate restriction sites. ZHX1-N and ZHX1-C overlapped and contained an endogenous *Eco*RI site (Fig. 1A).

cDNA was synthesized from total RNA, which was isolated from frozen paraneoplastic liver tissues. Using the cDNA as a template, ZHX1-N and ZHX1-C gene fragments were amplified with P1F/P1R and P2F/P2R, respectively. PCR mixtures were prepared in a total volume of 50 μ l, including 5.0 μ l of 10X buffer, 5.0 μ l dNTP mixture (2 mM), 3.0 μ l MgSO₄ (25 mM), 1.5 μ l of each paired primer (10 μ M), 2.0 μ l template cDNA, 1.0 μ l KOD DNA polymerase (1.0 U/ μ l) and double distilled water. Parameters for PCR were as follows: 5 min pre-denaturation at 94°C; 35 cycles of denaturation at 94°C for 45 sec, annealing at 58°C for 45 sec and extension at 72°C for 1 min (ZHX1-N) or 2 min (ZHX1-C), followed by a final 10-min extension at 72°C. To obtain poly(A) tails, the PCR products were mixed with an equal volume of 2X Taq DNA polymerase in a water bath at 72°C for 30 min. The PCR products were subjected to agarose gel electrophoresis and purified using the TIANgel Midi Purification kit, according to the manufacturer's instructions.

TA cloning and sequencing. ZHX1-N and ZHX1-C were respectively cloned into the pEASY-T1 vector by TA strategy resulting in pEASY-T1-ZHX1-N and pEASY-T1-ZHX1-C. DNA sequencing was used to analyze the two ZHX1 gene fragments.

Generation of recombinant plasmid pcDNA3-ZHX1. To obtain the recombinant expression vector containing the full length ZHX1 gene, *Bam*HI/*Eco*RI fragments of pEASY-T1-ZHX1-N were subcloned into pcDNA3 to produce pcDNA3-ZHX1-N. ZHX1-C was obtained by *Eco*RI and *Xba*I digestion with pEASY-T1-ZHX1-C and subcloned into *Eco*RI/*Xba*I of pcDNA3-ZHX1-N, yielding the recombinant eukaryotic expression plasmid, pcDNA3-ZHX1.

The construction strategy of recombinant eukaryotic expression plasmid pcDNA3-ZHX1 is presented in a schematic representation (Fig. 1B).

Cell culture and transient transfection. SMMC-7721 cells were cultured in RPMI-1640 medium supplemented with 10% FCS, in an incubator at 37°C, with 5% CO₂ and saturated humidity. The day prior to transfection, 2.5x10⁵ cells were inoculated in a 24-well plate. When cells reached 70-80% confluency, transfections were performed with pcDNA3 and pcDNA3-ZHX1, respectively, following the Lipofectamine 2000 manufacturer's instructions. Cells were collected 48 h following transfection.

Reverse transcription-PCR (RT-PCR) and qPCR for ZHX1 mRNA detection. RT-PCR was used to analyze the ZHX1

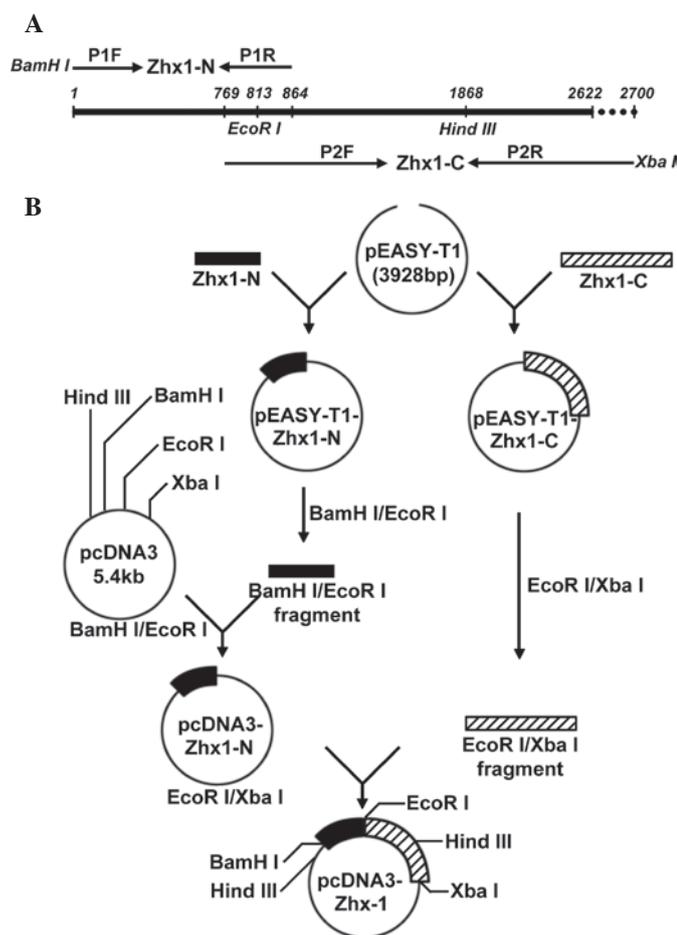


Figure 1. Schematic representation of the construction of the recombinant plasmid pcDNA3-ZHX1. (A) Two partial genetic fragments of hZHX1 were amplified by RT-PCR. (B) Two PCR fragments were first cloned into the pEASY-T1 vector and subcloned into the pcDNA3 plasmid. ZHX1, zinc-fingers and homeoboxes 1; hZHX1, human ZHX1; RT-PCR, reverse transcription-polymerase chain reaction.

mRNA expression in SMMC-7721 cells. Briefly, total RNA was extracted from transfected SMMC-7721 cells using TRIzol reagent, according to the manufacturer's instructions. cDNA was subsequently synthesized from the total RNA with the ReverTra Ace qPCR RT kit (Toyobo). Primers, designed using the software Primer Premier v5.0 (Premier Biosoft, Palo Alto, CA, USA), were as follows: Sense: 5'-GAAATCAAACCAGACCGTGAAGA-3' and antisense: 5'-ATGCAGCATTGTAGGTGGGAA-3' for ZHX1; and sense: 5'-AGTTGCGTTACACCCTTTC-3' and antisense: 5'-CCTTCACCGTTCAGTTT-3' for β -actin. PCR amplifications were performed for 26 (β -actin) or 30 (ZHX1) cycles of denaturation at 94°C for 35 sec, annealing at 58°C for 35 sec and extension at 72°C for 35 sec, followed by a final 10-min extension at 72°C. PCR products were separated on 1.5% agarose gel by electrophoresis and visualized by ethidium bromide staining.

To quantify the ZHX1 mRNA expression in HCC patients, qPCR was performed. Total RNA of liver tissue samples from 12 HCC patients was prepared using TRIzol reagent and converted to cDNA with the ReverTra Ace qPCR RT kit. qPCR was performed in a mixture of 20 μ l containing 10 μ l SYBR-Green PCR Master mix, 1.5-3 μ l cDNA and 0.4 μ l

of each paired primer (10 μ M). qPCR was performed on the LightCycler 2.0 Instrument (Roche Diagnostics GmbH, Mannheim, Germany). Parameters were as follows: A cycle of 95°C for 30 sec and 40 consecutive cycles of 5 sec at 95°C, 5 sec at 58°C (for ZHX1 and β -actin) and 30 sec at 72°C. The expression levels of ZHX1 gene in HCC patients were normalized to that of β -actin.

Western blot analysis for ZHX1 protein detection. Transfected SMMC-7721 cells were collected and lysed in cell lysis buffer. The protein concentration was measured with the bicinchoninic acid assay. Following boiling for 10 min, cell lysates with an equal quantity of protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene fluoride membranes. ZHX1 was immunoblotted with the rabbit anti-ZHX1 antibody as follows: Membranes were blocked with phosphate-buffered saline buffer containing 3% non-fat milk overnight, followed by incubation with polyclonal rabbit anti-ZHX1 antibody (1:1500) at room temperature for 1 h and subsequently incubated with the secondary horseradish peroxidase-Immunoglobulin G antibody (1:10,000) at room temperature for 1 h. After three washes, the immunoreactive proteins were visualized by enhanced chemiluminescence.

Cell growth curve assay. To evaluate the effect of ZHX1 on SMMC-7721 cell proliferation, a CCK-8 assay was employed. SMMC-7721 cells were seeded into 96-well plates with 6,000 cells/well and transfected with pcDNA3-ZHX1 (experimental group) or pcDNA3 (control group). The cell number was quantified daily by CCK-8 according to the manufacturer's instructions. Cell absorbance was read using a Model 680 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) with a 450 nm filter and expressed as optical density values.

Statistical analysis. GraphPad Prism (GraphPad Software, San Diego, CA, USA) was used for data analysis. Two-way analysis of variance was employed to analyze statistical differences between different groups of SMMC-7721 cells at various time points. Wilcoxon matched pairs test was applied to evaluate the differences between cancerous and adjacent cirrhotic liver tissues from patients. $P < 0.05$ or $P < 0.001$ was considered to indicate a statistically significant difference.

Results

Cloning of hZHX1 CDS and identification of recombinant plasmids. Two specific PCR amplifications were performed, one with P1F/P1R and another with P2F/P2R. The PCR products were subsequently analyzed by agarose gel electrophoresis. A band between 750 and 1 kb (ZHX1-N) and another at ~2 kb (ZHX1-C) were separated (Fig. 2A) and were consistent with the expected molecular mass. ZHX-N and ZHX-C were respectively cloned into pEASY-T1 vectors, which were identified by restriction enzymes and DNA sequencing. The identified ZHX1-N and ZHX1-C fragments were subcloned into a pcDNA3 vector to produce pcDNA3-ZHX1. To confirm the construction of pcDNA3-ZHX1, HindIII and BamHI/XbaI restriction enzymes and agarose gel electrophoresis was used.

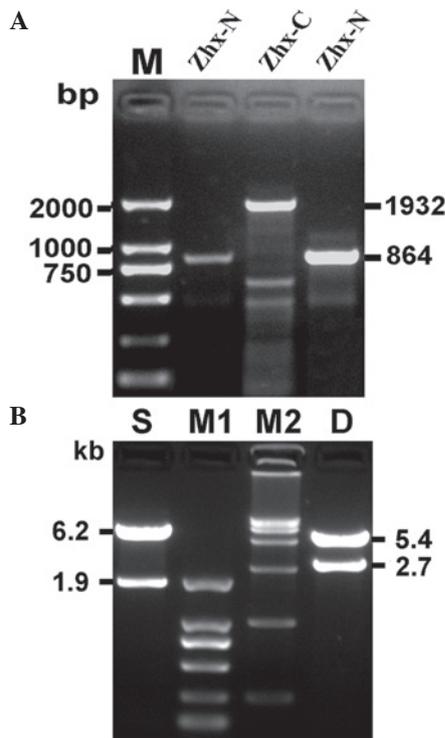


Figure 2. Cloning of hZHX1 coding sequence and identification of recombinant plasmid pcDNA3-ZHX1. (A) Electrophoresis of the target genetic fragments of hZHX1. (B) Identification of the recombinant plasmid pcDNA3-ZHX1 by single/double restriction endonuclease analysis. M, DNA marker DL2,000; ZHX-N, a target fragment of 864 bp; ZHX-C, a target fragment of 1932 bp; S, single restriction endonuclease analysis with *Hind*III; M1, DNA marker DL2,000; M2, DNA marker DL15,000; D, double restriction endonuclease analysis with *Bam*HI and *Xba*I. hZHX1, human zinc-fingers and homeoboxes 1; ZHX-N, N-terminal ZHX; ZHX-C, C-terminal ZHX.

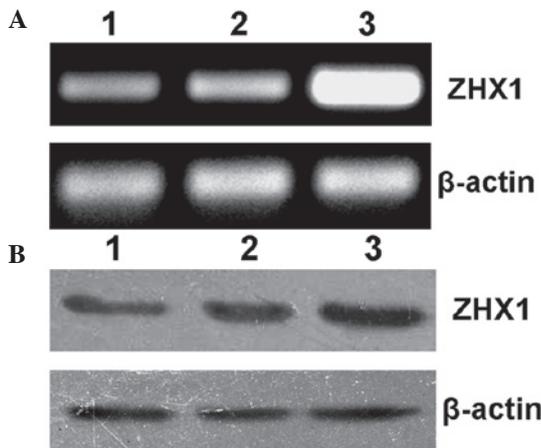


Figure 3. mRNA and protein expression of ZHX1 in different cell groups. (A) ZHX1 mRNA expression in SMMC-7721 cells. (B) ZHX1 protein expression in SMMC-7721 cells. Lane 1, no transfection; lane 2, transfected with pcDNA3; lane 3, transfected with pcDNA3-ZHX1. ZHX1, zinc-fingers and homeoboxes protein 1.

Two DNA bands with 6.2 and 1.9 kb were obtained with *Hind*III digestion and two DNA bands of 5.4 and 2.7 kb were obtained with *Bam*HI and *Xba*I digestion (Fig. 2B). DNA sequencing further confirmed the successful construction of pcDNA3-ZHX1 (data not shown).

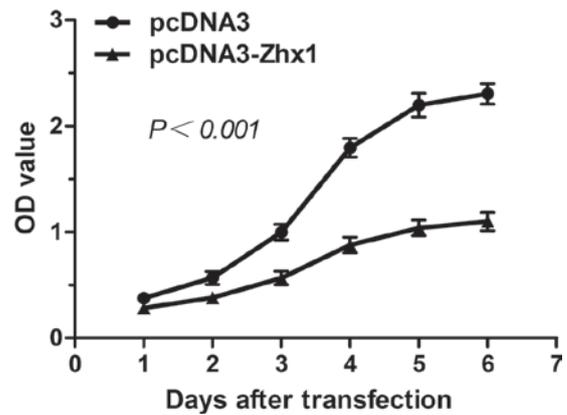


Figure 4. Growth curves of different cell groups. SMMC-7721 cells transfected with pcDNA3-ZHX1 exhibited a decreased rate of proliferation compared with cells transfected with pcDNA3. $P < 0.001$, tested by two-way analysis of variance. ZHX1, zinc-fingers and homeoboxes 1.

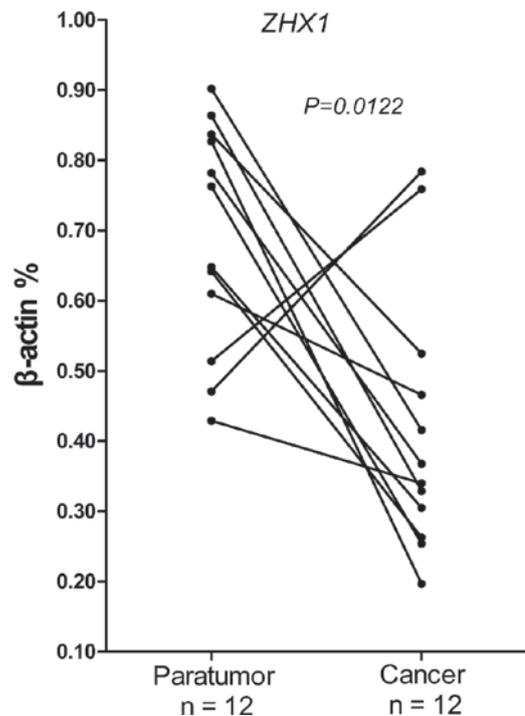


Figure 5. Reduced expression of ZHX1 transcripts in cancerous liver tissue compared with adjacent cirrhotic liver tissue (paratumor) from the same patients ($P = 0.0122$, $P < 0.05$, by a Wilcoxon matched pairs test). Data shows qPCR quantitation of paired samples from 12 HCC patients. The relative quantities are expressed as a percentage of β-actin. ZHX1, zinc-fingers and homeoboxes protein 1; qPCR, quantitative polymerase chain reaction; HCC, hepatocellular carcinoma.

Expression of the recombinant plasmid pcDNA3-ZHX1 in HCC cells. Forty-eight hours after transfection, SMMC-7721 cells were collected for RT-PCR and western blot analysis. As shown in Fig. 3A and B, SMMC-7721 cells transfected with pcDNA3-ZHX1 showed significantly increased levels of ZHX1 mRNA and protein expression, while cells without transfection or transfected with empty plasmid pcDNA3 showed low-level expression of endogenous ZHX1, indicating that a functional eukaryotic expression plasmid pcDNA3-ZHX1 was obtained.

Identification of biological activity by cell growth curve assay. To investigate the involvement of ZHX1 in HCC, a CCK-8 assay was employed to evaluate cell proliferation. As shown in Fig. 4, SMMC-7721 cells transfected with pcDNA3-ZHX1 exhibited a decreased proliferation rate compared with cells transfected with pcDNA3, indicating that the overexpression of ZHX1 may inhibit the proliferation of SMMC-7721 cells.

Reduced ZHX1 expression among cancer tissue from HCC patients. qPCR was used to evaluate the differential expression of ZHX1 in adjacent cirrhotic and cancer liver tissue from the same HCC patients. Following normalizing against the house-keeping gene, 7/12 cases showed a 2-5-fold reduction of ZHX1 mRNA compared with the adjacent cirrhotic liver tissues (Fig. 5). A significant difference was confirmed by Wilcoxon matched pairs test. These results demonstrated that reduced ZHX1 expression was widespread among cancer tissues from HCC patients and indicated that ZHX1 may be responsible for hepatocarcinogenesis.

Discussion

The ZHX1 gene was originally cloned by immunoscreening with a monoclonal B92 antibody (12). Similar to its family members, ZHX1 is expressed in various tissues, is localized in the cell nucleus and appears to act as a transcriptional repressor (12,14,15). It was previously reported that ZHX1, together with ZHX2 and ZHX3, were involved in regulating gene expression in podocyte disease (21,22). However, a number of studies indicated that ZHX1 may be involved in tumorigenesis, including in hepatocarcinogenesis (11,13,14,18-20). In the current study, the expression vector of hZHX1 was successfully prepared. Moreover, the present results demonstrated the expression pattern and involvement of ZHX1 in HCC.

To determine the involvement of ZHX1 in HCC, a vector containing the full length of hZHX1 CDS was required. However, due to the size of hZHX1 CDS, more than one amplification was necessary. A two-step strategy was employed to obtain the full length hZHX1 gene. Using NEBCutter analysis, an *EcoRI* site, one of the multiple cloning sites of pcDNA3, was identified in the gene sequence of hZHX1. Therefore, two pairs of primers were designed to obtain two partial genetic fragments of hZHX1; ZHX1-N and ZHX1-C, which had an overlapping sequence including the *EcoRI* site. The two partial genetic fragments were subcloned into the *EcoRI* site of pcDNA3, and pcDNA3-ZHX1 was successfully constructed and expressed in SMMC-7721 cells.

With the successful construction of pcDNA3-ZHX1, the potential involvement of ZHX1 in HCC was evaluated for the first time. Results of the CCK-8 assay showed that overexpression of ZHX1 may inhibit the proliferation of SMMC-7721 cells. This is consistent with the detected ZHX1 expression pattern in HCC. Compared with the adjacent cirrhotic tissues, ZHX1 expression was significantly lower in cancer tissues from patients with HCC (Fig. 5).

As a member of the ZHX family, ZHX1 contains two zinc finger and five homeobox domains, indicating the potential of ZHX1 interaction with DNA and proteins. Proteins known to interact with ZHX1 include ZHX2 (13), NF-YA (14) and DNMT 3B (18). ZHX2 prevents AFP expression but also

inhibits the proliferation of HCC cells (10,11). NF-YA is a subunit of NF-Y, which is an important transcription factor capable of binding to the CCAAT box to trigger transcription of numerous eukaryotic genes and is involved in tumorigenesis (19). DNMT3B is important in the development of tumorigenesis (23), including hepatocarcinogenesis (20,24). The interaction of ZHX1 with these tumor-associated proteins indicates that ZHX1 may be involved in hepatocarcinogenesis. However, the molecular mechanism underlying the involvement of ZHX1 in the pathology of hepatocarcinogenesis remains unclear, thus further studies are required.

In conclusion, the recombinant eukaryotic expression plasmid, pcDNA3-ZHX1, was successfully constructed. It contained the full length sequence of the hZHX1 gene and demonstrated that overexpression of ZHX1 may inhibit the proliferation of SMMC-7721 cells. This study provides preliminary data for further studies regarding the function and molecular mechanism of the ZHX1 gene in HCC.

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