The International Journal of Biochemistry & Cell Biology 55 (2014) 129–135

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Reduced nucleic ZHX2 involves in oncogenic activation of glypican 3 in human hepatocellular carcinoma

Fang Luan[a,b], Peng Liu[a], Hongxin Ma[a], Xuetian Yue[a], Jun Liu[c], Lifen Gao[a], Xiaohong Liang[a], Chunhong Ma[a,*]

[a] Key Laboratory for Experimental Teratology of Ministry of Education and Department of Immunology, Shandong University School of Medicine, 44 Wenhua Xi Road, Jinan, Shandong 250012, PR China
[b] Department of Clinical Laboratory, Provincial Hospital Affiliated to Shandong University, 324 Jingwu Road, Jinan, Shandong 250021, PR China
[c] Department of Surgery, Provincial Hospital Affiliated to Shandong University, 324 Jingwu Road, Jinan, Shandong 250021, PR China

ARTICLE INFO

Article history:
Received 16 March 2014
Received in revised form 23 August 2014
Accepted 27 August 2014
Available online 6 September 2014

Keywords:
ZHX2
GPC3
HCC
Transcriptional repressor
Nuclear translocation

ABSTRACT

Glypican 3 (GPC3) has been paid particular attention owing to its potential as diagnosis marker for hepatocellular carcinoma (HCC). Identifying the mechanisms regulating the reactivation of GPC3 in HCC appears to be clinically meaningful. Previous study identified zinc-fingers and homeoboxes 2 (ZHX2) as transcriptional factor responsible for postnatal repression of GPC3 in mice. Here, in this study, we provided the first evidence that down regulated ZHX2 is responsible for GPC3 reactivation in HCC. First, inverse correlation of ZHX2 with GPC3 expression was shown in cultured liver cell lines. Second, ZHX2 overexpression significantly decreased GPC3 expression, while ZHX2 knockdown effectively increased GPC3 level in different HCC cell lines. Consistently, dual luciferase and ChIP assay showed that ZHX2 dose-dependently suppressed GPC3 promoter activity by binding with the core promoter. More importantly, immunohistochemical staining demonstrated the inverse correlation between nuclear ZHX2 with GPC3 expression in HCC tissues. Further in vitro analysis showed that nuclear translocation was crucial for ZHX2 mediated repression on GPC3 transcription. Taken together, our results prove that ZHX2 suppresses GPC3 transcription by binding with its core promoter and reduced nucleic ZHX2 expression may be involved in GPC3 reactivation in HCC.

1. Introduction

Glypican 3 (GPC3) is a heparan sulfate proteoglycan that linked to the exocytoplasmic surface of the plasma membrane by a glycosyl-phosphatidylinositol (GPI) anchor (Lander et al., 1998; Veugelers and David, 1998; Filmus and Selleck, 2001; Filmus and Song, 2000), and a proportion of GPC3 can be released by lipases from the cell surface. Both the attached and secreted form of GPC3 can regulate different signaling pathways, including Wnt signaling, fibroblast growth factors signaling, bone morphogenetic proteins signaling and hedgehog signaling (Capurro et al., 2005; Kleeff et al., 1998; Steinfeld et al., 1996). Recently, GPC3 has been of particular interests as a specific marker for hepatocellular carcinoma (HCC) (Filmus and Capurro, 2004; Yamauchi et al., 2005). GPC3 is highly expressed in fetal liver and placenta but silenced in normal adult liver tissues (De Cat and David, 2001; Song and Filmus, 2002; Filmus and Selleck, 2001). In contrast, GPC3 is markedly overexpressed in a high proportion of HCC and promotes the growth of HCC by stimulating the canonical Wnt pathway (Filmus and Selleck, 2001; Luo et al., 2006; Capurro et al., 2005). GPC3 expression level significantly correlates to tumor grading and tumor aggressiveness in HCC individuals (Shirakawa et al., 2009; Zhang et al., 2012). More interestingly, GPC3 becomes reactivated in HCC as frequently as alpha-fetoprotein (AFP), the extensively used diagnosis indicator of liver cancer, and may be a more reliable early diagnostic marker than AFP (Llovet et al., 2006; Wang et al., 2006). However, the mechanisms that regulate the reactivation of GPC3 in HCC remain unclear.

Zinc-fingers and homeoboxes 2 (ZHX2) belongs to ZHX transcription factors family which contains two C2–H2 zinc-finger motif and four homeodomains. ZHX2 are ubiquitously expressed and localized to the nuclei of cells, and appear to function as transcriptional repressor (Yamada et al., 2002, 2003; Kawata et al., 2003).

Abbreviations: GPC3, glypican 3; HCC, hepatocellular carcinoma; ZHX2, zinc-fingers and homeoboxes 2; ECL, enhanced chemilluminescent; shRNA, small hairpin RNA.

* Corresponding author. Tel.: +86 531 88382038; fax: +86 531 88382052.
E-mail address: machunhong@sdu.edu.cn (C. Ma).

http://dx.doi.org/10.1016/j.biocel.2014.08.021
1357-2725/

Accumulated studies have shown that ZHX2 is responsible for postnatal repression of oncofetal genes GPC3, AFP and H19 in mice (Perincheri et al., 2005; Spear, 1999; Peterson et al., 2011; Morford et al., 2007). Our previous study further identified ZHX2 as the negative regulator for AFP in human HCC (Shen et al., 2008). More recently, we demonstrated ZHX2 as a tumor suppressor gene in HCC. Nucleic ZHX2 represses HCC cell growth by negative regulation of Cyclin A/E (Yue et al., 2012). Abnormal nucleic ZHX2 expression has been detected in HCC (Yue et al., 2012). Therefore, it is reasonable to speculate that GPC3 may also be regulated by ZHX2 in HCC.

To address our hypothesis, the expression of ZHX2 and GPC3 in both liver tumor cell lines and HCC tissues was examined in this study. We found that overexpression of human ZHX2 in HCC cell lines inhibited endogenous GPC3 gene expression, while shRNA-mediated inhibition of ZHX2 led to increased GPC3 expression. Reporter and ChIP assay suggested that ZHX2-mediated repression acted through the GPC3 core promoter. Immunohistochemical data showed that reduced expression of nuclei ZHX2 was correlated with GPC3's reactivation in HCC tissues. Consistently, co-transfection and dual-luciferase assay verified that translocation of ZHX2 from nuclei to cytoplasm completely destroyed its repression on GPC3.

2. Materials and methods

2.1. Tissue source

Thirty surgical samples of HCC were collected from Shandong Provincial Hospital affiliated to Shandong University (supplementary Table S1). The patients consisted of 28 men and 2 women, ranging in age from 34 to 79 years (mean ± SD, 54.33 ± 10.97 years). The diagnosis was confirmed histologically in all cases, based mainly on the examination of sections stained with H&E. Cell differentiation-based HCC tumor grading was determined as described by Edmondson and Steiner (1954). The HBV viral load was quantified using a HBV diagnostic kit (QIAgene, Shenzhen, China) according to the manufacturer's instruction. None of the patients was positive for hepatitis C antigen or human immunodeficiency virus antigen, which was determined using a standard serologic test. None of them consumed excessive quantities of alcohol. None of them had been treated with antiviral therapy or chemotherapy before surgery. Informed consent was obtained from all patients before the study was initiated with approval of the Shandong University Medical Ethics Committee in accordance with the Declaration of Helsinki.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2014.08.021.

2.2. Cell culture and transfection

HCC cell lines HepG2 (ATCC HB-8065), Hep3B and SMMC7721 were cultured in minimum essential medium (MEM) with sodium pyruvate. HepG2.2.15 cells harboring 4 copies of HBV DNA and expressing all viral proteins were cultured in MEM with 380 μg/ml G418 (Mary et al., 1987). Immortal liver cell line LO2 was cultured in RPMI1640. All the cells were purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. All the mediums were supplemented with 10% fetal calf serum. Transient transfections were performed with lipofectamine™2000 (Invitrogen, USA) according to the manufacturer's instruction.

2.3. Isolation of total RNA, semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted by TRIZOL (Sangon, Japan) from 5×10^5 cells or 100 mg tissues and quantified using a spectrophotometer (Eppendorf Co., Hamburg, Germany). Three micrograms of total RNA was reverse transcribed into cDNA using Murine Moloney Leukemia virus reverse transcriptase (Promega, USA). PCR was carried out according to the manufacturer's instructions. Sense and reverse primers: β-actin 5′-GGCATCGTGATGGACTCCG-3′, 5′-GCTGGAAGGTGGACAGCGA-3′; ZHX2 gene 5′-CCCCCAATGGTGCTCTGT-3′, 5′-TTGCTTTCCTTGCTACGG-3′; GPC3 gene 5′-GTTCTTTGCCCAGTCCTTC-3′, 5′-CGCCTCGCTTCCATTCTT-3′. Amplification was done at 95 °C for 3 min, 30 cycles of 95 °C for 1 min, 55 °C (β-actin), 55 °C (ZHX2) or 56 °C (GPC3) for 1 min, and 72 °C for 1 min, with final extension of 5 min at 72 °C.

2.4. Small hairpin RNA

Small hairpin RNA (shRNA) oligonucleotides, targeting regions centered on 1674nt and 2360nt of the transcribed region of the human ZHX2 gene (details for oligos are available upon request), were cloned into the vector pSilencer3.0 to get the shRNA expression vectors ps1647 and ps2360 (Shen et al., 2008). For shRNA knockdown assays, LO2 and SMMC7721 cells were seeded into 24-well plates and transfected with 1 μg of ps1674, ps2360 or the control shRNA vector as described above. Fifty-six hours after transfection, cells were harvested to assay ZHX2 and GPC3 mRNA expression as described above.

2.5. Construction of GPC3 core promoter plasmid and luciferase report assays

The human GPC3 promoter region containing 3 Sp1 binding sites (−218 to +76) was amplified from human genomic DNA by PCR using primers (P1, P2) and then inserted into the KpnI/HindIII sites of pGL3-basic (Promega, Jinan, China) to construct the reporter plasmid pGL3-GPC3-218. The three Sp1-binding sites (−92, −34, −14) on GPC3 core promoter were named as A, B and C. Series of single, dual or muti-sp1-mutant plasmids (pGL3-GPC3-A, pGL3-GPC3-B, pGL3-GPC3-C, pGL3-GPC3-AB, pGL3-GPC3-AC, pGL3-GPC3-BC, pGL3-GPC3-ABC) were constructed using the specially designed primers (A up/down, B up/down, C up/down) by site directed mutagenesis. All the primers were showed in supplementary Table S2.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2014.08.021.

In order to analyze the repression of nuclear ZHX2 on GPC3 promoter, HepG2 cells were cotransfected with GPC3 luciferase reporter plasmid pGL3-GPC3-218 (0.4 μg) and different doses of ZHX2 expression plasmid (0, 0.4, 0.8 or 1.6 μg) or different ZHX2 expression constructs (Yue et al., 2012) [ZHX2 full length/pEGFP-ZHX2 FL, truncated ZHX2 containing the nuclear localization signal (NLS)/pEGFP-ZHX2(242–446) and ZHX2 without NLS/pEGFP-ZHX2(242–439)]. Series of single, dual or muti-sp1-mutant GPC3 promoter plasmids (0.4 μg) were respectively transfected into HepG2 cells with ZHX2 expression plasmid (0.8 μg) to analyze the role of Sp1 binding site in ZHX2 repressing GPC3 promoter. 16 ng of SV40-Renilla (Promega, Beijing, China) per well was used to standardize the transfection efficiency in each experiment. Cells were harvested 48 h later in reporter lysis buffer. Luciferase assays were performed using the Dual-Luciferase Reporter Assay System according to the manufacturer's protocol (Promega, Bejing, China).