Down-regulation of PTEN expression due to loss of promoter activity in human hepatocellular carcinoma cell lines

Dong-Zhu Ma, Zhen Xu, Yu-Long Liang, Jian-Ming Su, Zeng-Xia Li, Wen Zhang, Li-Ying Wang, Xi-Liang Zha

Abstract

AIM: To investigate the regulation of phosphatase and tensin homolog deleted on chromosome ten (PTEN) gene expression in human hepatocellular carcinoma (HCC) cell lines.

METHODS: The mRNA and protein levels of PTEN were detected by Northern blot and Western blot in HCC cell lines, respectively. Plasmids containing different fragments of PTEN promoter with Luciferase reporter were constructed and transiently transfected into HCC cell lines to study the promoter activity. DNA analysis and RT-PCR were performed to detect the mutation of PTEN promoter and PTEN cDNA.

RESULTS: Either protein or mRNA levels of PTEN in L02 cells (as a control) were significantly higher than that in HCC cell lines. The profile of PTEN promoter activity in 8 cell lines was closely correlated with levels of PTEN mRNA and PTEN protein. Furthermore, the sequence analysis of 8 cells lines showed no mutation in the region of PTEN promoter and PTEN cDNA.

CONCLUSION: PTEN expression is down-regulated in HCC cell lines probably due to loss of activity of PTEN promoter.

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Key words: Phosphatase; Tensin homolog; Hepatocellular carcinoma

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INTRODUCTION

The tumor suppressor gene phosphatase and tensin homolog deleted on chromosome ten (PTEN), mutated in a wide range of human cancers[1,2], encodes a protein containing 403 amino acids with phospholipid and protein phosphatase activity[3-6]. Consequently, PTEN inhibits the generation of phosphatidylinositol 3,4,5-trisphosphate (PIP3) and then blocks the activation of proto-oncogene PKB/Akt[7]. The loss of PTEN in human tumors leads to an increase in PI(3,4,5)P3 and the uncontrolled stimulation of growth and survival signals[8]. PTEN also dephosphorylated focal adhesion kinase because of its tyrosine phosphatase activity[9], which might lead to the inactivation of Ras/mitogen-activated protein kinase(MAPK) pathway[12-18]. It is well known that both pathways mentioned above are intimately involved in control of cell growth and survival, so PTEN appears to impinge on cell proliferation, adhesion, cell migration, and cell invasion[14,15]. Moreover, germline mutations in PTEN cause Cowden disease, which is characterized by the formation of multiple hamartomas and increased susceptibility to skin, thyroid, and breast tumors[19]. Together, these findings suggest that loss of PTEN activity sensitizes cells to malignant transformation and PTEN is an important protein to regulate various physiological pathways. Despite extensive characterization of PTEN mutations in human cancers and relatively good understanding of the molecular roles of PTEN in the control of cellular processes, little is known about modes of PTEN regulation. Recently, scientists have paid more attention to the regulation of PTEN expression. It was reported that the transcription of PTEN could be regulated by p53 and Sp1[17,18]. In addition, 5′-untranslated region (5′-UTR) of PTEN gene was responsible for constitutive PTEN expression in mice[19]. Salvesen et al[20], found that PTEN promoter methylation was relatively frequent in endometrial carcinoma. Till now, the regulation of PTEN expression is still unclear especially in HCC cells. It is well known that the regulation of gene expression is a multi-step process in eukaryotes, and the transcriptional regulation plays a important role in it. So, we attempted to study the transcriptional regulation of PTEN expression in HCC cell lines.

MATERIALS AND METHODS

Cell culture

Human hepatocellular carcinoma (HCC) cell lines (SMMC-7721, BEL-7402, BEL-7404, and BEL-7405) and human liver immortal cell line L02, purchased from Institute of Biochemistry and Molecular Biology, Shanghai Medical College, Fudan University, Shanghai 200032, China.  xlzha@shmu.edu.cn

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of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China), were routinely maintained in RPMI 1640 (Gibco BRL, USA) supplemented with 100 mL/L fetal bovine serum (HyClone, USA) at 37 °C in a humidified atmosphere containing 50 mL/L CO₂ in air. HepG2 (human hepatoblastoma) was obtained from American Type Culture Collection (ATCC). HCC cell lines MHCC-97H and MHCC-97L kindly provided by Liver Cancer Institute of Zhongshan Hospital, Fudan University (Shanghai, China), were maintained in Dulbecco’s modified Eagle’s medium (Gibco BRL, USA) supplemented with 100 mL/L fetal bovine serum (HyClone, USA) at 37 °C in a humidified atmosphere containing 50 mL/L CO₂ in air.

**Western blot analysis**

After being grown to confluence, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in cold lysis buffer (50 mmol/L HEPES, pH 7.5, 150 mmol/L NaCl, 1.5 mmol/L MgCl₂, 1 mmol/L EDTA, 0.2 mmol/L EGTA, 10 mL/L NP-40, 100 g/L glycerol, 1 mmol/L dithiothreitol, 1 mmol/L phenylmethylsulfonyl fluoride, 20 mmol/L sodium fluoride, 5 mmol/L sodium orthovanadate, 10 g/L aprotinin, 10 g/L leupeptin, 2 g/L pepstatin, and 1 mmol/L benzamidine). Lysates were incubated for 20 min on ice and centrifuged at 12 000 g for 20 min. The supernatants were collected and protein concentration was determined by Lowry protein assay. Cell lysates were electrophoresed by SDS-PAGE and then transferred onto polyvinylidene fluoride (PVDF) membrane. The membranes were blocked with 50 g/L nonfat dry milk in PBST (PBS, 0.5 mL/L Tween-20) for 4 h at room temperature and incubated overnight at 4 °C with a mAb against human PTEN (Santa Cruz, CA, USA), followed by incubation with HRP-conjugated secondary antibody at room temperature for 1 h. Antibody binding was detected by enhanced chemiluminescence (ECL).

**Northern blot analysis**

Total RNA was isolated using the TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer’s directions. The 20 µg RNA was electrophoresed on a 12 g/L agarose/formaldehyde gel and blotted onto a nylon membrane (Schleicher & Schuell, Germany) by capillary transfer. Hybridization was performed in 0.2 mol/L NaHPO₄/Na₂HPO₄ (pH 7.2), 1 mmol/L EDTA, 10 g/L BSA, 70 g/L SDS and 150 mL/L formamide at 50 °C, and the filters were washed extensively with 40 mmol/L NaHPO₄/Na₂HPO₄ (pH 7.2), 1 mmol/L EDTA, 10 g/L SDS at 65 °C. A 1.2 kb DNA fragment representing the entire coding region of PTEN was used as a probe and was labeled by Prime-a-Gene labeling system (Promega, Madison, WI, USA). Air-dried blots were autoradiographed onto Kodak film (Eastman Kodak, Rochester, NY, USA) and the RNA signal was detected using an ImageMaster VDS system (Pharmacia Biotech, San Francisco, CA, USA) and normalized against the signal for β-actin using ImageMaster TotalLab 1D software.

**Isolation of 5’-flanking and promoter region of PTEN gene**

Based on the published sequence of PTEN (accession number AF067844), a 2.7 kb DNA fragment of PTEN containing 5’-flanking region, 5’-untranslated region (5’-UTR) and full-length of PTEN promoter region were obtained by PCR using primers 5’-GATATGATCTGGTG- GGTGCCTGGGTAGGATGC-3’ and 5’-GAGAGCTTCTGCGGCGGTTGCGATGTTTGC-3’. The fragment was subcloned into the luciferase reporter plasmid pGL3-basic (Promega, Madison, WI, USA) which was digested twice with BglII and HindIII restriction enzymes. Positive clones, pGL3-2768, from 8 cell lines were identified by restriction enzymes digest and DNA sequencing, and aligned with the GenBank databases.

**Reporter gene plasmids constructions**

Several specific primers containing BglII and HindIII restriction enzyme sites (listed below) were designed to clone the entire deletion fragment of PTEN using the following DNA fragments as probes in a cloning vector pGL3-2768 (-2 927/-160) as a template.

- 5’-GAGAGATCTCGCGGTGATGTGGCGATCAGTTCTCTCCT-3’ (-778)
- 5’-GAGAGATCTCGCGGTGATGTGGCGGCGTCTCAGTCTCTCT-3’ (-858)
- 5’-GCAAAACTGCTCGGCGTCTCAGGCTGACACTCTATGTTGTCGCTTTTGC-3’ (-1 230)
- 5’-GCAAAACTGCTCGGCGTCTCAGGCTGACACTCTATGTTGTCGCTTTTGC-3’ (-1 526)
- 5’-GCAAAACTGCTCGGCGTCTCAGGCTGACACTCTATGTTGTCGCTTTTGC-3’ (-1 016)
- 5’-GCAAAACTGCTCGGCGTCTCAGGCTGACACTCTATGTTGTCGCTTTTGC-3’ (-916)
- 5’-GCAAAACTGCTCGGCGTCTCAGGCTGACACTCTATGTTGTCGCTTTTGC-3’ (-858)
- 5’-GCAAAACTGCTCGGCGTCTCAGGCTGACACTCTATGTTGTCGCTTTTGC-3’ (-778)
- 5’-GCAAAACTGCTCGGCGTCTCAGGCTGACACTCTATGTTGTCGCTTTTGC-3’ (-458)
- 5’-GCAAAACTGCTCGGCGTCTCAGGCTGACACTCTATGTTGTCGCTTTTGC-3’ (-160)

**Transient transfections and luciferase activity assays**

Cells were seeded into 6-well plates at a density of 150 000 cells per well 1 day before transfection. The transfection was performed with the Lipofectamine™ 2000 transfection reagent (Invitrogen, CA, USA) according to the manufacturer’s guidelines. Typically, 3 µg of pGL3 vector and 1 µg of pGFP-β-Gal (a gift from Houyuan Song, Department of Molecular Genetics, Shanghai Medical College, Fudan University, Shanghai, China) were used per well. After 48 h, cells were lysed with lysis buffer (Promega, Madison, WI, USA). The mixtures were centrifuged at 12 000 g for 15 s at 4 °C, and the supernatant was preserved at -70 °C. Activities of firefly luciferases were measured in a luminometer Lumat LB 9507 using the luciferase assay system (Promega, Madison, WI, USA). β-Gal activity was measured by β-galactosidase enzyme assay system (Promega, Madison, WI, USA). Promoter activity was quantified by calculating the ratio of firefly luciferase activity/β-gal activity of the same sample. Transfection efficiency was determined through the positive cells with green fluorescence from the green fluorescence protein (GFP) under fluorescent microscope. All the luciferase assays were carried out at least in triplicate, and the experiments were repeated thrice.
RT-PCR and DNA sequencing of PTEN cDNA and PTEN promoter

Total RNA was isolated from cell lines using the TRIzol RNA isolation kit (Invitrogen, CA, USA) according to the manufacturer’s protocols. After synthesis of first strand cDNA using oligo-d(T)12-18 primer and moloney murine leukemia virus (M-MuLV) reverse transcriptase (Promega, Madison, WI, USA), PTEN cDNA was amplified using PCR with pyrococcus furiosus (Pfu) DNA-polymerase (Promega, Madison, WI, USA). The primer sequences were as follows: upper primer, 5′-ACAGGC-TCCCAGACATGACA-3′ and lower primer, 5′-TCAG-ACTTTTGTAATTTGTGTATG-3′. PCR amplification was carried out for 30 cycles under denaturing-annealing-extension conditions of 94 °C for 30 s, 60 °C for 1 min and 72 °C for 1 min, respectively. The PCR product was cloned into the T vector and was identified by DNA sequencing of at least three independent clones and aligned with the GenBank databases.

Statistical analysis

F test was used for statistical analysis.

RESULTS

PTEN protein and mRNA expression in 8 cell lines

Most mammalian cells containing the wild-type PTEN gene expressed detectable levels of PTEN mRNA and protein under normal growth conditions[17]. L02, a human liver immortal cell line, was used as a control in the present study. There was one 55 ku PTEN protein detected with various levels in 8 cell lines by Western blot analysis (Figure 1). The protein level of PTEN in L02 cells was the highest among the 8 cell lines, whereas the PTEN protein in HepG2 cells was almost undetectable. Simultaneously, Northern blot analysis showed a major 2.5-kb transcript and a lower abundance 5.0-kb transcript of PTEN mRNA in all 8 cell lines, which was consistent with previous reports[20] (Figure 2). The total mRNA of PTEN was calculated in both 5.0-kb and 2.5-kb transcripts. The mRNA level of PTEN was much higher in L02 cells than the other 7 HCC cell lines, especially in HepG2 cells (Figure 2). The mRNA level of PTEN in L02 cells was over five-folds than in HepG2 cells. However, the profile of PTEN protein level in each of 8 cell lines closely paralleled with its PTEN mRNA.

Absence of PTEN promoter and PTEN cDNA mutation in 8 cell lines

Deletions or mutations of PTEN encoding gene are associated with a variety of human cancers[12]. Furthermore, decreased expression of PTEN was associated with advanced glioma, melanoma, and prostate cancer, implicating losses of PTEN by mutation involved in tumor progression[21-23]. To investigate whether the deletion or mutation exists in PTEN gene, leading to the lost expression of PTEN in HCC cell lines, we analyzed the sequence of PTEN cDNA and PTEN promoter (-2 927/-160 bp) in 8 cell lines. We found no mutation in PTEN cDNA and PTEN promoter region of 8 cell lines (data not shown), indicating that the different levels of PTEN mRNA and protein in 8 cell lines were not caused by the mutation of PTEN cDNA and promoter region. It might be related to PTEN transcriptional or post-transcriptional regulation.

Core region of PTEN promoter identified in SMMC-7721 and L02 cell lines

It is well known that promoter plays the most important role in gene transcription. In an attempt to analyze the function of PTEN promoter, we isolated a DNA fragment containing 5′-flanking region and the 5′-untranslated region (5′-UTR) from PTEN gene, and performed a series of promoter deletion. Eleven fragments of PTEN gene promoter were constructed into pGL3-basic with luciferase reporter (Figure 3 A) and were transiently transfected into L02 and SMMC-7721 cell lines. It was found that the profiles of luciferase activities of various plasmids were the same in the two cell lines (Figures 3B and C). The 612-bp fragment (-1 389/-778) was sufficient to induce maximum luciferase activity in L02 and SMMC-7721 cell lines. The plasmid pGL3-2768 (-2 927/-160), which
Figure 4. Activity of PTEN promoter in 8 cell lines.

The profiles of PTEN protein and PTEN mRNA. These results demonstrated that the changes of PTEN protein and PTEN mRNA in 8 cell lines might result from the function of PTEN promoter.

**DISCUSSION**

Since the isolation of PTEN/MMAC1/TEP1 (acronyms for phosphatase and tensin homolog\(^2\), mutated in multiple advanced cancers\(^1\), and TGF-\(\beta\) (transforming growth factor-\(\beta\))-regulated and epithelial cell-enriched phosphatase\(^{[25]}\) as a candidate tumor suppressor gene, hundreds of reports have been published focusing on its structure and function, as well as on mutations that cause human diseases\(^{[26]}\).

Mutations of the PTEN gene arise during cancer progression in a remarkable variety of cancers, including brain, prostate, breast, endometrial cancers and melanoma\(^{[27]}\). The frequency of PTEN mutations observed in endometrial tumors\(^{[20]}\), malignant glial tumors\(^{[27]}\), malignant melanoma cell lines\(^{[28]}\) and metastatic prostate carcinomas\(^{[27]}\) was about 45%, 24%, 40%, and 10%, respectively. In addition, germline mutations in the PTEN gene have been associated with Cowden syndrome and a significantly increased risk of certain tumors, including cancer of the breast and thyroid\(^{[21,28]}\). These data further support that PTEN is a tumor suppressor gene.

The structure of PTEN contains a phosphatase domain that has a structure resembling tyrosine phosphatase and a C2 domain appears to bind PTEN to the plasma membrane, which might orientate the catalytic domain appropriately for interactions with phosphatidylinositol 3,4,5-trisphosphate (PIP3) and other potential substrates\(^{[29]}\). A PDZ binding motif in the tail might also play a role in altering the balance of PTEN effects on potential downstream signaling targets such as Akt\(^{[30]}\). PTEN is also known to be critically important both during embryonic development and in mature organisms as a tumor suppressor\(^{[29,31]}\). Studies of PTEN functions have provided a novel insight into the regulation of apoptosis, migration and tumor progression. PTEN appears to serve as a hub or switchpoint linking complex signaling pathways\(^{[32,33]}\).

HCC presents a major health threat in South-East Asia, especially in China. It ranks the third among all malignancies both in incidence and mortality in China and accounts for approximately 42.5% of the total incidence worldwide\(^{[34]}\).
As a tumor suppressor gene, PTEN expression is downregulated in tumors and tumor cell lines by genetic and epigenetic mechanisms\textsuperscript{[12]}. Therefore, it is very important to study the regulation of PTEN expression in human HCC cell lines. In this study, the results of Western blot analysis demonstrated that the protein level of PTEN in L02 cells was the highest among 8 cell lines, whereas there was almost undetectable PTEN protein expression in HepG2 cells. Northern blot analysis showed that the profile of PTEN mRNA in 8 cell lines almost parallelized to the profile of PTEN protein, indicating that the variation of PTEN protein was mostly dependent on the change of PTEN mRNA. Moreover, deletions or mutations of PTEN gene are associated with a variety of human cancers\textsuperscript{[12]}. Does such deletion or mutation of PTEN gene exist in HCC cell lines, causing the cut down of PTEN expression in HCC cell lines? The sequence analysis of PTEN cDNA and PTEN promoter region showed no mutations in these HCC cell lines. Hence the downregulation of PTEN expression in HCC cell lines probably existed in transcriptional or post-transcriptional levels.

The deletion analysis of PTEN promoter showed that the fragment of 612 bp (-1389/-778) could produce maximum promoter activity in 8 cell lines and the core region of PTEN promoter was within the 341 bp (-1118/-778) fragment. The full-length fragment possessing low activity indicated that the double ends of the 612 bp fragment contained suppressive elements or special structures. We used the Genomatix Suite/MatInspector software\textsuperscript{[6]} to analyze the potential binding sites in PTEN core promoter region and its downstream DNA sequence (-1118/-160 bp), and found a variety of binding sites for p53, NF-kappaB, Ap2, MAZ, Sp1, E4F and Egr-1. Particularly, there were five MAZ binding sites in core promoter region (-1118/-778) area and 11 Egr-1 in its downstream area (-779/-160). Our results suggested that these two transcription factors might play an important role in control of PTEN expression. After the transfection of pGL3-612 (-1389/-778), which could produce maximum promoter activity into 8 cell lines, we found the profile of PTEN promoter activity was almost parallelized with the profiles of PTEN mRNA and PTEN protein in 8 cell lines. Furthermore, a recent study reported that the PTEN had no internal ribosome entry site (IRES) that could mediate cap-independent initiation of translation\textsuperscript{[18]}. Taken together, we conclude that the downregulation of PTEN expression in 7 HCC cell lines may not be responsible for the mutation of PTEN, but mainly contribute to the loss of PTEN promoter activity.

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