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

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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.

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)[7] and then blocks the activation of proto-oncogene PKB/Akt[8,9]. 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[10]. *PTEN* also dephosphorylated focal adhesion kinase because of its tyrosine phosphatase activity[11,12], which might lead to the inactivation of Ras/mitogen-activated protein kinase(MAPK) pathway[12-15]. 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[16]. 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[18]. Salvesen *et al*[19], 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 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 ℃ in a humidified atmosphere containing 50 mL/L CO_2 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 ℃ in a humidified atmosphere containing 50 mL/L CO_2 in air.

Western blot analysis

After being grown into 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 $MgCl_2$, 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 centrifugated 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 ℃ with a mAb against human *PTEN* (Santa Cruz, CA, USA), followed by incubation with HRP-conjugated secondary antibody at room temperature for 3 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 Na_2HPO_4/NaH_2PO_4 (pH 7.2), 1 mmol/L EDTA, 10 g/L BSA, 70 g/L SDS and 150 mL/L formamide at 50 ℃, and the filters were washed extensively with 40 mmol/L Na_2HPO_4/NaH_2PO_4 (pH 7.2), 1 mmol/L EDTA, 10 g/L SDS at 65 ℃. 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'-GATAGATCTGGGTG-GGGTGCGGGGTAGGAGTGC-3' and 5'-GAGAAG-CTTGCTGCGGCGGCTGCTGGATGGTTG-3'. The fragment was subcloned into the luciferase reporter plasmid pGL3-basic (Promega, Madison, WI, USA) which was digested twice with *Bgl*II and *Hin*dIII 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 *Bgl*II and *Hin*dIII restriction enzyme sites (listed below) were designed to amplify serial deletion fragment of *PTEN* using the clone pGL3-2768 (-2 927/-160) as a template.

5'-CGGAGATCTGTGTTTGGATGTGGGTGCTTTT-3' (-2 403),
5'-GCTAGATCTTCATTTAGATAGGTGCCCTTTGG-3' (-1 794),
5'-GAGAGATCTGCGTGGTCACCTGGTCCTT-3' (-1 389),
5'-CTGAGATCTCTCAGTAGAGCCTGCGGCTTGG-3' (-1 118),
5'-GGCAGATCTGCGGTGATGTGGCGGGACTCTT-3' (-916),
5'-GCGAGATCTCGCGACTGCGCTCAGTTCTCTCCT-3' (-858),
5'-CCGAAGCTTGGCCTCGCCTCACAGCGGCTCAACT-3' (-778),
5'-CAGAGATCTGGTCTGAGTCGCCTGTCACCATTT-3' (-458),
5'-GAGAAGCTTGCTGCGGCGGCTGCTGGATGGTTG-3' (-160).

Fragments of 2 234 (-2 403/-160), 1 635 (-1 794/-160), 1 230 (-1 389/-160), 1 526 (-2 403/-778), 1 016 (-1 794/-778), 612 (-1 389/-778), 341 (-1 118/-778), 139 (-916/-778), 81 (-858/-778) and 299 (-458/-160) were cloned into the vector pGL3-basic at the *Bgl*II and *Hin*dIII sites to individually generate pGL3-2234, pGL3-1635, pGL3-1230, pGL3-1526, pGL3-1016, pGL3-612, pGL3-341, pGL3-139, pGL3-81, pGL3-299, respectively. These constructs were sequenced and aligned with the GenBank databases.

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 Houyan 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 centrifugated at 12 000 *g* for 15 s at 4 ℃, and the supernatant was preserved at -70 ℃. 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 ℃ for 30 s, 60 ℃ for 1 min and 72 ℃ 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 parallelized 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

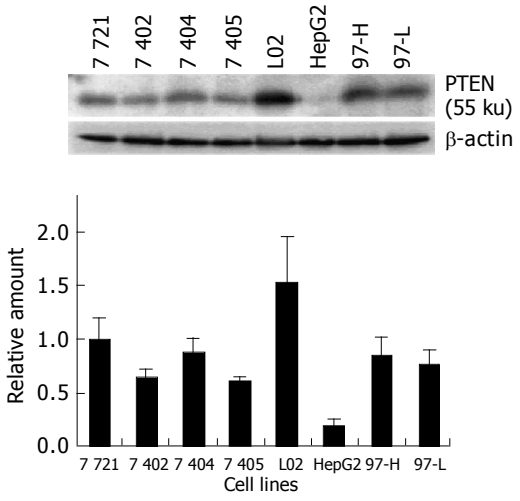

Figure 1 Western blot analysis for *PTEN* protein expression in 8 cell lines under normal growth conditions.

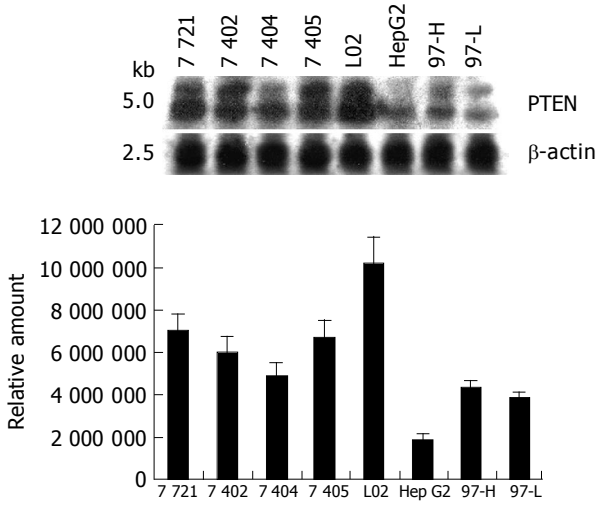

Figure 2 Northern blot analysis of *PTEN* mRNA expression in 8 cell lines under normal growth conditions.

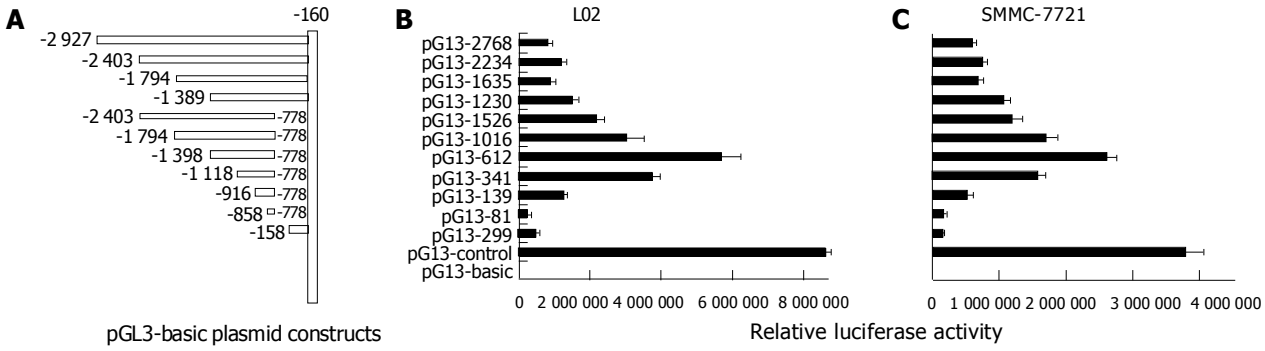

Figure 3 *PTEN* promoter deletion analysis in SMMC-7721 and L02 cell lines. **A**: Schematic representation of serial deletion constructs of human *PTEN* genomic locus; **B**: promoter activity of serial deletion constructs of *PTEN* in L02 cell lines; **C**: promoter activity of serial deletion constructs of *PTEN* in SMMC-7721 cell lines.

contained full-length promoter, appeared to have lower activity than that of pGL3-612 (-1 389/-778), indicating that the suppressor elements or special DNA structure may exist in double ends, especially in 3' end fragment (-777/-160). The 341 bp fragment (-1 118/-778) possessed over 60% of the promoter activity of the 612 bp fragment. The 81-bp fragment (-858/-778), however, appeared to be insufficient for inducing transcriptional activation of *PTEN*. The 299-bp downstream fragment (-458/-160) possessed no significant promoter activity in L02 and SMMC-7721 cells. No further increase in activity was observed when longer fragments than 612 bp were transfected, which was consistent with previous report[24]. Taken together, these data showed that the 612-bp fragment (-1 389/-778) had optimal promoter activity and the core region of *PTEN* promoter was located within the -1 118 to -778 region.

Different activities of PTEN promoter in 8 cell lines

When transfecting the plasmid pGl3-612 that possessed maximum *PTEN* promoter activity into the 8 cell lines, we found the activity of pGl3-612 in SMMC-7721 and L02 cells were much higher than that in others (Figure 4), while the activity of pGl3-612 in HepG2 cells was the lowest, only 17% of that in L02 cells. The activities of pGl3-612 in BEL-7402, BEL-7404, BEL-7405, MHCC-97H and MHCC-97L were 55%, 65%, 62%, 45%, and 39% of that in L02 cells, respectively. The profile of *PTEN* promoter activity in 8 cell lines was mostly parallelized to 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.

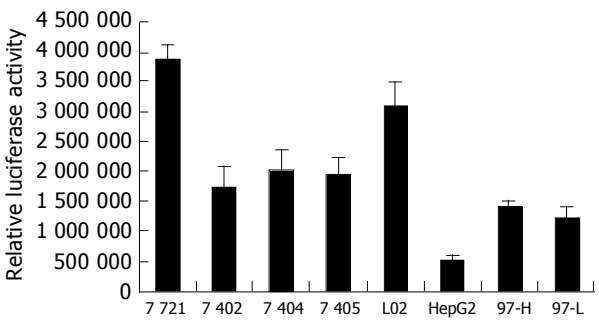

Figure 4 Activity of *PTEN* promoter in 8 cell lines.

DISCUSSION

Since the isolation of *PTEN/MMAC1/TEP1* (acronyms for phosphatase and tensin homolog[2], mutated in multiple advanced cancers[1], and TGF-β (transforming growth factor-β)-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[26]. The frequency of *PTEN* mutations observed in endometrial tumors[26], malignant glial tumors[25], malignant melanoma cell lines[26] 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[16]. *PTEN* is also known to be critically important both during embryonic development and in mature organisms as a tumor suppressor[30-32]. 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[33,34].

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[35].

As a tumor suppressor gene, *PTEN* expression is downregulated in tumors and tumor cell lines by genetic and epigenetic mechanisms[26]. 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[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 (-1 389/-778) could produce maximum promoter activity in 8 cell lines and the core region of *PTEN* promoter was within the 341 bp (-1 118/-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[36] to analyze the potential binding sites in *PTEN* core promoter region and its downstream DNA sequence (-1 118/-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 (-1 118/-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 (-1 389/-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[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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