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Effect of overexpression of *PTEN* on apoptosis of liver cancer cells

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ABSTRACT. Liver cancer is a common malignant tumor associated with a short-survival period and high-mortality rate, and its prevalence in China is particularly high. This study aimed to investigate the effect of overexpressing the phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*) gene on liver cancer cell apoptosis and provide new insight into the treatment of this disease. The experimental design included four treatment groups, consisting of HHCC and H22 cells transfected with *PTEN* recombinant plasmids (HHCC+PTEN, H22+PTEN), and those transfected with control plasmids (HHCC+NC, H22+NC). The expression of *PTEN* mRNA was determined by quantitative polymerase chain reaction, and protein levels were examined by western blot. Cell apoptosis was measured using flow cytometry and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling. *PTEN* mRNA expression

in cells transfected with pcDNA3.1-PTEN was significantly increased compared to the control groups ($P < 0.05$). In addition, western blotting revealed PTEN protein expression in the treatment groups to be significantly elevated in comparison to control cells ($P < 0.05$). Flow cytometry showed that apoptosis rates of both HHCC+PTEN (approximately 21.9%) and H22+PTEN (approximately 41.0%) cells were significantly higher than those of the control groups ($P < 0.05$). Moreover, the difference in apoptosis rate between experimental and control groups was significant ($P < 0.05$). In this study, HHCC and H22 cells were successfully transfected with pcDNA3.1-PTEN *in vitro*. We conclude that overexpression of *PTEN* can effectively inhibit proliferation of these cells and promote their apoptosis.

Key words: *PTEN*; Transfection; Liver cancer; Apoptosis

INTRODUCTION

Liver cancer is a fatal malignant disease and constitutes the third leading cause of tumor mortality worldwide (Thomas et al., 2010). The prevalence of this disease is high in China, with both the number of new cases and deaths accounting for 54% of the global total (Wang et al., 2015). Liver cancer has become a major health concern in China. Currently, surgery remains the conventional approach to treatment. Owing to its high invasiveness, most cases of this disease have already progressed to intermediate and advanced stages by the time of diagnosis, meaning that the optimal time for surgery is missed. The resection rate for liver cancer is lower than 15%, while the postoperative recurrence rate is approximately 50% (Siegel et al., 2015).

With the rapid development of molecular biological techniques, great advances have been made in our understanding of liver cancer at the molecular level. Moreover, the study of cancer cell proliferation and migration is of great significance to the diagnosis, treatment, and prognosis of related malignancies (Gupta and Massagué, 2006). Phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*) was one of the first observed tumor suppressor genes with phosphatase activity. Located on chromosome 10q23 and 200 kb in length, *PTEN* is a member of the protein tyrosine phosphatase gene family. It plays an important role in physiological activities involving cell apoptosis, adhesion, and migration (Shearn and Petersen, 2015). The frequency of loss of heterozygosity of *PTEN* in hepatocellular carcinoma is reported to be around 33% (Yamazaki et al., 2015). Deletion and mutation of this gene may occur at high frequency during the progressive stage of metastatic malignancies affecting the brain (Carletti et al., 2015), endometrium (Clements et al., 2015), prostate (Manda et al., 2015), breast (Chen et al., 2016), lung (Dragoj et al., 2015), bone (Xi and Chen, 2015), colorectum (Atreya et al., 2013), and liver (Sendor et al., 2015). The loss of *PTEN* expression has been shown to be associated with aggressive tumor behavior. Therefore, we investigated the effect of overexpression of this gene on liver cancer cell apoptosis.

In this study, a eukaryotic expression vector carrying *PTEN* was transfected into human hepatic carcinoma cells (HHCC) and mouse hepatoma H22 cells to explore the effect of its overexpression on apoptosis, and further investigate its role in the development of liver cancer, to provide a primary scientific basis for the prevention and treatment of this disease.

MATERIAL AND METHODS

Instruments

Polymerase chain reaction (PCR) thermal cycler (ABI 9700; Applied Biosystems, Foster City, CA, USA), real-time PCR instrument (Mx3000P; Stratagene, La Jolla, CA, USA), gel scanner (DF-23B; UVP, Cambridge, UK), flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA), fluorescence microscope (LSM 510; Carl Zeiss, Oberkochen, Germany).

Reagents

TRIzol RNA extraction reagent (Invitrogen, Waltham, MA, USA), RNasin (Promega, Madison, WI, USA), fetal calf serum (Gibco, Waltham, MA, USA), annexin V/propidium iodide (PI) apoptosis kit (MultiSciences Biotech, Hangzhou, China), SYBR Green PCR Master Mix (purchased from Applied Biosystems), Lipofectamine transfection reagent (Invitrogen), antifade mounting medium and 4',6-diamidino-2-phenylindole (DAPI)-staining solution (Sigma-Aldrich, St. Louis, MO, USA), DL2000DNA marker (Dalian Proteri Biotechnology Co. Ltd., China), terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) reagent kit (Roche, Basel, Switzerland).

PTEN vector construction

Genomic DNA was prepared from peripheral leukocytes. The primers (synthesized by Sangon Biotech, Shanghai, China) used to amplify *PTEN* were as follows: F, 5'-CCG GAA TTC ATG GCC ATG GCA ACC AAA GG-3'; and R, 5'-CCC AAG CTT TCA GAC TTT TGT AAT TTG TGT ATG C-3'. The reaction system was provided by Takara Bio Inc., and the product was run on a 1% agarose gel, from which it was extracted using a Gel Extraction Kit (Omega Bio-Tek Inc., Norcross, GA, USA). The product was then cloned into the pcDNA3.1 vector using *Hin*dIII and *Eco*RI restriction enzymes.

Cell culture and transfection

HHCC and H22 cells were purchased from ATCC (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum at 37°C in a 5% CO_2 atmosphere at saturated humidity. During the logarithmic phase, Lipofectamine 2000 was used to transfect empty pcDNA3.1 vectors and a pcDNA3.1-PTEN construct. The experiment comprised four treatment groups: pcDNA3.1 empty vector + H22 cells (H22+NC), pcDNA3.1-PTEN vector + H22 cells (H22+PTEN), pcDNA3.1 empty vector + HHCC cells (HHCC+NC), and pcDNA3.1-PTEN vector + HHCC cells (HHCC+PTEN).

Quantitative PCR (qPCR)

Forty-eight hours after transfection, cells from each group were transferred into 1 mL TRIzol for total RNA extraction. Reverse transcription was then used to generate complementary DNA, 1 μL of which was used to measure *PTEN* mRNA expression. The SYBR Green method was applied to perform qPCR, in which each sample was tested in triplicate. The primers used for qPCR are shown in Table 1.

Table 1. Primers used for *PTEN* quantitative polymerase chain reaction.

PTEN F	5'-TAG ACC AGT GGC ACT GTT GT-3'
PTEN R	5'-TGG CAG ACC ACA AAC TGA GGA T-3'
GAPDH F	5'-CAG CCT CAA GAT CAT CAG CA-3'
GAPDH R	5'-TGT GGT CAT GAG TCC TTC CA-3'

F = forward; R = reverse.

Western blot

Forty-eight hours following transfection, cells from each group were collected by centrifugation. Radioimmunoprecipitation assay lysis buffer (100 μL) was then added to lyse the cells on ice for 20 min. Total protein concentration was measured by bicinchoninic acid assay prior to loading samples to ensure that all wells contained the same quantity of protein (30 mg). Proteins were separated on a 10% separation gel before being wet-transferred to a polyvinylidene difluoride (PVDF) membrane for 30 min at 300 mA. The primary antibody (anti-PTEN) was diluted 1:4000 in phosphate-buffered saline (PBS), and the PVDF membrane was then incubated with this antibody for 1 h at ambient temperature. Following this, Tris-buffered saline with Tween 20 (TBST) was used to wash the membrane three times (5 min/wash). The secondary antibody was then added and incubated with the membrane for 1 h at ambient temperature before three further TBST washes.

Cells apoptosis detection

Forty-eight hours after plasmid transfection, cells were treated with 0.5% pancreatin and washed with 1X PBS. Cells were then resuspended in pre-cooled 1X binding buffer solution for a concentration of 1 x 10^6 cells/mL. Fluorescein isothiocyanate (FITC)-conjugated annexin V (1.25 μL) was added and reacted with cells in a light-free environment for 15 min. The cells were then centrifuged for 5 min at ambient temperature and resuspended in 0.5 mL pre-cooled 1X binding buffer solution. PI (10 μL) was then added and cells were kept on ice in a dark place before being analyzed by flow cytometry.

TUNEL assay

The culture medium was discarded and cells were transferred to a plate and washed three times with PBS before being fixed with 4% paraformaldehyde for 20 min. After three further PBS washes, the fixed cells were incubated with 0.3% PBS-Triton X-100 for 20 min and washed again three times with PBS. A mixture of TUNEL enzyme solution and TUNEL label solution (1:9) was then prepared, 50 μL of which was added to each well and incubated with cells at 37°C for 1 h, prior to being washed three times with PBS. DAPI-staining solution was added and incubated for 10 min at ambient temperature, and cells were then analyzed under a fluorescence microscope.

Statistical analysis

Statistical significance was defined as $P < 0.05$, and data were analyzed by SPSS version 10.0 (SPSS Inc., Chicago, IL, USA). The *t*-test was used to make comparisons between the two groups, while multi-group data were analyzed with a multi-factor *Q*-test.

RESULTS

PTEN vector construction

PCR products were examined by 1% agarose gel electrophoresis (Figure 1), which showed an objective band of 1212 bp. The subsequently constructed plasmid was sent to Life Technologies Corporation (Shanghai, China) for sequencing, the results of which were verified using the Basic Local Alignment Search Tool and GenBank.

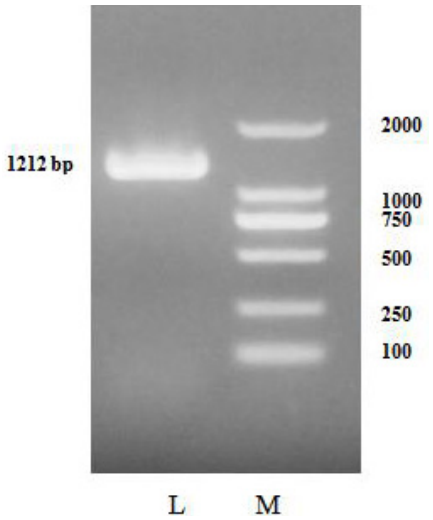

Figure 1. Agarose gel electrophoretogram of the *PTEN* polymerase chain reaction (PCR) product. *Lane M* = DL2000 DNA marker; *lane L* = PCR product.

PTEN expression in H22 and HHCC cells measured by qPCR

Forty-eight hours after transfection, *PTEN* expression in each group was determined by qPCR (Figure 2). Compared to the HHCC+NC group, relative *PTEN* expression in the HHCC+PTEN group was increased more than 9-fold (9.26 ± 0.4674). Moreover, this result was statistically significant (t = 17.02, P = 0.0034). Relative *PTEN* levels in H22+PTEN cells were more than 1800 times higher (1877.93 ± 191.043) than those of H22+NC cells, and this difference was also found to be significant (t = 30.63, P = 0.0011).

PTEN expression in H22 and HHCC cells measured by western blot

Forty-eight hours after transfection, the expression of PTEN protein in each group was examined by western blot (Figure 3). In the HHCC+NC and HHCC+PTEN groups, values were relative to GAPDH expression, PTEN expression was found to be 0.072 ± 0.0096 and 0.134 ± 0.0094, respectively. Statistical analysis revealed this to be a significant difference (t = 8.14, P = 0.0012). Meanwhile, H22+NC and H22+PTEN cells demonstrated relative PTEN levels of 0.056 ± 0.0086 and 0.149 ± 0.0077, respectively. This difference between treatment and control groups was also found to be statistically significant (t = 14.04, P = 0.0001).

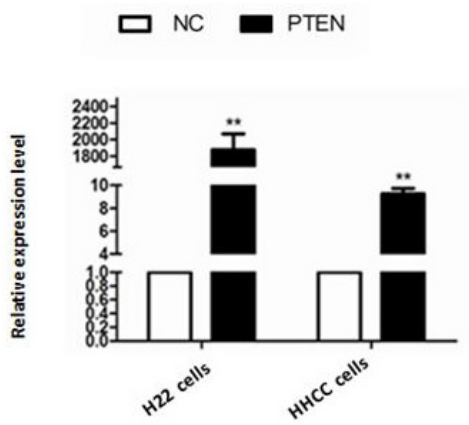

Figure 2. *PTEN* expression level in each group, measured by quantitative polymerase chain reaction. NC = transfected with empty vector, PTEN = transfected with *PTEN* vector. **P < 0.01.

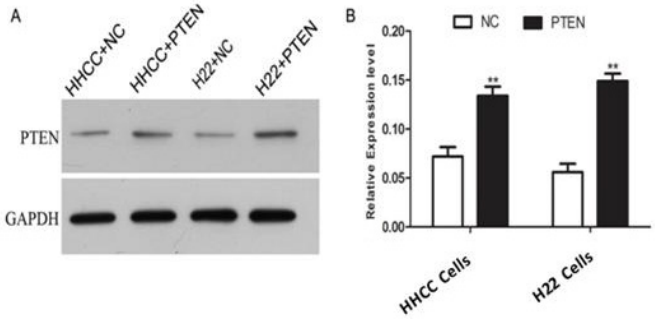

Figure 3. PTEN protein expression in each group, measured by western blotting. **A.** Western blot showing PTEN expression in each group. **B.** Quantified PTEN western blot, using gray values of each band; NC = transfected with empty vector, PTEN = transfected with *PTEN* vector.

Effect of *PTEN* overexpression on H22 and HHCC cell apoptosis

Forty-eight hours after transfection, cells from each group were stained with FITC-conjugated annexin V, and apoptosis was detected by flow cytometry (Figure 4). The apoptosis rate of cells in the H22+PTEN group was revealed to be 41.0%, significantly higher than that of the H22+NC group (chi-square = 34.07, P = 0.000). Apoptosis of HHCC+PTEN cells was measured as 21.9%, a rate significantly higher than that of cells in the HHCC+NC control group (chi-square = 12.37, P = 0.001).

Effect of *PTEN* overexpression on H22 and HHCC apoptosis measured by TUNEL assay

Forty-eight hours following transfection, the effect of *PTEN* overexpression on H22 and HHCC cell apoptosis was detected using a TUNEL assay (Figure 5). Compared to their corresponding controls, apoptosis rates in H22 (t = 45.12, P = 0.000) and HHCC (t = 22.47, P = 0.008) *PTEN*-overexpression groups were significantly increased.

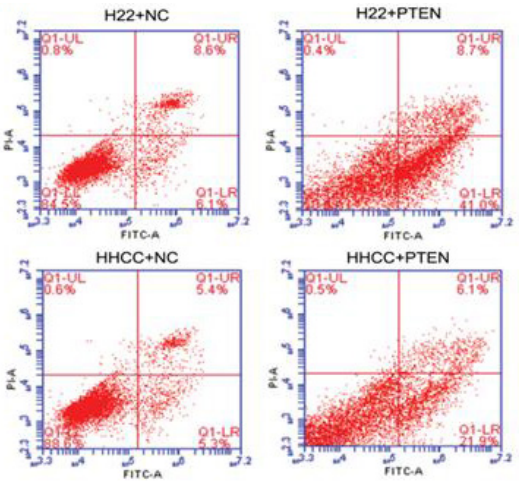

Figure 4. Cell apoptosis measured by flow cytometry. PI = propidium iodide; FITC = fluorescein isothiocyanate.

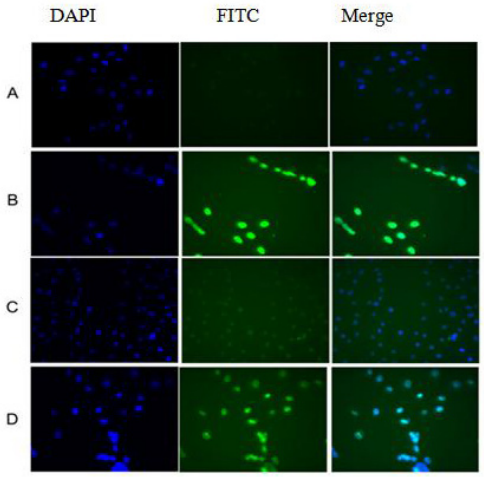

Figure 5. Effect of *PTEN* overexpression on H22 and HHCC cell apoptosis, measured by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling assay. **A.** H22 control group, **B.** H22 *PTEN* overexpression group, **C.** HHCC control group, **D.** HHCC *PTEN* overexpression group. DAPI = 4',6-diamidino-2-phenylindole; FITC = fluorescein isothiocyanate.

DISCUSSION

PTEN can induce apoptosis, inhibit invasion and metastasis of tumor cells and tumor angiogenesis, and maintain immune system stability, among other functions (Zhao et al., 2013). It has been shown that the tumor suppressive effect of *PTEN* is principally achieved through regulation of the PI3K/AKT signaling pathway and inhibition of those involving FRAP/mTOR, FAK/P130Cas, and APK (Ohta et al., 2015). The *PTEN* gene has been reported to regulate the proliferation and apoptosis of liver cancer cells, and can maintain normal cellular physiology,

including metabolism, proliferation, differentiation, and apoptosis by inhibiting AKT activity in the PI3K/AKT signaling pathway (Yamamoto et al., 2013). As a result of *PTEN* mutation or deletion, PI3K signal transduction may be enhanced, and AKT excessively activated, resulting in hepatocyte overproliferation or even vicious transformation (Yan et al., 2015). Furthermore, *PTEN* can reduce the phosphorylation of FAK, regulate P130Cas levels, and inhibit cell adhesion and metastasis. As a result, this gene may prevent tumor migration and diffusion (Ohta et al., 2015). Microvessel density is significantly higher in cancer tissue with inactive *PTEN* than in that with normal *PTEN*. Moreover, it has been demonstrated that the mutation or inactivation of this gene can enhance expression of endothelial growth factors (including VEGF) and matrix metalloproteinases, and augment the formation of tumor vasculature (He et al., 2016). Liu et al. (2012) explored the effect of docetaxel and a mannan-modified adenovirus encoding PTEN on tumor growth in a murine model, finding that such combined treatment may constitute an effective potential adjuvant therapy for hepatocellular carcinoma.

In this study, we constructed a eukaryotic expression plasmid containing the *PTEN* gene. The sequence of this construct was found to correspond exactly to that given in GenBank, confirming its successful assembly. Our experiment included four groups, based on two different expression vectors (empty or carrying *PTEN*) and two cell types (H22 and HHCC). Our results showed that *PTEN* mRNA was strongly expressed in cells treated with pcDNA3.1-PTEN, demonstrating successful transfection of both HHCC and H22 cells. In addition, PTEN protein levels were high in HHCC and H22 cells transfected with pcDNA3.1-PTEN, and significantly so compared to the control groups consisting of cells expressing the pcDNA3.1 empty vector. The apoptosis rate of HHCC cells transfected with the pcDNA3.1-PTEN construct was approximately 21.9%, while that of H22 cells treated with the same plasmid was around 41.0%. These values were significantly higher than those of the corresponding control groups transfected with the empty vector.

In conclusion, transfection can be successfully used to import a recombinant plasmid containing the *PTEN* gene into HHCC and H22 cells. Overexpression of *PTEN* can inhibit proliferation of these cells and effectively promote their apoptosis. Therefore, it is possible to use *PTEN* transfection to provide new insights into the treatment of liver cancer.

Conflicts of interest

The authors declare no conflict of interest.

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