# Elucidation of functions of PTEN in pancreatic carcinogenesis and its therapeutic application

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# Elucidation of functions of PTEN in pancreatic carcinogenesis and its therapeutic application

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This certifies that the Doctorial Dissertation of Seungmin Bang is approved.

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Seungmin Bang

# Table of contents

# LIST OF FIGURES

# LIST OF TABLES

**ABSTRACT**

Elucidation of functions of PTEN in pancreatic carcinogenesis and its therapeutic application

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(Directed by Professor Si Young Song)

PTEN has been emerged as a tumor suppressor gene frequently deleted or mutated in various human cancer. Recently, PTEN in hematopoiteic stem cells is suggested as the key for maintaining normal hematopoietic stem cell population and preventing the development of leukemia-initiating cells. However, the mutation or deletion of PTEN in pancreatic cancer is reportedly very rare. Although PTEN deletion is reported to be related to the centroacinar cell expansion and emergence of metaplastic duct and pancreatic cancer via transgenic mouse experiment, it does not seem to be physiologic in pancreatic cancer. So this study was intended to analyze the real condition of PTEN and relating molecular event in pancreatic cancer. On

the contrary to the previous reports, PTEN is overexpressed in pancreatic cancer cell lines and human pancreatic cancer. Furthermore, pancreatic cancer with overexpressing PTEN showed poorer prognosis. With these controversial data, further experiments were performed with hypotheses that the overexpressed PTEN in pancreatic cancer might be related to the unexpected tumor promoting acitivity, so called “antagonistic duality”. For validating this hypothesis, a transient knock down of PTEN via siRNA technique was used. After knocking down of PTEN, pancreatic cancer cells showed two different molecular and phenotypical changes. Interestingly, PTEN knock down pancreatic cancer cells harboring wild p53 (HPAC) showed the predicted activation of Akt and its downstream signals. On the contrary, pancreatic cancer cells harboring gain of function mutation or frame shift mutation did not show the activation of Akt. And GSK3 was inactivated which in turn, result in activation of p21. Furthermore, these mutant p53 pancreatic cancer cells showed decreased cell proliferative activity in the condition of PTEN knock-down. With these findings, PTEN can be thought to have tumor promoting activity in specific conditions including gain of function mutation of p53. Considering the current paradigm of anti-cancer treatment is shifting to target the specific tumor associated pathway including PTEN pathway, this experimental outcome should be considered for the

therapeutic strategies that aim at manipulating PTEN or p53 in human pancreatic cancer.

Key words: Pancreatic cancer, PTEN, antagonistic duality, p53

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## I. INTRODUCTION

Ductal adenocarcinoma of the pancreas is an aggressive disease characterized by its invasiveness, rapid progression, and resistance to treatment.[1] There is currently no effective treatment option except curative operation in cases of early lesions. Combined with this frustrating situation is a real sense of urgency. From the decade of the 1990s, several dramatic advances in pancreatic carcinogenesis have been reported. Among these advances, consensus for pancreatic intraepithelial neoplasia (PanIN) as a direct noninvasive neoplastic precursor to human cancer shed the light in the field of pancreatic carcinogensis.[2,3] The identification of PanIN as a relevant precursor lesion of pancreatic cancer further allowed the ordering of characteristic genetic events in a step-wise carcinogenesis, in which KRAS mutation and telomere

shortening were characterized as early events, loss of p16INK4a as an intermediate event, and loss of p53 and DPC4 as late events.[4-8] This consensus is supported by the transgenic mouse model of Hingorani et al, in which they reported the generation of progressive PanIN lesions and low-frequency progression to invasive and metastatic adenocarcinoma following activation of oncogenic KRAS in mouse pancreas.[9] However, more sophisticated and concrete molecular network should be needed even though the progress just mentioned above.

Phosphatase and tensin homologue on chromosome 10 (PTEN) is a well known tumor suppressor gene. In 1997, PTEN was cloned and mapped to cytoband 10q23, a region undergoing frequent somatic deletion in tumors.[10-12] The protein encoded by the PTEN gene is a phosphatase-an enzyme that facilitates the removal of phosphate groups from macromolecules. Its primary biochemical and physiological targets are highly specialized plasma membrane lipids.[13] These lipid, phophatidyl-inositol-3,4,5-triphosphate (PIP3) and phosphatidylinositol-3,4-bisphosphate are produced during cellular signaling events by the action of the lipid kinase phosphor-inositide 3-kinase (PI3K).[14] Thus, an elegant on-off switch has been evolved where the switch moves to "on" position when PI3K deposits a phosphate group on the D3 position of inositol ring and is turn "off" when PTEN removes the phosphate group from the same position. A critical role for this switch in both the normal response to growth signals and in the abnormal response to transforming signals was evident in the mid 1980s when PIP3 was first discovered as an evanescent molecule, the abundance of which was significantly upregulated on growth factor stimulation. At

the same time, elevated PI3K activity was linked both to transformation by onco-genes, such as polyoma middle T antigen, and to mitogenic stimulation through the platelet derived growth factor receptor.[15-17] In other words, the discovery of PTEN's lipid phosphatase activity, and its ability to act as an "off" switch for PI3K signaling, suggested that PTEN functioned as a tumor suppressor by directly antagonizing the activity of the PI3K signaling pathway.

Recently, PTEN has been spotlighted as a key factor for carcinogenesis and cancer stem cell.[18-20] Yilmaz et al[19] reported that intact PTEN drives the self-renewal of normal hematopoietic stem cells (HSCs). However, deletion of PTEN in HSCs drives the depletion of normal HSCs and formation of leukemia initiating cells. Furthermore, the blocking the mTOR, which are one of the downstream of PTEN and related to cell cycle progression and carcinogenesis, can restore the HSCs and prevent the development of leukemia-initiating cells.

Aberrant PTEN signaling in cancerous diseases is associated with genetic alterations and transcriptional/translational modifications. Genetic mutations of PTEN are reported as 50% of endometrial cancer, 30% of glioblastoma, 10% of prostate cancer and 5% of breast cancer.[11,12,21] Aside from the genetic alterations, expression of PTEN gene is also frequently diminished by DNA methylation, transcriptional repression and micro-RNA directed mRNA degradation and translational disruption appear to reduce PTEN expression in numerous cancers.[22,23] In the field of pancreatic carcinogenesis, the role of PTEN has not been validated well. Asano et al[24] reported that 60% of pancreatic cancer cell lines and human

cancer tissues showed reduced expression of PTEN. And this reduced expression of PTEN in pancreatic cancer cell lines was related to the promoter methylation. Furthermore, Stanger et al[25] reported the progressive proliferation of metaplastic duct from centroacinar cells, which are referred as pancreas-specific stem cells and low-frequency progression of pancreatic adenocarcinoma, following deletion of PTEN in mouse pancreas.

With this shortage of the fine molecular phenomena about PTEN in pancreatic cancer, the real molecular status of PTEN and the function of PTEN in pancreatic cancers were analyzed in this study.

## II. MATERIALS AND METHODS

### 1. Cell lines and reagents

Human pancreatic cancer cell lines (Aspc-1, CFPAC-1, BxPC-3, Capan-1, 2, MiaPaca-2, HPAC and Panc-1) were purchased from American Tissue Culture Collection (Manassas, VA, USA). Aspc-1 and BxPC-3 cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). Capan 1 cells were grown in Iscove's Modified Dulbeco's Medium (IMDM) supplemented with 20% FBS. Capan 2 cells were grown in McCoy'5A supplemented with 10% FBS. CFPAC-1 cells were grown in IMDM supplemented with 10% FBS. Hpac cells were grown in Dulbecco's Modified Eagle's-Ham's F12 medium (DMEM-F12) supplemented with 10% FBS. Miapaca2 and Panc-1 cells were grown in DMEM supplemented with 10% FBS. Normal human pancreatic ductal epithelial cells (HPDE-6) were a generous gift from Dr Ming-Sound Tsao (Toronto, Canada). HPDE cells were grown in keratinocyte serum-free medium supplemented with 4 mM L-glutamine and adjusted to contain 0.2 ng/ml EGF, 30 ug/ml bovine pituitary extract.[26,27] All cells were maintained in a humidified incubator with 5% $CO_2$ and 95% air.

### 2. Western blotting

Western blotting was performed using antibodies specific for PTEN, phospho-PTEN, p53, phopho-AKT, phosphor-Glycogen synthesis kinase (pGSK), phosphorylated S6 ribosomal protein (Ser235/236), GAPDH, mdm2, p21 and cyclin

D1. Tissues of pancreatic adenocarcinoma and normal pancreas were collected from surgical specimen of 12 patients. And 8 cancer cell lines (-1, CFPAC-1, BxPC-3, Capan-1, 2, MiaPaca-2, HPAC and Panc-1) and immortalized normal pancreatic ductal epithelial cells (HPDE) were used for blotting. The tissues were lysed in buffer containing 70mM β-glycerophosphate (pH 7.2), 0.6mM sodium vanadate, 2mM $MgCl_2$, 1mM EGTA, 1mM DTT, 0.5% Triton X 100, 0.5% NP-40, 0.2mM PMSF and protease inhibitors. In case of cell line, the buffer solution without 0.5% NP-40 was used. The purified proteins were separated by 12% SDS-PAGE and transfered for 1 hour onto PVDF membrane (Millipore corperation, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk in Tris-buffered saline/0.05% Tween-20 (TBS-T) for 1hr at room temperature and probed overnight with specified antibodies at 4℃. After washing with TBS-T, the membranes were treated with horseradish peroxidase-conjugated secondary antibody for 1hr and washed several times. Proteins were detected using enhanced chemiluminescence system (PIERCE, Rockford, IL, USA). The concentration of primary antibodies were 1:1,000 dilution in 0.5% non-fat milk in TBS-T for anti-PTEN antibody (Cell signaling technology, USA), 1:500 dilution for phospho-PTEN (Cell signaling technology, USA), 1:1000 dilution for p53 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:1000 dilution for pAKT (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:500 dilution for pGSK3 a/b (cell signaling technology, USA), 1:500 dilution for pS6 (cell signaling technology, USA), 1:500 dilution for p21 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:500 dilution for cyclinD1 (Santa

Cruz Biotechnology, Santa Cruz, CA, USA), 1:5000 dilution for GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

### 3. Reverse transcriptase – polymerase chain reaction (RT-PCR) for PTEN

Standard RT-PCR was conducted using total RNA prepared from 8 pancreatic cancer cell lines and HPDE cells. Reverse transcription was conducted for 60 min at 42℃ from 2 μg of purified total RNA in a 20 μl volume of reverse transcription system reaction mixture (Invitrogen, Carpinteria, CA, USA ). Reverese transcription was followed by 25 cycles of standard PCR (30 sec denaturation at 94℃, 30 sec annealing at 55℃, and 30 sec extension at 72℃). Primer sequences and PCR condition were described in Table 1. Amplified products were separated on 1.5% agarose gels and visualized by ethidium bromide.

**Table 1. Primer sequence and PCR condition**

| Gene | Primer sequence | Tm | Cycles | Accession number |
|---|---|---|---|---|
| PTEN | Forward:<br>5’- GGACGAACTGGTGTAATGAT-3’<br>Reverese:<br>5’- CAGACCACAAACTGAGGATT-3’ | 55℃ | 25 | NM-000314 |
| β-actin | Forward:<br>5’-ATGATATCGCCGCGCTCGTCGTC-3’<br>Reverse:<br>5’-GCTTCGGCCGTGGTGGTGAA-3’ | 55℃ | 25 | |

## 4. Immunohistochemical staining

A set of pancreatic adenocarcinoma samples was randomly obtained from individuals who had undergone surgical resection for pancreatic cancer between January 2001 and Decemder 2007 at Severance Hospital, Yonsei University College of Medicine. All patients underwent pancreatic resection for curative purposes and gave informed consent for using tissue samples. Fifty two pancreatic cancer tissue samples were collected. A pathological grading was made, and the tumor stage of the tissue samples was determined according to the AJCC staging system. The Ethics Committee for the Clinical Research of the Institutional Review Board of Yonsei Medical Center, Korea, approved this study protocol.

Immunostaining with a mouse-derived monoclonal PTEN antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:200 was performed on whole tissue slides, using standard procedures. Initially, slides were deparaffinized in xylene and rehydrated in graded alcohol. Endogenous peroxidase activity was blocked by incubating slides with methanol containing 0.3% hydrogen peroxide at room temperature for 20 min. Microwave antigen retrieval was performed in sodium citrate buffer (0.01 M, pH 6.0) for 3 min. To block non-specific staining, slides were incubated with 10% normal donkey serum solution for 1 hour. Blocked sections were incubated in primary antibody overnight at 4˚C. Slides were then treated using a DAKO Real™ Envision™ kit (Dako Cytomation, Carpinteria, CA, USA), according to the manufacturer's recommended protocol. Slides were developed by incubating them with 3,3' Diaminobenzidine and then counterstained

with haematoxylin.

The islets in normal pancreas were used as a positive control, which were stained strongly on both cytoplasm and nuclei with PTEN antibody. Samples were evaluated by two pathologists who were blinded to patients' clinical information. Intensities were scored as 0, no staining; 1, weak; 2, moderate; or 3, strong.

## 5. Cytoplasmic and nuclear protein extraction

Cytosolic and nuclear fractions were isolated using a commercialized kit (NE-PER nuclear and cytoplasmic extraction reagents, Thermo scientific, Rockford, IL, USA) according to manufacturer's directions. In brief, cells were harvested with trypsin-EDTA and then centrifuge at 500 x g for 5 min. Before adding CER-I, the cells were washed with PBS and collected with centrifugation at 500 x g for 3 min. After vortex for 15 sec to fully suspend the cell pellet, incubate the cells for 10 min. CER-II reagent was added and then incubate for 1 min after vortex. In this process, supernant was collected as the cytoplasmic fraction. The remaining insoluble fraction was treated with NER reagent. With this step, nuclear fraction was separated. All steps were performed in ice-cold. Western blotting was performed just same as above.

## 6. RNA interference (siRNA)-induced gene silencing

Three Pancreatic cancer cell lines (Aspc-1, HPAC, Panc-1) were chosen for transient transfection with PTEN siRNA. These cells were transfected with PTEN

siRNA or control non-specific siRNA using Stealth™ siRNA Duplex Oligoribonucleotides (Invitrogen, Carlsbad, CA, USA). Human pancreatic cancer cells were seeded at a density of 2 x $10^5$ cells per per well of a 6 well plate in medium with 10% FBS and without antibiotics, and allowed to adhere overnight before transfection. At initial screening for choosing the most efficient siRNA against PTEN, three different sequences of siRNA were used (Table 2). For further experiment, the P1 siRNA was used. PTEN siRNA (100 pmol) or control siRNA were added to 250 µl of Opti-MEM-I Reduced Serum Medium, while 5 µl of Lipofectamine™ RNAiMAX (Invitrogen, carlsbad, CA, USA) was added to 250 µl Opti-MEM-I Reduced Serum Medium. Each was mixed gently and incubated for 5 min, after which diluted siRNA and diluted Lipofectamine™ RNAiMAX were then combined, gently mixed, and allowed to incubate for 20 min at room temperature. The siRNA plus Lipofectamine 2000 complex were added directly to the culturing cells. Complete medium with 10% FBS was added and cells were cultured for another 48 hours.

**Table 2. Primer sequences of siRNA for PTEN**

| siRNA | Primer seuqences |
|---|---|
| P1 | forward: 5’- AAUUGCUGCAACAUGAUUGUCAUCU-3’ |
| | Reverse: 5’-AGAUGACAAUCAUGUUGCAGCAAUU-3’ |
| P2 | forward: 5’-AGUACAUGAACUUGUCUUCCCGUCG-3’ |
| | reverse: 5’-CGACGGGAAGACAAGUUCAUGUACU-3’ |
| P3 | forward: 5’-UUAGCCUGGCAGACCACAAACUGAGG-3’ |
| | reverse: 5’-CCUCAGUUUGUGGUCUGCCAGCUAA-3’ |

## 7. Cell cycle analysis with phospho-histone H3 immunofluorescence staining

The pancreatic cancer cells were cultured for 24 h in culture media on 8 well chamber slide, fixed with 100% methanol, and then permeabilized with 0.1% Triton X-100. After blocking with 0.2% BSA in PBS, the cells were incubated with rabbit polyclonal antibodies against phosphorylated histone H3 (Ser10) at 4 ◦C overnight. The cells were washed with PBS containing 0.1% Triton X-100 and incubated with Cy2-labeled affinity purified antirabbit IgG secondary antibody. DNA was counterstained with PI. The images were captured and observed using fluorescence microscope (Olymphus, Japan).

## 8. TdT-mediated dUTP Nick End Labeling (TUNEL) assay

After exposure to 200 μmol/L $H_2O_2$ for 6 h, cells grown on 8 well chamber slide ($10^4$ cells/well) were washed with PBS/1% BSA and fixed in paraformaldehyde solution (4% in PBS) for 30 min at room temperature.

The cells were permeabilized in a solution containing 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice, followed by incubation in freshly prepared TUNEL reaction mixture (Roche, Mannheim, Germany) for 60 min at 37℃ in the dark. The coverslips were washed with PBS and analyzed by fluorescence microscopy.

## III. RESULTS

### 1. PTEN expression of pancreatic cancer tissues

For the validation of PTEN protein expression status in pancreatic cancer, pancreatic cancer tissues from 12 patients of histologically confirmed pancreatic ductal adenocarcinoma were collected during the curative surgery. On the contrary to our prediction, PTEN protein level of pancreatic cancer tissues was relatively higher than the adjacent non timorous tissues of the same patients. Among the all of 12 tissues sample, nine (75%) showed stronger expression of PTEN protein in pancreatic cancer tissues (Fig. 1).

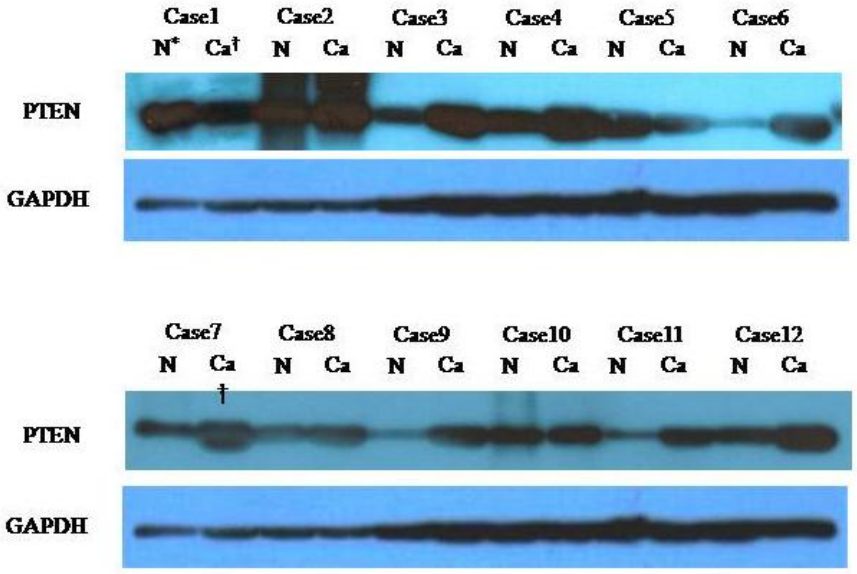


**Figure 1. PTEN protein expression in pancreatic cancer tissues**

Among 12 samples of pancreatic cancer and normal pancreas from same patients, 9 cases showed relatively high level of PTEN protein in pancreatic cancer tissues than the adjacent non tumorous pancreas. * Non tumorous pancreas; † Pancreatic cancer.

## 2. PTEN expression in pancreatic cancer cell lines

With the above debatable data, the PTEN mRNA and protein expression level in established pancreatic cancer cell lines was analyzed. In this experiment, 8 of commercialized pancreatic cancer cell lines (Aspc-1, CFPAC-1, BxPC-3, Capan-1, 2, MiaPaca-2, HPAC and Panc-1) were used. For the control, the immortalized human pancreatic ductal cell lines – HPDE was chosen. In the RT-PCR experiment, all of 8 pancreatic cancer cells were confirmed to express mRNA of PTEN. Compared with the control cell – HPDE, the mRNA expression in pancreatic cancer cell lines were not suppressed (Fig. 2).

With the above experiment, PTEN protein expression in pancreatic cancer cell lines were shown as being increased rather than the normal ductal cell line of pancreatic epithelium (Fig. 2). For semiquantitative densitometric analysis of western blot of PTEN revealed that all of the eight pancreatic cancer cell line showed overexpression of PTEN, compared to the normal pancreatic ductal cell line-HPDE.

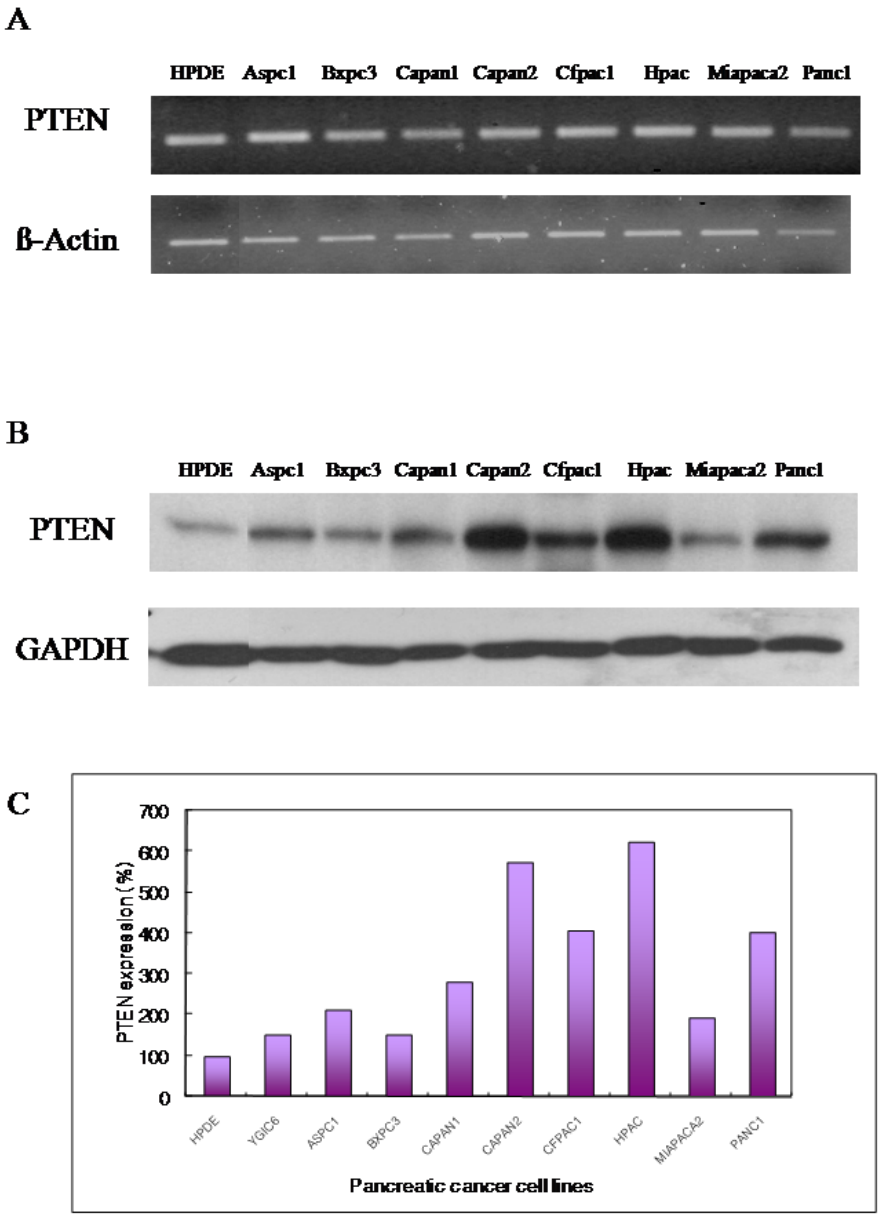


**Figure 2. PTEN expression status in pancreatic cancer cell lines**

A. mRNA of PTEN expression was not decreased in various pancreatic cancer cell lines.

B. Furthermore, the protein level of PTEN in pancreatic cancer were relatively increased, compared with control – HPDE cells.

C. In densitometric analysis, all of the 8 pancreatic cancer cell lines over-expressed PTEN compared with the control cell line-HPDE.

### 3. Immunohistochemical staining of PTEN

The expression of PTEN was double checked with immunohistochemical (IHC) staining of pancreatic cancer slide. For this experiment, a set of pancreatic cancer tissue slide was made of paraffin-embaded pancreatic cancer tissue blocks from 52 patients after pathologic review. The clinicopathologic characteristics of these samples were in table 3. The islet cells, which served as internal positive control, showed moderate expression of PTEN in both cytoplasm and nucleus (Fig 3). Among the all of 52 pancreatic cancer samples, only 11 samples were negative for PTEN immunostaining. Of the remaining 41 samples, fifteen showed weak staining and the others had moderate or strong expression level (Table 4).

**Table 3. The clinicopathologic characteristics of the pancreatic cancer samples (N=52)**

| | |
|---|---|
| Age, median (range) | 64 years (42-78) |
| Sex, Male:Female (%) | 35:17 (67.3:32.7) |
| Primary site | |
| Pancreatic Head | 34 (65.4) |
| Pancreatic Body | 6 (9.7) |
| Pancreatic Tail | 12 (19.4) |
| Stage (AJCC, $6^{th}$) | |
| IB | 2 (3.2) |
| IIA | 18 (29.0) |
| IIB | 28 (45.2) |
| IV | 4 (6.5) |
| Histologic differenciation (%) | |
| Well differentiated | 5 (8.1) |
| Moderate differentiated | 42 (67.7) |
| Poor differentiated | 5 (8.1) |
| Overall survival, median (range) | 722.5 days (30-2,749) |

**Table 4. The outcome of immunohistochemical staining for PTEN in pancreatic cancer tissues**

| Staining intensity | Cases | % |
|---|---|---|
| Negative | 11 | 17.7 |
| Weak | 15 | 24.2 |
| Moderate | 21 | 33.9 |
| Strong | 5 | 8.1 |

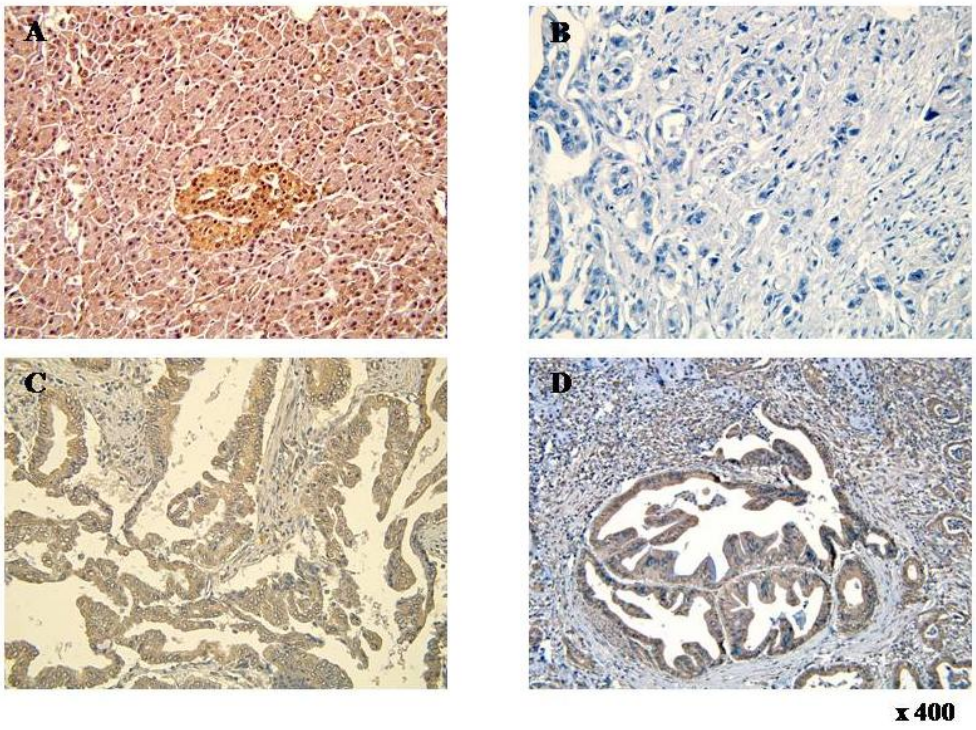


**Figure 3. Immunohistochemical staining of PTEN.**

A. normal islet cells showed moderate expression of PTEN. B. a pancreatic cancer showing negative for PTEN. C and D. 26 showed strong expression of PTEN among 52 samples of pancreatic cancer,.

## 4. Analysis of subcellular location of PTEN

For analysis of the difference in the expression of PTEN between cytoplasm and nucleus, two experiments were planned. The one was for analyzing the difference of PTEN protein expression along with subcellular location. In this western blotting, there was any difference in PTEN expression between normal pancreatic ductal epithelium and 4 pancreatic cancer cell lines (BxPC3, Miapaca-2, Capan-2 and HPAC). In all of the cell lines including the control cell line, cytoplasmic expression of PTEN and phosphorylated PTEN was stronger than the nucleus (Fig. 4). And visual analysis with confocal microscope also revealed similar pattern of PTEN expression to the above experiment (Fig. 5).

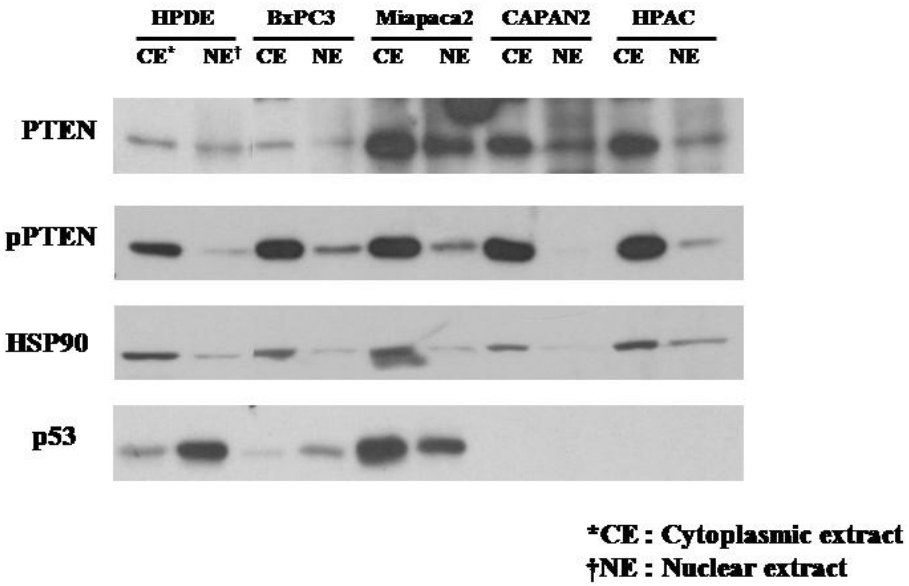


**Figure 4. PTEN expression in both nucleus and cytoplasm**

The PTEN expression in cytoplasm was stronger than nucleus in both normal control (HPDE) and pancreatic cancer cells. HSP90 was used as a cytoplasmic control and p53 was used as a nuclear control protein.

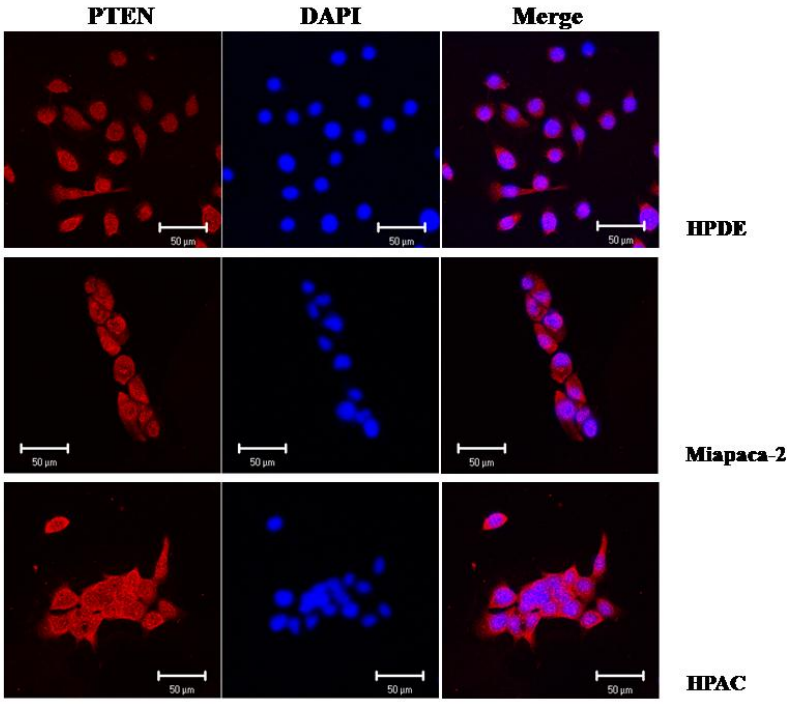


**Figure 5. Visual analysis of subcellular localization of PTEN**

In both the normal control cell (HPDE) and two pancreatic cancer cells, major fraction of PTEN was located in cytoplasm and the remaining was in nucleus.

## 5. The molecular effect of PTEN in pancreatic cancer

With the above evidence that PTEN is not suppressed in most of pancreatic cancer, the tumor suppressive function of PTEN in pancreatic cancer was validated via RNA interference technique. For the establishment of transient PTEN knock-down cell lines, 3 siRNA sequences were validated. Among the three sequences, sequence # showed the most potent effect of PTEN knock-down. And for evaluation of association of PTEN with other genetic mutations including p53 mutational status, 3 pancreatic cancer cell lines were chosen on the base of previously reported mutational status of pancreatic cancer cell lines (Table 5).[28] HPAC cell line is reported to have wild type p53 and Aspc-1 is known to harbor frameshift mutation of p53.

**Table 5. Various mutational statuses of pancreatic cancer cell lines**

| Cell line | Histology | K-ras | p16 | p53 |
|---|---|---|---|---|
| AsPC-1 | Moderate differentiated | G12D (GAT)* | HD† exon 2,3 | HD exon5 |
| MiaPaca-2 | Undifferentiated | G12C (TGT)* | HD† exon 2,3 | R248W (CGT to TTG) |
| BxPC3 | Moderate | No mutation | HD† exon 2,3 | Y220C (TAT to TGT) |
| Panc-1 | Undifferentiated | G12D (GAT)* | HD† exon 2,3 | R273H (CGT to CAT) |
| Capan-1 | well differentiated | G12C (TGT)* | HD† exon 1,2 | Unknown |
| Capan-2 | well differentiated | G12T | HD† exon 1,2 | splicing mutation on codon 125 (ACG to ACT) |
| HPAC | Unknown | G12D | Unknown | Wild |
| CFPAC | Unknown | G12T | Unknown | Unknown |

*Loss of the wild-type allele, †HD, homozygous deletion

With functional knock-down of PTEN gene, the three pancreatic cancer cell lines showed some interesting and differentiated molecular changes (Fig. 6). In HPAC cell lines which harbor wild p53 gene, after blocking PTEN, the AKT and its downstream signals were activated as expected. However, in the other cell lines with mutant p53, the blockage of PTEN showed unexpected phenomena. Especially, activation of AKT via phosphorylation was decreased in p53 null Aspc-1. With inactivation of AKT, normally inactivated GSK3 was released to activate p21.

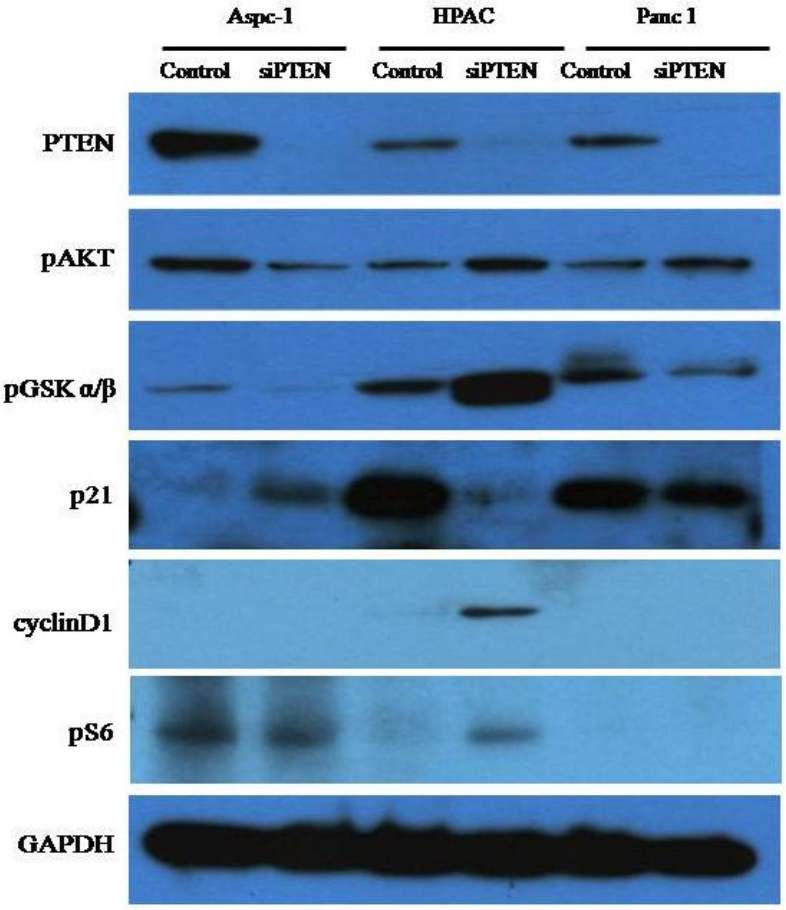


**Figure 6. PTEN has some oncogenic properties in condition of deranged p53 function.**

In condition of PTEN knock-down with siRNA, pancreatic cancer cells with p53 mutations (Aspc-1, Panc-1) showed inactivation of AKT and thus restoration of GSK for p21 activation. On the contrary, pancreatic cancer cell with wild p53 lead expectant activation of AKT signaling.

**6. The effect of PTEN in pancreatic cancer cell prolieferation**

The effects of PTEN knock-down on cell proliferation and apoptosis in pancreatic cancer cells were analyzed. In the experiment of phospho-histone H3 immunofluorescence staining, the mitotic index of mutant p53 pancreatic cancer cell lines (Aspc-1, Panc-1) showed significantly higher in intact PTEN control group than in PTEN knock-down group (Fig. 7). On the other hand, HPAC cells with wild p53, the mitotic index of PTEN knock-down group was significantly increased compared with intact PTEN group.

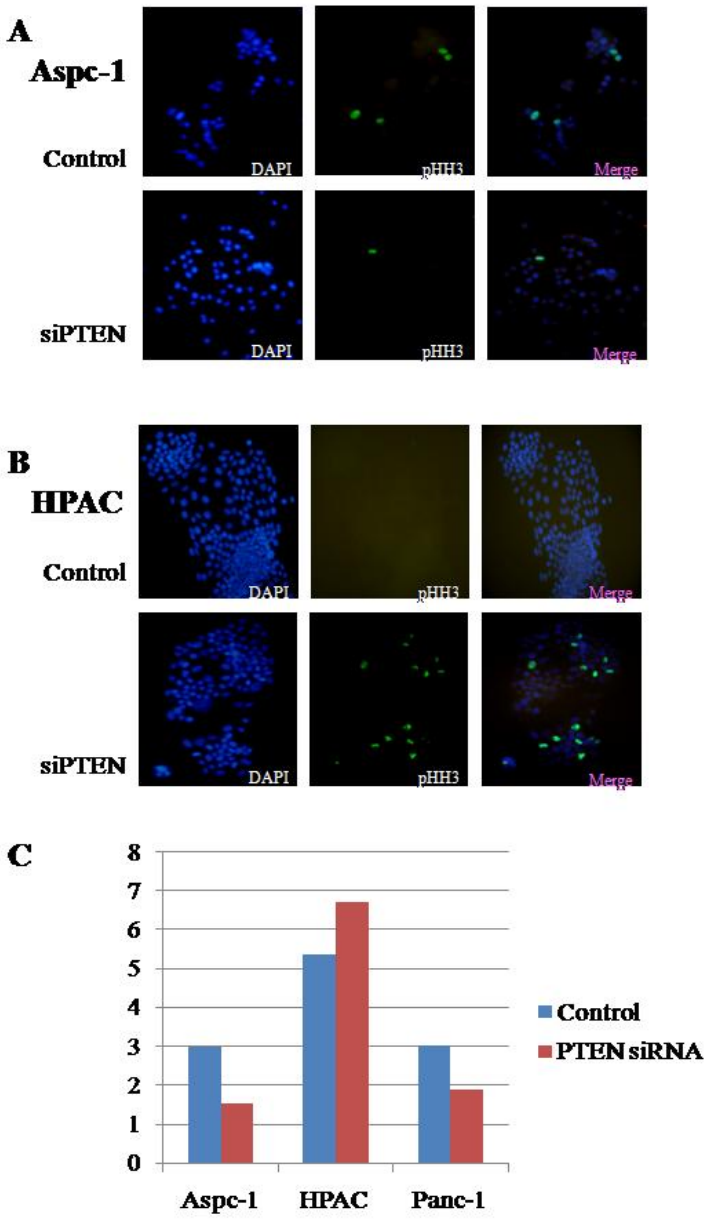


**Figure 7. The effect of PTEN in pancreatic cancer cell proliferation.**

A. In pancreatic cancer cell harboring mutant p53, knock-down of PTEN with

siRNA decrease the mitosis. B. However, in pancreatic cancer cell with wild p53, the induction of mitosis can be lead by silencing PTEN. C. In mitotic index analysis, HPAC cell lines harboring wild p53 showed increased cell proliferative activity after PTEN knock down with siRNA. On the contrary, mutant p53 cell lines – Aspc-1 and Panc-1 showed decreased cell proliferation after PTEN knock down.

7. The effect of PTEN in apoptosis of pancreatic cancer

In the experiment for validating the apoptotic sensitivity to $H_20_2$, pancreatic cancers with mutant p53 showed increased vulnerability to nonspecific apoptotic stimuli in condition of PTEN knock-out. And the pancreatic cancer cell with wild p53 showed vice versa phenomenon (Fig. 8).

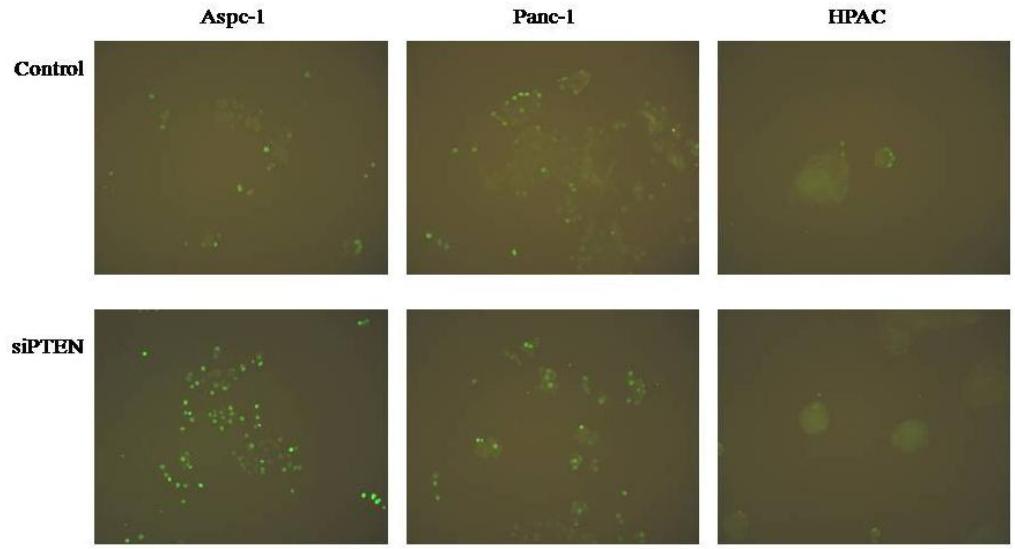


**Figure 8. TUNEL assay of pancreatic cancer cell lines.**

In condition of mutant p53 (Aspc-1, Panc-1), transient PTEN blockage with siRNA increased vulnerability for nonspecific apoptotic stimulus with $H_20_2$ (200 μM/L). However, HPAC which harbor wild p53 expressed decreased vulnerability for apoptosis when PTEN function was knocked down with siRNA.

## IV. DISCUSSION

Most of studies related PTEN in oncologic field have suggested functional loss of tumor suppressive properties of PTEN contributes carcinogenesis or poor prognosis in various cancers. In pancreatic cancers, a few reported suggested the similar phenomena. Furthermore, transgenic animal model study with pancreas specific knock-out of PTEN showed loss of PTEN in centroacinar cell, which are suggested as a pancreatic stem cell, can lead to the formation of timorous condition. However, there were any definite evidences that support the suppression of PTEN is one of the important molecular mechanism or event for pancreatic cancer development. So, we tried to evaluate the practical situation of PTEN in pancreatic cancer.

First, in the experiment for analysis of the expression of PTEN in pancreatic cancer with the tissue samples obtained from the pancreatic cancer patients, the PTEN protein was increased in most of pancreatic cancer tissue comparing to the nearby noncancerous pancreatic tissues of the same patients. This arguable phenomenon was confirmed by the cell line evaluation. For this analysis, the HPDE cell line, which is immortalized normal human pancreatic ductal epithelial cell and Dr Tsao MS kindly gifted, was used as a control cells. Compared with the control cells, all of the eight commercialized pancreatic cancer cells showed increased expression of PTEN in the level of mRNA and protein. Even though these data were confirmed by repetition at least three times, another study with immunohistochemical staining of pancreatic cancer tissues was performed for the confirmation. Among the all of 52 tissue slides of pancreatic cancer, 41 showed

PTEN protein expression same or stronger than the control intensity of pancreatic islet cells.

With the above evidence that PTEN is not suppressed in most of pancreatic cancer, I hypothesized that overexpressed PTEN protein might not function due to abnormality in subcellular translocation. Recently, PTEN-mediated cell cycle arrest and apoptosis were reported to be compartmentalized. Cytoplasmic PTEN downregulates phosphorylation of AKT and upregulates p27, whereas nuclear PTEN downregulates cyclin D1 and prevent the phosphorylation MAPK. Additionally, nuclear PTEN is reported to be required for cell cycle arrest and cytoplasmic PTEN is required for apoptosis.[29,30] For valiation of abnormality in subcellular localization of PTEN, two experiments were performed. Cytoplasmic and nuclear protein was separated and isolated for checking the difference in PTEN expression according the the subcellular location. In this experiment, any difference in PTEN expression between the control cell-HPDE and cancer cells according to the subcellular location was not found. PTEN was more expressed in the cytoplasm than in the nucleus of all of the cell lines including control cells. Immunofluoresent imaging analysis with confocal microscope was done for further validation. In this exam, the situation of PTEN in pancreatic cancer cells was not different from the control.

With the outcome of the above experiment, I hypothesized that PTEN in pancreatic cancer might have some different functions, rather than the previously well-known tumor suppressive functions in pancreatic cancer. This controversial

oncogenic activity of PTEN was recently reported by Li et al.[31] They showed wild PTEN has a tumor promoting properties by enhancing gain of function mutation of p53. As already known, p53 mutations are so frequent in pancreatic cancer, up to 75%. For further validation, transient PTEN knock-down cell lines with siRNA technique was established. For evaluating the association of PTEN with p53 mutational status, 3 pancreatic cancer cell lines were chosen on the base of mutational status of p53.[28] HPAC cell line is reported to have wild type p53 and Aspc-1 is known to harbor frameshift mutation of p53. The Panc-1 has the same mutation of p53 (R273H) with the glioma cell line of Li et al's experiment.[31]

With functional knock-down of PTEN gene, the three pancreatic cancer cell lines showed some interesting and differentiated molecular changes. In HPAC cell lines which harbor wild p53 gene, after blocking PTEN, the AKT and its downstream signals were activated as expected. However, in the other cell lines with mutant p53, the blockage of PTEN showed unexpected phenomena. Especially, activation of AKT via phosphorylation was decreased in p53 null Aspc-1. With inactivation of AKT, normally inactivated GSK3 was released to activate p21.

Even though we could not find a fine molecular link between PTEN and p53 in this study, we found some interesting molecular phenomenon supporting our hypothesis. In cell proliferation analysis, PTEN knock down condition can lead to the inhibitory activity of cell proliferation if combined with p53 mutation. In other words, wild PTEN can have oncogenic activity in the situation of p53 mutation. However, PTEN showed the predicted tumor suppressive activity in combination of

wild p53. Furthermore, in mutated condition of p53, PTEN showed antiapoptotic activitiy.

## V. CONCLUSION

To be summarized, PTEN activity is not suppressed in most of pancreatic cancer, which is contrary to the previous studies. And in condition of gain of function mutation or deletion of p53, PTEN can show unpredicted tumor promoting activity in pancreatic cancer. Actually, this bidirectional activity, so called "antagnostic duality" was reported in some of the known tumor suppressor gene including p21.[31,32] Especially, Li Y et al[31] showed PTEN can have the tumor promoting activity in combination of the gain of function mutation of p53, which is supported by our study. They showed the dependency of the tumor promoting effect of PTEN enhanced mutant p53 protein levels via inhibition of mutant p53 degradation by Mdm2 and possibly also via direct protein binding. Even though the fine molecular mechanism between PTEN and p53 need to be further validated, this study can shed a light for the unexpected oncogenic activity of PTEN in pancreatic cancer. Considering the current paradigm of anti-cancer treatment is shifting to target the specific tumor associated pathway including PTEN pathway, this experimental outcome should be considered for the therapeutic strategies that aim at manipulating PTEN or p53 in human pancreatic cancer.

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**ABSTRACT (IN KOREAN)**

췌장암 발암과정에 있어 PTEN 유전자의 역할 규명 및 치료적 접근

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방 승 민

PTEN은 인체에 발생하는 악성 종양에서 흔하게 돌연변이가 발생하는 종양억제유전자로 알려져 있다. 최근에는 조혈모세포의 유지를 위해 필수적이며, PTEN유전자의 결손이나 돌연변이를 통한 기능 소실은 백혈병을 유발하는데 중요한 역할을 하는 것이 규명되었다. 그러나 췌장암에서 PTEN유전자의 기능소실을 유발하는 돌연변이나 결손은 매우 드문 것으로 보고되고 있다. 물론, PTEN의 췌장 발생시 선택적 결손을 유발시킨 형질전환백서 실험에서 췌장 선방세포의 팽창과, 췌장내 화생관 (metaplastic duct) 의 출현이 나타나는 것이 확인 되었고, 일부에서 췌장 선암이 발생하는 것을 보고하였으나, 이는 실제 췌장암 환자에서 PTEN의 돌연변이가 매우 드문 것임을 감안한다면 생리적인

현상으로 췌장암의 발암과정에 PTEN의 역할이 중요하다는 것을 대변하기에는 논란이 있을 수 있다. 이에 연구자는 실제 췌장암에서 PTEN과 이와 관련된 분자생물학적 현상이 어떠한가를 알아보고자 하였다.

먼저 수술로 절제된 조직을 확보한 12명의 췌장암 환자의 췌장암 조직 및 주변 정상조직을 통한 PTEN단백 발현분석에서 75%의 환자에서 췌장암 조직 내 PTEN단백 발현이 주변 정상조직에 비해 증가되어 있음을 확인하였다. 또한 8개의 췌장암 세포주 및 불멸화된 정상 췌관 상피 세포주 (HPDE)를 통한 PTEN단백 및 mRNA발현 실험에서도 정상 HPDE세포에 비해 8개의 췌장암세포주의 PTEN단백 및 mRNA발현이 증가되어 있음을 확인하였다. 이러한 논란의 소지가 있는 실험결과를 재차 확인하기 위해 총 52개의 췌장암 조직 슬라이드를 가지고 PTEN에 대한 면역형광염색을 시행하였다. PTEN면역형광염색실험에서도 총 41개 (81.3%)의 슬라이드에서 내부 양성 표지 강도인 췌도세포내 PTEN 염색 강도와 동일하거나 강한 염색강도를 나타냄을 확인하였다. 이를 토대로 PTEN유전자가 췌장암에서 종양억제유전자의 기능보다는 오히려 종양촉진유전자로써의 기능을 할 수도 있을 것이라는 가설을 세우고 이를 증명하기로 하였다. 이를 위해 3개의 췌장암세포주 (HPAC, Aspc-1, Panc-1)에 대해 siRNA기법을 통한 PTEN유전자의 기능억제를 시도하였다. 우선 PTEN의 기능이 억제된 3개의 세포주를 확립하였고, 이를 통한 PTEN 신호체계 분석을 시도하였다. 흥미롭게도, PTEN의 기능을 억제하였을 때,

p53유전자가 정상적인 종양억제유전자로써의 기능을 수행하는 HPAC세포주는 예상한 데로 PTEN에 의해 억제되어 있던 Akt의 인산화를 통한 활성화가 증가되어 세포분열이 증가하고, 세포의 사멸반응이 억제됨을 확인하였다. 그러나, p53유전자의 돌연변이 및 결손을 가진 Aspc-1, Panc-1 세포주는 PTEN의 기능을 억제하였을 때, Akt의 활성화에는 큰 영향이 없이, GSK3의 활성화가 감소하고, p21의 발현이 증가되는 종양억제 효과가 나타났다. 또한 PTEN의 기능을 억제하였을 때 대조군에 비해 세포분열능이 감소하였고, $H_2O_2$에 대한 비특이적 세포사멸반응도 증가하였다. 이러한 현상은 PTEN유전자가 p53유전자의 돌연변이과 같은 특수한 상황에서는 소위 “antagnositc duality”라 하는 종양 억제 기능이 아닌 종양 촉진 기능을 보일수 있음을 시사하는 것이라 하겠다. 그러나 본 연구를 통해 PTEN유전자와 p53사이의 분자생물학적 상관관계에 대한 연구가 수행되지 않았으므로, 향후 이에 대한 추가적인 연구가 필요할 것이다.

핵심되는 말: 췌장암, PTEN, p53, antagnostic duality