Anti-tumor effect and radiation response modification by tyrosine kinase inhibitor imatinib mesylate on pancreatic cancer cells

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Anti-tumor effect and radiation response modification by tyrosine kinase inhibitor imatinib mesylate on pancreatic cancer cells

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The Doctoral Dissertation submitted to the Department of Medicine, the Graduate School, Yonsei University in partial fulfillment of the requirements for the degree of Doctor of Philosophy of Medical Science

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Finally, I am dedicating this thesis to my parents.

Hye Won Chung

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Abstract

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Pancreatic cancer has a very poor prognosis with less than 20% of 1-year survival. Conventional oncologic strategies, such as chemotherapy and radiotherapy are resistant. Imatinib mesylate (STI-571, Gleevec®, Glivec®), is a selective signal transduction inhibitor of the c-Kit, Bcr-Abl, and platelet-derived growth-factor receptor (PDGFR) tyrosine kinases. Recently, target-therapy concept is prevalent and this drug has been actively investigated. Here, we tried imatinib mesylate to pancreatic cancer cells and investigated the anti-tumor effect and its mechanism. And, for clinical usefulness, we evaluated the radiosensitizing effect of imatinib mesylate to pancreatic cancer cells. In result, imatinib mesylate inhibited the growth of pancreatic cancer cells in dose and time-dependent manners with the range of 18-47μM of IC_{50} after 48 hours and 15-39μM after 72 hours exposure. Imatinib mesylate caused the programmed cell death through the interruption of function of c-Kit or PDGF receptor and its downstream proteins PI3K/Akt and PLCγ. Slight delay of S-phase progression was

also observed. For the evaluation of radiosensitizing effect, pancreatic cancer cells was incubated for 1 hour with 5, 10, 20μM of imatinib mesylate and 1, 3, 6 Gy of irradiation was delivered. Clonogenic survival assay revealed the novel radiosensitizing effect of imatinib mesylate in pancreatic cancer cells, and suggested the potential clinical usefulness.

These results suggest a promising therapeutic impact of low dose imatinib mesylate in combination with radiation in pancreatic cancer.

Key words: pancreatic cancer, imatinib mesylate, c-Kit, PDGFR, apoptosis, radiosensitizing effect

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I. INTRODUCTION

Imatinib mesylate (STI-571, Gleevec®, Glivec® [Novartis, Basel, Switzerland]) is a selective signal transduction inhibitor of the c-Kit, Bcr-Abl, and platelet-derived growth-factor receptor (PDGFR) tyrosine kinases. Because this compound inhibits Abl kinase activity[1] at concentrations that are otherwise nontoxic to normal hematopoietic cells[2] and has shown impressive activity in early-phase clinical trials in patients with chronic myelogenous leukemia (CML),[3] this compound has been actively used in the treatment of CML.[4-8]

Recently, the spectrum of diseases for clinical trials of this compound is growing based on its ability to inhibit other tyrosine kinases (c-Kit, and PDGFR). For example, based on the identification of c-Kit mutations in gastrointestinal stromal tumors (GIST) and the ability of imatinib mesylate

to inhibit c-Kit, clinical trials of this compound were begun for patients with unresectable GIST.[9] Actually, many clinical trials has shown remarkable responses in patients with GIST.[10-12] The Kit tyrosine kinase is also expressed in 70% of small cell lung cancers (SCLC), and imatinib mesylate has been demonstrated to have cytostatic effect to SCLC.[13]

c-Kit, a 145 kD transmembrane glycoprotein, is a product of the c-Kit gene and is considered as a receptor for stem cell factor (SCF).[14] Normal functional activity of the c-Kit is absolutely essential for maintenance of normal hematopoiesis, melanogenesis, gametogenesis, and growth and differentiation of mast cells and interstitial cell of Cajal. In other hands, overexpression or constitutive activation of c-Kit is known to be associated with various malignancies as soft tissue sarcomas, GIST, SCLC et al.[10-13] Proliferation of tumor cell growth mediated by c-Kit occurs either by a specific mutation of the c-Kit that results in ligand-independent activation or by autocrine stimulation of the receptor.[15,16] Therefore imatinib mesylate, a potent inhibitor of c-Kit kinase, may be useful in treating various tumors that are partly or completely dependent on c-Kit for proliferation or survival.[10-13,15,17] Relationship between pancreatic cancer and c-Kit expression is not fully understood. However, Esposito et al. reported that human pancreatic cancer overexpress c-Kit.[18] And our preliminary data showing overexpression of c-Kit in BOP-induced pancreatic carcinogenesis model of Syrian Golden hamster may suggest the potential role of imatinib mesylate in the treatment of human pancreatic cancer.

Blockage of PDGFR by imatinib mesylate in various malignancies, such as glioblastoma, is also of interest.[19,20] PDGF is one of the first identified polypeptide growth factors that signals through a cell surface tyrosine kinase receptor to stimulate various cellular functions, including cell growth, proliferation, and differentiation. When the PDGF binds to the extracellular domain of receptor, the receptor undergoes dimerization and autophosphorylation with tyrosine kinase activation. The biological role of PDGF signaling can vary from autocrine stimulation of cancer cell growth to subtler paracrine interactions involving adjacent stroma and angiogenesis. In pancreatic cancers, the mRNA level of PDGFR-alpha and PDGFR-beta is markedly increased comparing with normal pancreas, and both PDGF and PDGFR are over-expressed in cancer cells.[21]

Pancreatic cancer is a devastating disease characterized by low responsiveness to conventional oncological strategies, such as chemotherapy, radiotherapy or systemic use of monoclonal antibodies.[22] One-year survival after diagnosis is less than 20%. Only radical resection provides the chance for cure.[23] However, resectable cases are only 10-15% of pancreatic cancers.[24] Furthermore, most patients develop recurrent disease after surgical resection. Although gemcitabine was the first cytotoxic agent to show some efficacy in reducing symptoms and prolonging survival, the prognosis of patients with pancreatic adenocarcinoma remains dismal.[25]

Molecular targeted treatment based on the advance in new technology and cancer biology has been challenged. Recently various molecular

biologic characteristics of pancreatic cancer has been revealed by novel techniques such as DNA chip and proteomic analysis.[26] Human pancreatic cancer has been known to overexpress a number of important tyrosine kinase growth factor receptors and their ligands.[27,28] These growth factors act in an autocrine and/or paracrine manner to stimulate cancer cell growth. Binding of growth factors to their receptors results in receptor auto-phosphorylation and subsequent signal transduction via an array of different molecules. Thus, increasing attention has been directed towards the role of growth factors in the treatment of pancreatic cancers.

Protein kinases play a crucial role in signal transduction as well as in cellular proliferation, differentiation, and various regulatory mechanisms, and disregulation of these signaling pathways is frequently observed in malignancies. Therefore, the inhibition of tyrosine kinases might provide new tools in cancer treatment. Because a number of tyrosine kinase growth factor receptors and their ligands are overexpressed in pancreatic cancer, selective tyrosine kinase inhibitor might provide a new challenge for the patients with pancreatic cancer. Human pancreatic cancer overexpresses PDGFR and c-Kit,[18,21] the anti-tumor effect of imatinib mesylate may be expected in pancreatic cancer.

Therefore, we investigated the anti-tumor effect of imatinib mesylate in pancreatic cancer cells cultured in vitro and evaluated the possibility of clinical use of imatinib mesylate for the patients with pancreatic cancer.

II. MATERIALS AND METHODS

1. Cell lines and culture

Four pancreatic cancer cell lines were used in current study as follows: BxPC-3, HPAC, MiaPaCa-2, and Panc-1. BxPC-3 was cultured in RPMI-1640 medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco-BRL), containing 100 IU/ml of penicillin and 100μg/ml of streptomycin. HPAC was cultured in DMEM/F12 (Gibco-BRL), and MiaPaCa-2 and Panc-1 in DMEM (Gibco-BRL). c-Kit and PDGFR negative normal cell line, NIH3T3, was cultured in DMEM (Gibco-BRL). All cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA).

2. Antibodies and chemical

Anti-c-Kit antibody, anti-stem cell factor (SCF) antibody, anti-PDGFR (α or β) antibody, anti *p*-PDGFR (α or β), antibody, anti-total Akt antibody, anti-*p* Akt antibody, anti *p*-erk antibody, anti-caspase 3 antibody, anti-Bcl-2 antibody, and anti-BAX antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-*p*-c-kit antibody was purchased from Abcam Inc. (Abcam Inc., Cambridge, USA). For secondary antibody, anti-rabbit, anti-goat, and anti-mouse IgG-HPRO peroxidase-linked antibodies were purchased from Santa Cruz Inc., and ECL immunoblotting

detection reagents from Amersham Biosciences Inc. (Amersham Life Science, Arlington Heights, IL, USA). Annexin V-FITC conjugate was purchased from Invitrogen Co. (Invitrogen Co., California, USA).

Imatinib mesylate was kindly provided by Novartis Pharma AG (Novartis Basel, Switzerland). It is a 2-phenylaminopyrimisine derivative small molecule. Its molecular weight (MW) is 590. Figure 1 shows its chemical structure. Stock solutions at 10 mM were prepared with 100μl of filtered DMSO and frozen at -20^{o}C until use.

3. Immunohistochemistry

Immunohistochemical staining was performed by the avidin-biotin peroxidase complex method (Dako Co., Hamburg, Germany). Tissues were fixed in 4% paraformaldehyde and embedded in paraffin, and cut into 5 mm. The sections were deparaffinized in xylene for 20 min ×2, hydrated in 100% ethanol for 5 min ×3, 90%, 80%, 70%, 50%, and 30% ethanol

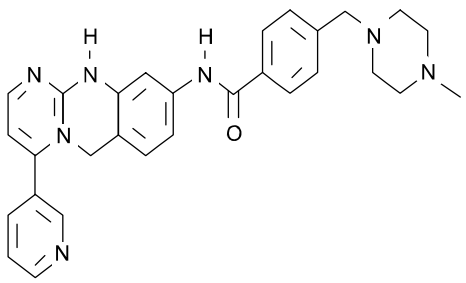

Figure 1. Chemical structure of imatinib mesylate, a 2-phenylaminopyrimisine derivative (STI-571, Gleevec®, Glivec®)

for one minute each other, and finally, washed in phosphate-buffered saline (PBS). The slides were incubated with 3% H_2O_2 in methanol for 20 min to block endogenous peroxidase. Antigen retrieval was performed by pressure cooking slides in 10 mM citrate buffer, pH 6.0, and then leaving them to cool at room temperature for 20 min. The slides were then incubated overnight at 4^{o}C with a primary antibody, followed by 1-hour incubation with biotin-labeled mouse anti-rabbit IgG antibody and 1 hour incubation with horseradish peroxidase-conjugated streptavidin. Among these three incubations, the cells were rinsed with calcium and magnesium-free PBS to remove any unbound antibodies. The slides were then subjected to enzymatic reaction by incubation with chromogen for 5 min, followed by washing with PBS and counterstaining with hematoxylin. Cells were then observed under a microscope. Each section was scored according to the intensity of labeling, from 0 (no staining) to 3 (strong staining). Labeling of any intensity in $>$10% of the cells were considered to be expression. Sections without the primary antibody were used as negative controls.

4. Western blot analysis

Cells were collected with a cell scraper and lysed in NP-40 lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 0.5% NP-40, 50 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 25μg/mL leupeptin, 25 μg/mL aprotinin). The supernatants were cleared by centrifugation at 4^{o}C.

Equal 30μg of cell lysates were separated on SDS-polyacrylamide gels

and electroblotted onto nitrocellulose membranes. Membranes were then incubated in blocking solution (5% nonfat-milk in 20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20) (TBS-T), followed by incubation with the indicated primary antibodies at 4^{o}C overnight. The membranes were then washed in TBS-T and incubated at room temperature with IgG-HPRO peroxidase-linked secondary antibodies (anti-rabbit, anti-goat or anti-mouse IgG; (Santa Cruz, CA, USA) for 1 hour. The signals were detected using the enhanced chemiluminance assay (Amersham Life Science Inc., Arlington Heights, IL, USA), according to the manufacturer's instructions.

5. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay

The anti-proliferative effect of imatinib mesylate on human pancreatic cancer cells was evaluated using MTT assay.[29] MTT is a measure of mitochondrial dehydrogenase activity within the cell and thereby provides an indication of cellular proliferation status. Briefly, 4×10^3 cells/well of pancreatic cancer cells were seeded into 96-well plates with 100 l of the growth medium and incubated for 24 hour in medium. After then, they were cultured with growth medium containing imatinib mesylate at 0, 0.01. 0.1, 1, 5, 10, 25, and 50μM for 48 hour or 72 hour at 37^{o}C in 5% CO_2. After exposure of imatinib mesylate to the cells for 48 or 72 hour, 200μl of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, 1 mg/ml) solution was added to each well and cells were incubated for 4

hour at 37°C to allow MTT to form formazan crystals by reacting with metabolically active cells. The formazan crystals were solubilized in 100μl of DMSO after 5-minute incubation at 37°C. ELISA well measured the absorbance of each at 570 nm. The percentage cell growth was calculated by comparison of that of control. The concentration of drug that inhibited cell survival by 50% (IC_{50}) was determined from cell survival plots.

6. Assessment of apoptosis by DAPI staining

Single cell suspensions were washed with 1× PBS, transferred to 1.5-ml microtubes, fixed with 70% ethanol for 20 min at room temperature and washed with 1× PBS. Cells were then treated with DAPI (1μg/ml, Sigma Chemical Co.) for 12 min, and washed again with 1X PBS for 5 min. Cell suspensions were mounted on slide glasses and subjected to fluorescence microscopic examination. (Provis AX70; Olympus Optical Co., Japan). Stained nuclei were visualized under a fluorescence microscope (×200).

7. Annexin V/propidium iodide binding assay using flow cytometry

Apoptosis was assessed using Annexin-V/propidium iodide (PI) staining kit (Annexin Apoptosis Detection Kit I; BD Biosciences, Heidelberg, Germany). To assess the peak apoptotic index, cells were harvested at various intervals after treatment (6-72 hour) and resuspended in PBS. Both adherent and floating cells were harvested for the assay. The cells were then suspended in 1-2 ml of FITC-Annexin V solution. Propidium iodide

was added to a final concentration of 1μg/ml. This was analyzed by flow cytometry (Becton Dickinson, San Jose, CA, USA) using blue light excitation, and green fluorescence of FITC was measured at 530±20 and red fluorescence of was measured at ＞600.

8. Cell cycle analysis by flow cytometry

Control and imatinib mesylate-treated cells were harvested by trypsinization, washed with PBS, then fixed in ice-cold 100% ethanol, and stored at 4°C before DNA analysis. After the removal of ethanol by centrifugation, cells were washed with PBS. After then, cells were stained for total DNA content with a solution containing 5 mg/ml of propidium iodide and 20 mg/ml RNase I in PBS for 30 min at 37°C. The cells were then analyzed with a FACS flow cytometry (Becton Dickinson) for cell cycle distribution and analyzed by Modfit (Verity Software House, Inc., Topsham, ME) for the proportion of cells in sub-G0, G1, S, and G2-M phases of the cell cycle.

9. Analysis of radiosensitizing effect of imatinib mesylate in pancreatic cancer cells

After HPAC cells were plated and incubated for 24 hours, imatinib mesylate was added in concentrations of 5, 10, 20μM. After pre-incubation of imatinib mesylate for 1 hour prior to irradiation, cells were exposed to 1, 3, 6 Gy ionizing radiation using a 60 Co source (γ-cell irradiator, MDS

Nordion. Ottawa, Canada). After irradiation, about 100 cells were plated into 60 mm-dishes. The medium without imatinib mesylate was renewed at every 3 days. Clonogenic survival assay was performed after 14 days incubation with 10% FBS contained medium. Colonies were stained with crystal violet in 100% ethanol and manually counted. Colonies consisting of 50 cells or more were scored.

The clonogenic survival curve for each condition was fitted to the linear-quadratic model. The plating efficiency (PE) means the percentage of cells seeded that grow into colonies under a specific culture condition of a given cell line. Therefore, the plating efficiency was defined as the percentage of cells plated that formed colonies in the un-irradiated dishes. The survival fraction, expressed as a function of irradiation, was calculated as follows: survival fraction=number of colonies formed/umber of cells seeded×plating efficiency (PE)/100. PE is defined as 'the percentage of % of cells plated that formed colonies in the un-irradiated dish.'

To demonstrate the synergistic effect of imatinib mesylate with radiation, sensitizer enhancement ratios (SER) were calculated. SER is defined as 'dose of radiation required to kill χ% of control cells/dose of radiation required to kill χ% of the cells exposed to the drug.' 'χ' means to be 37, and 50% survival of cells. SER $>$1.1 was considered statistically significant radiosensitization. The experiment was repeated for a total of three times and means value was used for calculation of survival fractions.[30-32]

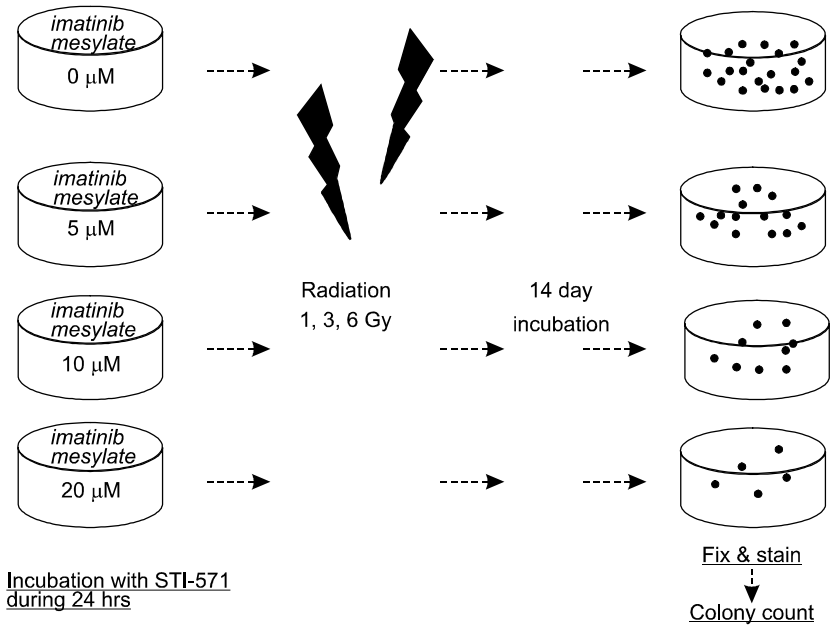

Figure 2. Analysis of radiosensitizing effect of imatinib mesylate in HPAC cells. HPAC cells were plated and incubated for 24 hours. After incubation, imatinib mesylate was added in concentrations of 5, 10, 20 μM. After pre-incubation of imatinib mesylate for 1 hour, irradiation was delivered by the doses of 1, 3, 6 Gy. After cells were replated, clonogenic survival was analyzed after 14 days. Colonies were stained with crystal violet in 100% ethanol and manually counted.

10. Statistical analysis

The statistical software program used was the Statistical Package for the Social Sciences (SPSS/PC+ 10.0, Chicago, IL, USA). A standard t test analysis was used to test for statistical significance.[33] The clonogenic survival analysis data were fitted to the linear-quadratic model according to the rules suggested by Fertil and Malaise.[34] All assays were repeated at least three times. Data are expressed as means with corresponding standard deviations. Significance was accepted at a *p*-value of less than 0.05.

III. RESULTS

1. Human pancreatic cancer cells overexpress the c-Kit and PDGFRα,β; immunohistochemistry and western blot

To determine whether c-Kit and PDGFR are overexpressed in pancreatic cancer cells, immunostaining for c-Kit, PDGFR α, and PDGFR β was performed using paraffin-embedded cancer tissues. The immunostaining was considered to be positive when intensity score is ≥ 1. Those cases with only faint immunostaining were regarded as negative (intensity score < 1).

We examined the c-Kit, PDGFR α, and PDGFR β expression in total 33 cases with the pancreatic ductal adenocarcinoma. As shown in figure 3A, c-Kit was widely expressed in the cytoplasm and/or on the plasma membrane of pancreatic cancer cells. To a lesser extent, it was also expressed in PanIN lesions. However, no expression was noted in normal pancreatic tissues. Among 33 cases, the expression rate of c-Kit was 69.7% (23/33) and the score of intensity was 3 in ductal adenocarcinoma cells. PanINs were observed in 72.7% (24/33) concomitantly in 33 pancreatic adenocarcinoma cases. The expression rate of c-Kit was 83.3% (20/24) in PanINs, and the score of intensity was 1 to 2. The intensity was increased correspondent to the degree of PanINs. In most PanIN-1 lesions, the score of intensity was 1. In other hands, in PanIN-2 or -3 lesions, it was 2 to 3.

PDGFR α (Fig. 3B) and β (Fig. 3C) were expressed in cytoplasm and/or

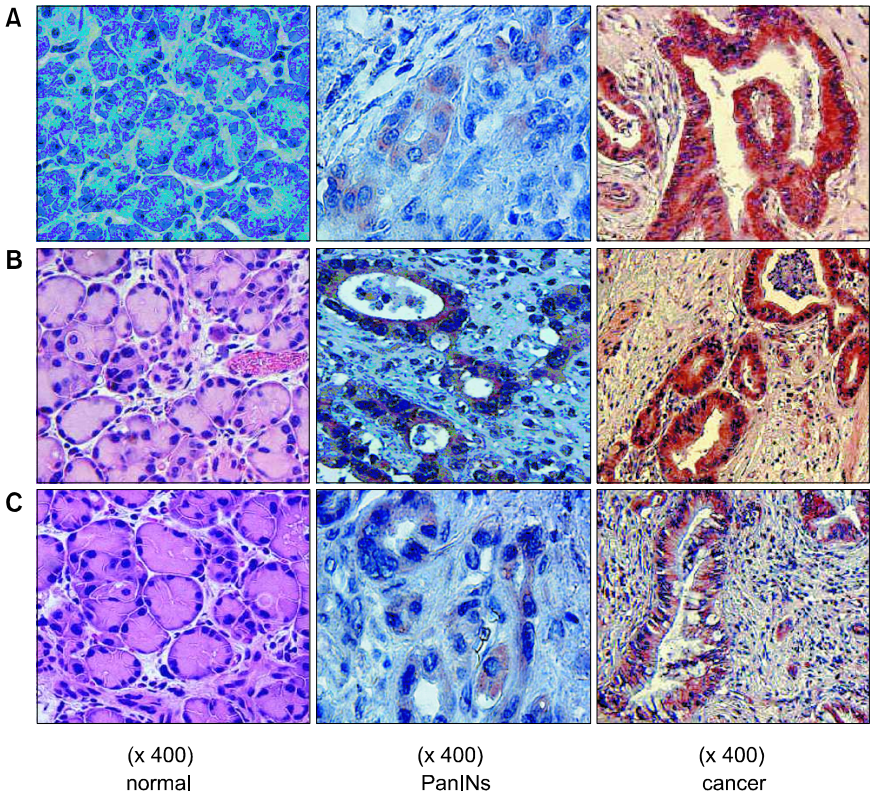

Figure 3. Expression of c-Kit (A), PDGFR α (B), and PDGFR β (C) in human pancreatic cancer tissue. Immunohistochemistry was performed for human pancreatic cancer tissues including adjacent normal pancreatic tissues and PanINs using anti-c-Kit antibody, anti-PDGFR α antibody, and anti-PDGFR β antibody, respectively. ×400).

on the cell membrane of pancreatic cancer cells and PanIN lesions similar to c-Kit, and were not expressed in normal pancreatic tissues. Among 33 cases which were examined, the expression rate of PDGFR α was 75.8% (25/33) and 78.8% (26/33) in PDGFR β. Score of intensity was 3 in ductal adenocarcinoma cell and 1 to 2 in PanINs in both PDGFR α and PDGFR β.

Western blot analysis of pancreatic cancer cell lines with an anti-c-Kit antibody showed that c-Kit was strongly expressed in four pancreatic cancer cell lines (BxPC-3, HPAC, MiaPaCa-2, and Panc-1) with different expression levels. However, as a negative control, NIH3T3 cells were not expressed the c-Kit. Similar results were observed in immunoblotting for PDGFR α and PDGFR β. However, PDGFR α and PDGFR β were not expressed in Panc-1 cells (Fig. 4).

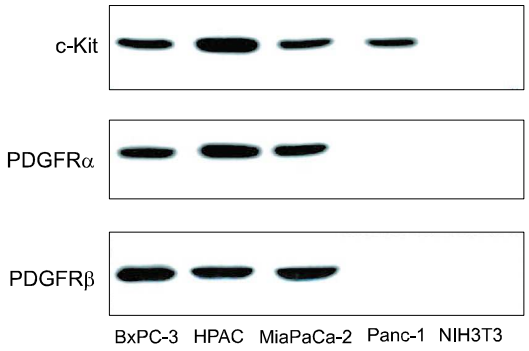

Figure 4. The expression of c-Kit and PDGFRα,β proteins were assessed by western blot analysis. c-Kit and PDGFRα, β proteins were expressed in pancreatic cancer cell lines, BxPC-3, HPAC, MiaPa Ca-2, and Panc-1. c-Kit and PDGFR were not expressed in normal cell lines, NIH 3T3.

2. Cytotoxicity assay of imatinib mesylate in pancreatic cancer cells

The anti-proliferative effect of imatinib mesylate was analyzed by MTT assay using four pancreatic cancer cells, BxPC-3, HPAC, MiaPaCa-2 and Panc-1. The IC_{50} was defined as the concentration causing a 50% decrease compared to untreated control. As seen from table 1 and figure 5, imatinib mesylate inhibited the growth of pancreatic cancer cells in dose and time-dependent manners in all tested pancreatic cancer cell lines, with IC_{50} in the range of 18-47μM (10% FCS) after 48 hours and 15-39μM (10% FCS) after 72 hours. However, the proliferation inhibitory effect of imatinib mesylate varied according to the cell line. BxPC-3 cell line was the most sensitive (15μM at 72 hr), and Panc-1 was the most resistant (37μM at 72 hr). In other hands, IC_{50} for NIH3T3 cells was beyond 50μM in both 48 and 72 hours. However, the IC_{50} for pancreatic cancer cells appeared to be higher than that of other reported cancer cells, such as small cell lung cancer cells (2-5μM), colon cancer cells (6μM), gastric stromal cells (1-2

Table 1. Value of IC_{50} by MTT assay

IC_{50}	BxPC-3	HPAC	MiaPaCa-2	Panc-1	NIH3T3
48 hrs* (μM)	18	22	23	41	71
72 hrs (μM)	15	17	19	30	65

*hrs; hours. Cells were cultured in complete medium with 10% FBS and 1% antibiotics.

μM), or CML cells (0.5-1μM), and out of the range of the safe plasma concentration under clinical conditions.[3-8,13,54]

Cells were cultured in complete medium with 10% FBS and 1% antibiotics.

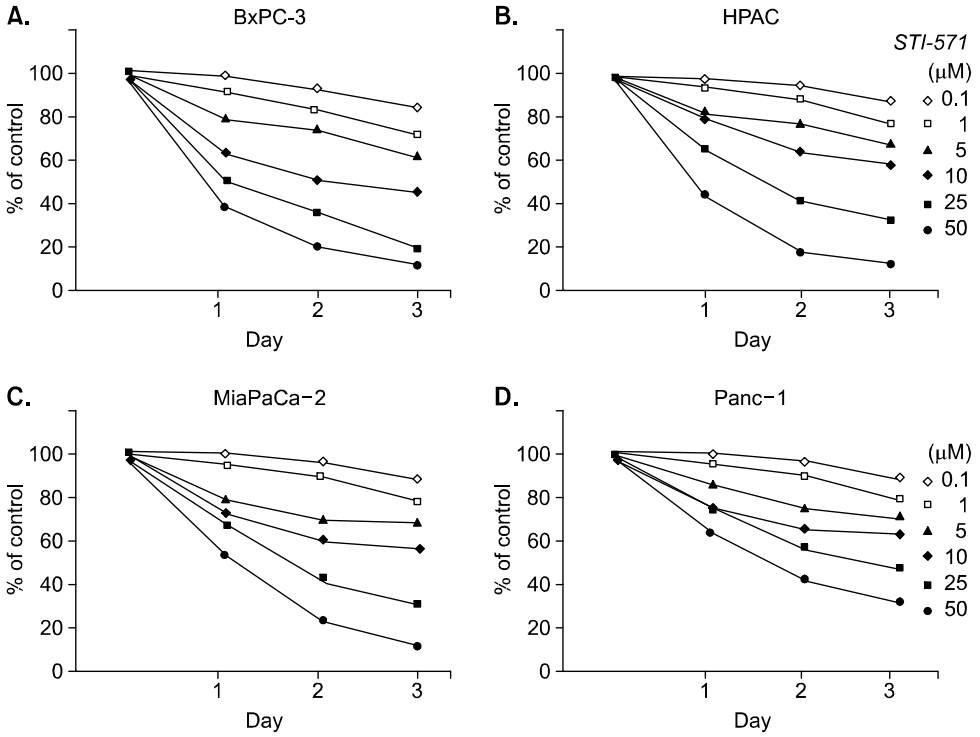

Figure 5. Cytotoxicity of imatinib mesylate was assessed by MTT assay. The graph shows the results of MTT assay in four pancreatic cancer cell lines, BxPC-3, HPAC, MiaPaCa-2, and Panc-1. Cells were cultured in complete medium with 10% FBS and 1% antibiotics. The dose-dependent growth inhibitory effect of imatinib mesylate on four pancreatic cancer cell lines is demonstrated.

3. Mechanisms of cytotoxicity of imatinib mesylate

We evaluated the cellular mechanism of cytotoxicity of imatinib mesylate in pancreatic cancer cells. For these evaluations, we used two pancreatic cell lines (BxPC-3, HPAC), which were relatively sensitive to imatinib mesylate based on MTT assay, and NIH3T3 cells were used as a control.

A. Cell morphology study by DAPI staining; detection of apoptotic body

We observed the cellular and nuclear morphologic changes of imatinib mesylate treated cells with a fluorescent DNA-binding agent, DAPI at 48 hours. BxPC-3 and HPAC cells treated with 20μM of imatinib mesylate displayed typical morphological features of cells in apoptotic process (apoptotic body): cell swelling, cell shrinkage, rounded up, detachment of the cells from the plate (floating), and highly condensed chromatin or nuclear fragmentation. In contrast, NIH3T3 cells treated with 20μM imatinib mesylate displayed similar morphology to untreated control cells (Fig. 6).

For quantitative analysis of apoptosis, we counted the number and calculated the proportion of apoptotic bodies in each group. In imatinib mesylate treated BxPC-3 and HPAC cells, approximate 3-4-fold increment of apoptotic cells were measured compared with untreated cells ($P < 0.05$). In other hands, in NIH3T3, the number of observed apoptotic cells showed

no significant difference ($P > 0.05$) (Fig. 7).

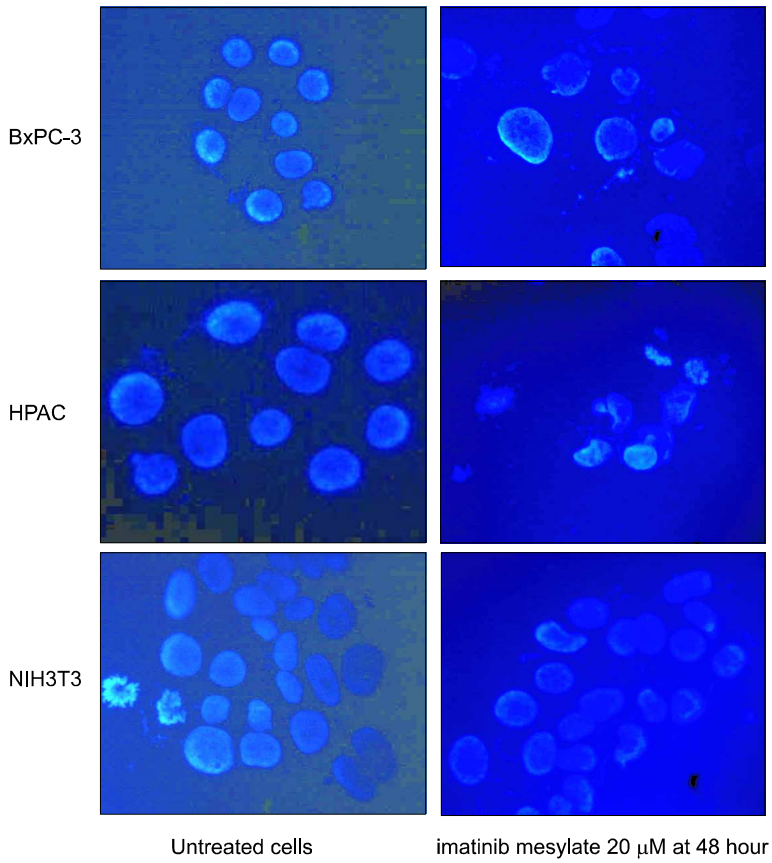

Figure 6. Detection of apoptotic bodies by DAPI staining in imatinib mesylate treated pancreatic cancer cells and NIH3T3 cells. Apoptotic bodies are observed in imatinib mesylate-treated pancreatic cancer cells whereas minimal morphologic change was observed in NIH3T3 cells after imatinib mesylate treatment.

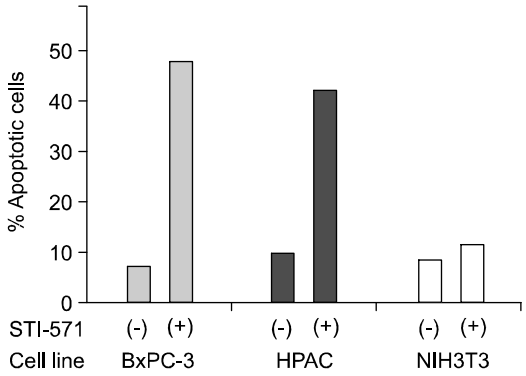

Figure 7. Quantitative analysis of apoptosis observed by DAPI staining. Difference between treated and untreated group is statistically significant in BxPC-3 and HPAC ($P < 0.05$).

B. Cell cycle distribution analyzed by Fluorescence-Activated Cell-Sorting analysis (FACS) in imatinib mesylate treated BxPC-3 and HPAC cells

The cell cycle distribution of imatinib mesylate treated BxPC-3 and HPAC cells was analyzed by FACS; they were treated with 20μM of imatinib mesylate and analyzed after 24, 48 and 72 hours of incubation. After 24 and 48 hours, minimal S-phase delay was observed. Between 48 and 72 h, the subdiploic fraction suggesting apoptotic cells was increased from 24.1% to 33.52%. Thus, we conferred that there was no significant change on the cell cycle progression in the presence of 20μM of imatinib mesylate. However, subdiploic cell population suggesting apoptotic cells was increased with incubation times compared with untreated cells (Fig. 8).

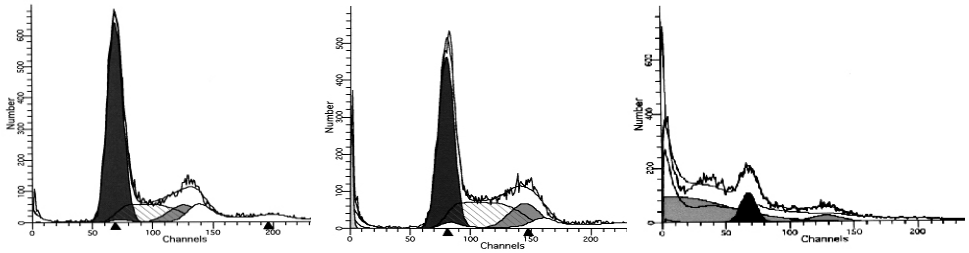

Figure 8. Cell cycle distribution in imatinib mesylate-treated HPAC cells. Concentration of imatinib mesylate was 20 μM (IC_{50}). There was not significant change on the cell cycle distribution in imatinib mesylate-treated HPAC cells.

C. Demonstration of apoptosis in imatinib mesylate-treated pancreatic cancer cells by annexin V/PI analysis

According to previous two experiments, the cellular mechanism of cytotoxicity of imatinib mesylate was suggested to be programmed cell. Thus, we performed simultaneous annexin V/PI analysis to determine whether the inhibitory effect of imatinib mesylate was caused by the induction of apoptosis as our previous results.

Two imatinib mesylate sensitive pancreatic cancer cell lines, BxPC-3 and HPAC cells were used in this experiment. NIH3T3 was used for normal control group. They were cultured in complete medium with 10% FBS, and treated with IC_{50} concentrations of imatinib mesylate (20μM) for 72 hours.

Cells were collected after 48 and 72 hours of exposure to IC_{50} of imatinib mesylate (20μM) and were washed and stained with Annexin-V-FITC (apoptotic death) or PI (necrotic death) according to the manufacturer's instructions (Roche, Mannheim, Germany).

Figure 9 demonstrates the results of annexin-V/PI analysis. Right lower quarter area (annexin-V positive area) means early apoptosis portion, (apoptosis events was observed only nucleus) and right upper quarter area (simultaneous annexin-V/PI positive area) means late apoptosis including non-viable non-adherent cells through induction of anoikis (cellular apoptosis). Left upper quarter area (PI positive area) means necrotic cell death. As shown from the results of Annexin-V/PI analysis for BxPC-3

Table 2. The proprotion of early and late apoptotic cells after treatment of imatinib mesylate observed by annexin V/PI staining

		Control	48 hours	72 hours
BxPC-3	Live	90.08%	48.86%	38.75%
	Early apoptosis	5.38%	28.26%	37.72%
	Late apoptosis	4.23%	21.95%	21.63%
HPAC	Live	87.18%	52.22%	45.26%
	Early apoptosis	5.14%	25.75%	33.30%
	Late apoptosis	5.13%	15.79%	21.52%
NIH3T3	Live	94.36%	87.84%	81.13%
	Early apoptosis	3.41%	2.73%	7.76%
	Late apoptosis	1.88%	8.55%	7.21%

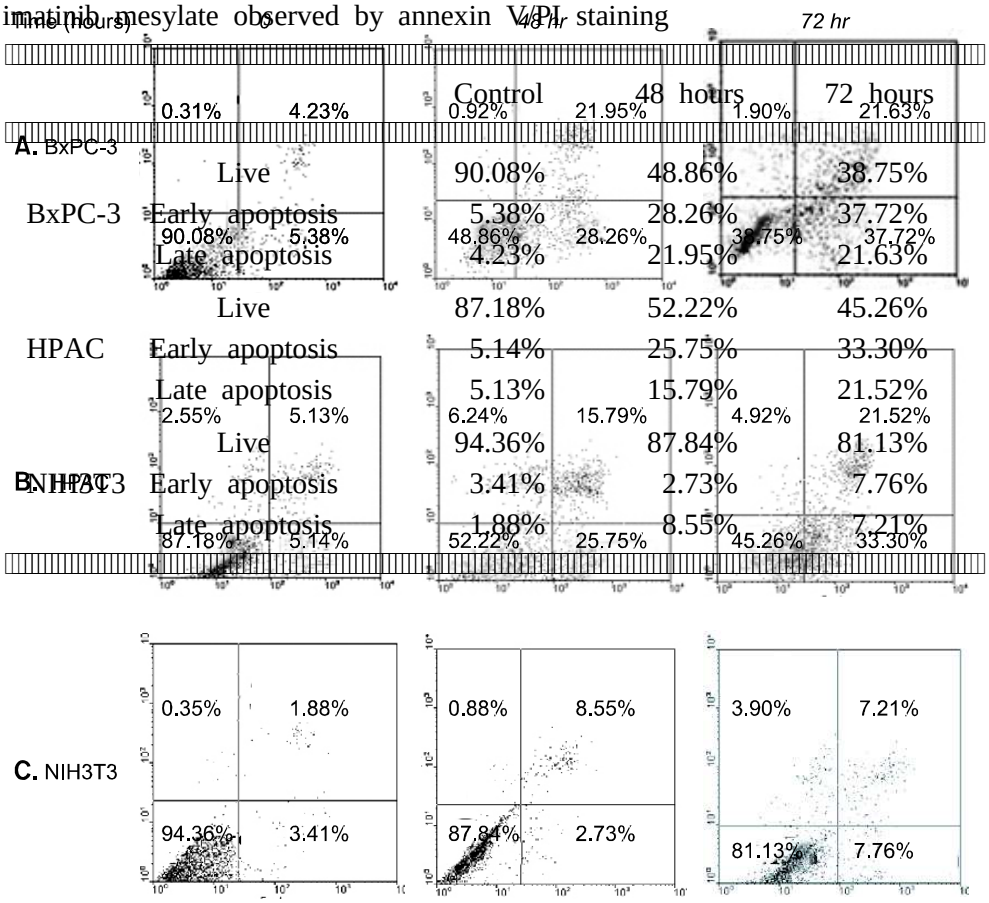

Figure 9. The proportion of early and late apoptotic cells after treatment of imatinib mesylate in BxPC-3 (A), HPAC (B), and NIH3T3 (C) analyzed by annexin V/PI staining.

cells (Fig. 9A, Table 2), the annexin-positive and PI-positive cells were progressively increased with prolonged exposure time of imatinib mesylate (48 hours ＜72 hours). The population of apoptotic cells was 9.6% in untreated cells whereas 50.1% for 48 hours ($P<0.05$) and 58.3% for 72 hours ($P<0.05$) in imatinib mesylate treated BxPC3 cells. For HPAC cells (Fig. 9B, Table 2), the proportion of apoptotic cells was 10.2% in untreated

condition, whereas 41.5 % for 48 hours ($P < 0.05$) and 54.8% for 72 hours ($P < 0.05$) exposure with imatinib mesylate. In contrast, the rate of apoptotic cells was 4.6% in untreated cells and 11.3% for 48 hours and 15.0% for 72 hours in imatinib mesylate treated cells in NIH3T3 cells ($P > 0.05$) (Fig. 9C, Table 2). However, the necrotic cell death was not significant in both untreated and treated cells.

D. The changes of apoptotic signaling proteins after treatment of imatinib mesylate in pancreatic cancer cells.

To conform the apoptotic event in imatinib mesylate treated cells in molecular level, we assessed the changes of expression of apoptosis-related proteins, including caspase-3 (pro), caspase-9 (pro), FASL, Nf-κB, *p*-JNK, Bcl-2 and BAX by western blot analysis. In this evaluation, BxPC-3 and HPAC cells were used. They were incubated with 20μM of imatinib mesylate.

Caspase-3, the final executioner in caspase-dependent apoptotic pathway was strongly over-expressed in untreated cells, and it was markedly decreased in imatinib mesylate treatment after 6 hours. However, after then, it was progressively increased with time (0, 6, 12, 24, 48, 72 hours) (Fig. 10A). Because we used the antibody for pro-caspase-3 in this experiment, the result may mean that the cleavage of caspase-3 was evident in imatinib mesylate treated cell after 6 hours and it was recovered with time. That is, apoptotic process was started and actively processed at early time after

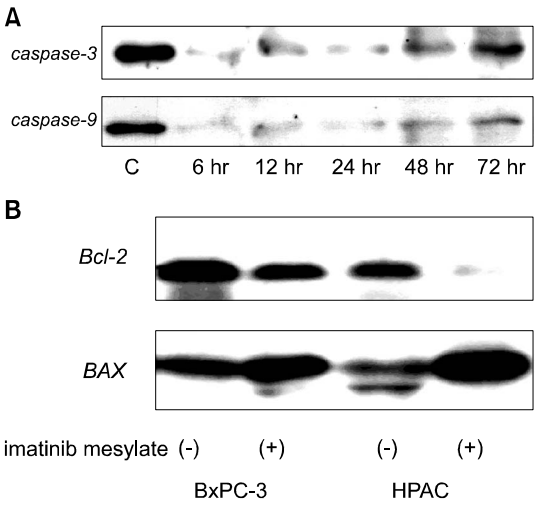

Figure 10. A. The expression change of caspase-3 and caspase-9 with time after imatinib mesylate treatment was analyzed by western blot in HPAC cells. The concentration of imatinib mesylate was 20 μM. B. The expression change of Bcl-2 and BAX after imatinib mesylate treatment was analyzed by western blot after 72 hours in BxPC-3 and HPAC cells. The concentration of imatinib mesylate was 20 μM. C: untreated control. hr: hour

exposure to imatinib mesylate, and it was progressively decreased with time. However, its expression was remained to be decreased state in treated cells compared with untreated cells after 72 hours. The expression of caspase-9 was showed similar pattern to caspase-3 (Fig. 10A).

Among the proteins we examined, the BAX expression (pro-apoptotic protein) was increased in cells in response to imatinib mesylate at 72 hours after imatinib mesylate treatment. In other hands, the Bcl-2 expression

(anti-apoptotic protein) was decreased (Fig. 10B).

With these results, we hypothesized that pathway of imatinib mesylate induced apoptosis is associated with caspase-dependent DNA fragmentation pathway.

4. Imatinib mesylate blocks the phosphorylation of c-Kit and PDGFR in pancreatic cancer cells

To determine whether imatinib mesylate modulated the expression of SCF and c-Kit protein, the membrane was probed with an anti-SCF primary antibody and an anti-c-Kit primary antibody. In this evaluation, BxPC-3 and HPAC cells were used. As shown the results, the total amount of expressed SCF and c-Kit protein did not change after exposure to 20μM (IC_{50}) of imatinib mesylate.

Next, to determine whether imatinib mesylate modulated the phosphorylation of c-Kit, the membrane was probed with an anti-*p*-c-Kit primary antibody. In contrast to total SCF and c-Kit protein level, the phosphorylation of c-Kit (*p*-c-Kit) was inhibited after imatinib mesylate treatment. (Fig. 11).

Similarly, the phosphorylation of PDGFRα,β (*p*-PDGFRα, *p*-PDGFR β) was inhibited after imatinib mesylate treatment although total protein level of PDGFRα,β did not change after exposure to 20μM (IC_{50}) of imatinib mesylate. (Fig. 11).

5. Imatinib mesylate block the phosphorylation of PI3K/Akt kinase,

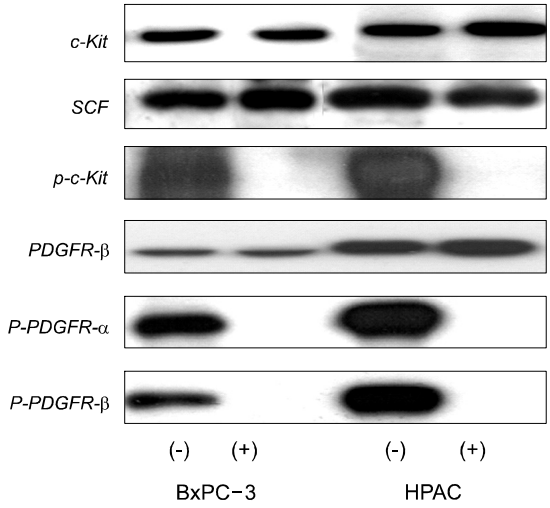

Figure 11. The expression changes of c-Kit, SCF, p-c-Kit, PDGFRb, *p*-PDGFRα,β were analyzed by western blot after imatinib mesylate treatment in pancreatic cancer cells. The concentration of imatinib mesylate was 20 μM.

but did not influence on MAPK kinase activation in pancreatic cancer cells

Activation of c-Kit receptor results in activation of phosphatidylinositol-3 kinase (PI3K).[35,36] One of the downstream events of the PI3K signal-transduction cascade is phosphorylation and resultant activation of the proto-oncogene Akt.[35,36]

To assess the effect of imatinib mesylate on this pathway, we extracted the lysates from BxPC-3 and HPAC cells treated with 20μM (IC_{50}) of imatinib mesylate, and probed with a specific antibody for the activated (phosphorylated) form of Akt. As shown the result, the phosphorylation of

Akt was inhibited after imatinib mesylate treatment. In conclusion, inhibition of phosphorylation of c-Kit and/or PDGFR by imatinib mesylate causes the block of Akt kinase (Fig. 12).

MAPK is one of the downstream events of c-Kit or PDGFR, and it is strongly activated in pancreatic cancer cells. Thus, to assess the effect of imatinib mesylate on this pathway, we probed with a specific antibody for the phosphorylated form of erk (*p*-erk). However, *p*-erk was not inhibited by imatinib mesylate. That is, imatinib mesylate did not influence on MAPK kinase activation in pancreatic cancer cells (Fig. 12).

6. Imatinib mesylate block the phosphorylation of PLCɣ in pancreatic cancer cells

Recent studies demonstrated that PI3K and phospholipase-C ɣ (PLCɣ) were found critical for Kit signaling propagation.[37] PLCɣ greatly contributes to mitogenesis and cellular transformation. According to previous studies, caspase-mediated cleavage of PLCɣ1 is required for apoptosis induced by drugs and TNFa, whereas PLCɣ1 tyrosine phosphorylation confers significant protection against this inducers.[38] PLCɣ is also one of downstream pathway protein of PDGFR. Thus, we assessed the effect of imatinib mesylate on this protein. We probed with a specific antibody for the phosphorylated form of PLCɣ1. As shown the result, the phosphorylation of PLCɣ1 was inhibited after imatinib mesylate treatment (Fig. 12).

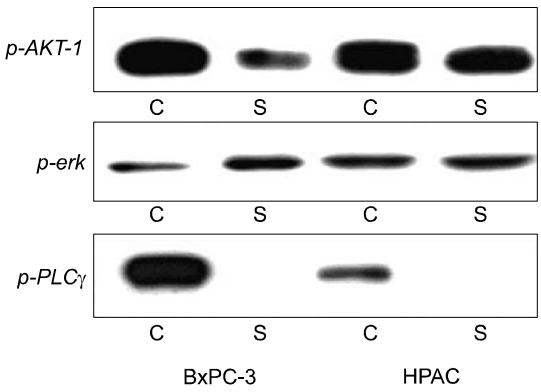

Figure 12. The expression change of PI3K/AKT, MAPK, and PLC γ pathway after imatinib mesylate treatment were analyzed by western blot using specific antibody for *p*-AKT, *p*-erk and *p*-PLC γ 1 in pancreatic cancer cell lines. The concentration of imatinib mesylate was 20 μM.

7. Imatinib mesylate modulate the radiation response in pancreatic cancer cells; radiosensitizing effect of imatinib mesylate

According to above our results, the IC_{50} of imatinib mesylate for pancreatic cells appeared to be higher than the IC_{50} for other reported cancer cells. This fact means the clinical application of imatinib mesylate alone in pancreatic cancer is therefore rather doubtful although imatinib mesylate was demonstrated the dose-dependent, time-dependent inhibitory effects in all tested pancreatic cancer cell lines. Thus, trial of combination treatment may be required. In previous report, investigators suggested that PLCɣ dependent c-Kit signaling may inhibit irradiation triggered apoptotic

signals and PLCγ inhibition results in radiosensitization of Kit-activated cells. Thus, we assessed the synergism of imatinib mesylate with radiation.

HPAC cells were incubated with 5, 10, 20μM of imatinib mesylate for 1 hour prior to irradiation. After then, it was exposed to 1, 3, 6 Gy doses of radiation. After radiation, each group was incubated without imatinib mesylate for 14 days. After then, survival fraction and sensitizer enhancement ratios (SER) calculated at a survival fraction of 37% (IC_{37}), and 50% (IC_{50}). We found that exposure of imatinib mesylate prior to radiation

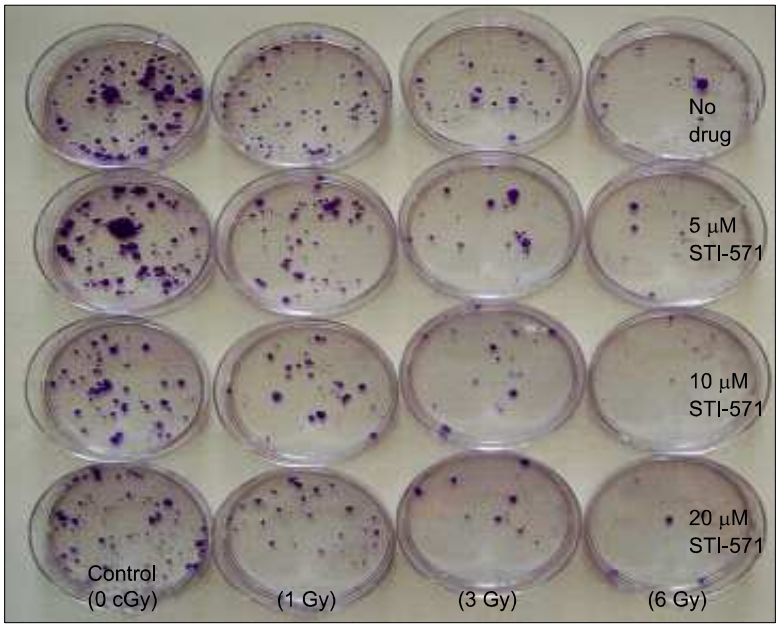

Figure 13. Radiosensitization assay. After incubation with 5, 10, 20 μM of imatinib mesylate and treatment with 1, 3, 6 Gy dose of irradiation, replated cells were re-cultured for 14 days. After then, increased colonies were observed by manual crystal violet staining in 100% ethanol. Colonies consisting of 50 cells or more were counted.

enhanced the cytotoxicity compared with radiation alone-treated cells (Fig. 13, 14). Increasing doses of imatinib mesylate resulted in increased cell death at the same dose of radiation. Clonogenic efficiency was about 50%. The radiation dose for IC_{50} was 1.50 Gy, 1.41 Gy, and 1.06 Gy, respectively for 5μM, 10μM and 20μM of imatinib mesylate pretreatment whereas 1.84 Gy for control. The radiation dose for IC_{37} was 2.00, 1.87 and 1.55, respectively for 5μM, 10μM and 20μM of imatinib mesylate whereas 2.45 Gy for control. The SER at IC_{50} was 1.2, 1.3 and 1.7 for 5 μM, 10μM and 20μM of imatinib mesylate, respectively. The SER at IC_{37} was 1.23, 1.31 and 1.6 for 5μM, 10μM and 20μM of imatinib mesylate, respectively (Table 3).

The radiosensitizing effect of imatinib mesylate was maximized after incubation for 2 hour prior to irradiation, and the effect decreased after that time (Fig. 15).

Table 3. The results of sensitizer enhancement ratios (SER)

Imatinib mesylate concentration	0 μM	5 μM	10 μM	20 μM
IC_{37} (SER)*	2.45 Gy	2.00 Gy (1.20)†	1.87 Gy (1.30)	1.55 Gy (1.70)
IC_{50} (SER)	1.84 Gy	1.50 Gy (1.23)	1.41 Gy (1.31)	1.06 Gy (1.60)

*sensitizer enhancement ratios=dose of radiation required to kill χ% of control cells/dose of radiation required to kill χ% of the cells exposed to the drug.□□□□χ□□ means to be 37, and 50% survival of cells.

†(SER); SER >1.1 were considered statistically significant.

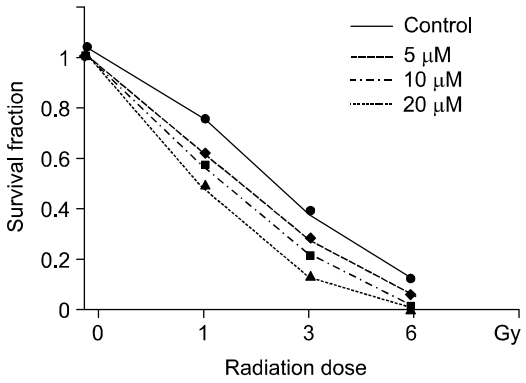

Figure 14. Clonogenic survival in HPAC cells following treatment with imatinib mesylate and ionizing radiation. Radiosensitizing effect of HPAC cells was accessed 1 hour after pre-treatment of imatinib mesylate. The clonogenic assay was performed as previously described. Cells were exposed to 0, 1, 3, or 6 Gy ionizing radiation using a 60 Co source. Mean data (SD) are shown for a typical experiment repeated at least three times.

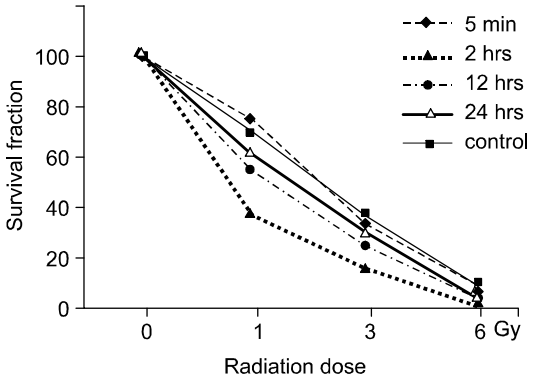

Figure 15. Clonogenic survival of pancreatic cancer cells according to the time of exposure to imatinib mesylate prior to ionizing radiation. The clonogenic assay was performed as previously described. Mean data (SD) are shown for a typical experiment repeated at least three times. Cells were treated with 10μM imatinib mesylate for 5 min to 24 hours prior to radiation. Radiation doses were 0, 1, 3, 6 Gy.

IV. DISCUSSION

Protein kinases play a crucial role in signal transduction as well as in cellular proliferation, differentiation, and various regulatory mechanisms. Disregulation of those signaling pathways is frequently observed in malignant transformation. Therefore, the inhibition of tyrosine kinases might provide new tool in cancer treatment. Actually, the protein tyrosine kinase inhibitor imatinib mesylate, a selective inhibitor of bcr-Abl, PDGFR and c-Kit, has been effective in GIST as well as CML. In this study, we demonstrated that human pancreatic cancer cells express c-Kit and SCF in about 70%. Thus, imatinib mesylate is expected to be effective in growth inhibition of pancreatic cancer.

Recent evidence suggests that, in the pancreas, there is a neoplastic progression similar to the adenoma-carcinoma sequence in the colon.[39] According to many recent studies, however, they suggested another process to be existed in pancreatic cancer development unlike other cancer models. That is, the transdifferentiation process of acinus to duct is needed in early process of carcinogenesis. c-Kit is a development-associated gene. Actually, c-Kit is observed in the fetal pancreas and the normal process of pancreatic development. In this study, I can observe the overexpression of c-Kit in the premalignant PanINs-like metaplastic ductal lesions. In addition, our group observed c-Kit expression at the early phase of pancreatic ducts development from acini in primary cell culture of rat acini (data did not be

shown). In this background, we hypothesized that the constitutive activation of the c-Kit tyrosine kinase may play an important role in early process of carcinogenesis in pancreatic cancer.

However, this study demonstrated that total expression level of c-Kit was not influenced by imatinib mesylate, and the level of c-Kit expression was not correlated with the level of growth inhibition. In other hands, the phosphorylation of c-Kit (*p*-c-Kit) was strongly inhibited by imatinib mesylate (Fig. 11). Because c-Kit must be phosphorylated for the proper biologic function (activated form), the inhibition of c-Kit phosphorylation by imatinib mesylate represents the inhibition of the function of c-Kit and the anticancer activity of imatinib mesylate.

Signaling from c-Kit involves the activation of Jak kinases (Jak2), phospholipase C (PLCγ), phosphoinositol-3-kinase (PI3K) and the Ras/Raf/ mitogen-activated protein (MAP) kinase kinase/MAP kinase cascade.[35-37] Therefore, the changes of expression level of *p*-PLCγ, *p*-Akt and the *p*-erk were evaluated in BxPC-3 and HPAC cells (Fig. 12). The expression of *p*-PLCγ, *p*-Akt was inhibited by imatinib mesylate whereas *p*-erk was not affected. These results suggest that the downstream target of imatinib mesylate may be related to the *p*-PLCγ or PI3K/*p*-Akt pathway rather than MAPK cascade.

PI3K and PLC γ were found to be critical components in Kit signaling propagation and in the protective effect of SCF against serum with-drawal-induced apoptosis.[37,42-44] This mechanism has been also proposed for

Kit-mediated radioprotection in epithelial cells.[45] PI3K is a well-known survival factor in apoptosis. PLCγ induces phosphatidylinositol (PI) 4,5-biphosphate hydrolysis and production of DAG and inositol 1,4,5-triphosphate (IP3). These two messengers induce PKC activation and intracellular Ca^{++} release, respectively.[46] Recent studies suggest that this enzyme is an important regulator of cell survival. For example, it has already been documented that caspase-mediated cleavage of PLCγ1 is required for apoptosis induced by drugs and tumor necrosis factor α, whereas PLCγ tyrosine phosphorylation confers significant protection against these inducers.[38] Moreover, PLCγ confers significant protection against apoptosis induced by UV-C and H_2O_2.[47,48] In this study, imatinib mesylate induced apoptosis is associated with the caspase-dependent DNA fragmentation by the inhibition of *p*-PI3K or *p*-PLCγ. In addition, according to previous studies, c-Kit also regulates cell-matrix adhesion through two independent pathways of PI3K and PLCγ associated with PDGFR.[36] Therefore, the cytotoxic effect of imatinib mesylate on pancreatic cancer cells can be hypothesized by the implication of c-Kit receptor in cell survival and/or adhesion through PI3K/Akt and PLCγ signaling. Other study investigating for anti-tumor effect of imatinib mesylate in colon cancer demonstrated that imatinib mesylate inhibited the cell adhesion and finally induced programmed cell death.[49]

The effects of imatinib mesylate on the cell growth are dependent on the type of cancer cells originating from. For example, in bcr-abl^+ leukemia

cells, the proliferation was inhibited and apoptosis was induced.[42] In contrast, in small cell lung cancer cells, proliferation was inhibited, but no apoptosis induced.[13] This study indicated that in pancreatic cancer cells, the growth-suppressive effect of imatinib mesylate is directly connected with its ability to induce programmed cell death, provened by Annexin-V/propidium critical component iodide flow cytometric assay.

However, although a growth inhibition of pancreatic cancer cells via apoptosis was observed in this study, the cell growth was inhibited marginally at a low concentration (1μM) and significant growth inhibition was observed at relatively higher concentrations (10 and 25μM). Thus, IC_{50} for growth inhibition of pancreatic cancer cells estimated as to be 10-20 times higher than that for the inhibition of bcr-abl expressing CML cells,[3-8] 10 times higher than for the inhibition of c-Kit^{+} small cell lung cancer cell lines,[13] and 1.6 times higher than for the inhibition of c-Kit^{+} colorectal cancer cell.[49] However, compared with c-Kit negative normal cell line (NIH3T3), reduction of cellular number did not ever exhibit at the lowest concentration. Only minimal reduction was observed at 10 and 25 μM. At least, at $>$50μM, a considerable reduction of cellular number was observed. However, at this dose, cytotoxic effect was observed in all cell lines non-specifically. The fact that pancreatic cancer cells are relatively resistant to imatinib mesylate compared with other cell lines expressing c-Kit may suggest that there are additional drug targets except c-Kit partially responsible for growth inhibition by imatinib mesylate in

pancreatic cancer.

Pancreatic cancer cells also express PDGFR. PDGFR signal is also associated with PI3K/Akt and PLC γ in cell survival. In our study, *p*-PDGFRα/β were inhibited by imatinib mesylate. Furthermore, *p*-PDGFR α/β were not expressed in Panc-1 cells which was the most resistant cell line to imatinib mesylate among 4 cell lines examined (Fig. 11). Thus, it is possible that PDGFR is a candidate for additional drug target of imatinib mesylate in pancreatic cancer. In previous studies investigating the anti-tumor effect and mechanism of imatinib mesylate in pancreatic cancer, inhibition of PDGFR was suggested to be an important mechanism of the anti-tumor effect of imatinib mesylate for pancreatic cancer.[50] However, IC_{50} for inhibition of cellular PDGFRα/β tyrosine kinase were only 0.1 μM (Table 4).[51] This result did not explain the relative resistance of imatinib mesylate in pancreatic cancer. Thus further investigation needs. The identity of this hypothetical additional drug targets could not investigate at this time.

This higher IC_{50} value in pancreatic cancer leads us to test a combination therapy with imatinib mesylate in vitro. If imatinib mesylate monotherapy is considered, it may not be possible to achieve therapeutically relevant concentrations without causing significant side effects.[2] However, dose reduction by combination therapy might be possible without causing severe side-effects.

According to previous studies, c-Kit activation confers radioprotection in

vitro. The mechanism of this phenomenon has been suggested that c-Kit tyrosine kinase receptor exerts its radioprotective effect by stimulating DNA repair by activation of *p*-PLCγ or PI3K.[45] That is, PLCγ or PI3K dependent c-Kit signaling may inhibit irradiation triggered apoptotic signals. This background may explain the possible mechanism of radio-resistance in pancreatic cancer, and *p*-PLCγ inhibition by imatinib mesylate results in radiosensitization of c-Kit activated pancreatic cancer cells. In addition, it offers a promising approach for future pharmacologic manipulation to improve the efficiency of radiotherapy in radio-resistant pancreatic cancer cells. We investigated the effect of combination therapy, radiation and imatinib mesylate.

In phase I-II studies of imatinib mesylate in CML patients, there was a higher frequency of nausea, vomiting, muscle cramps, edema, fatigue, and diarrhea at greater than 750 mg.[52] According to the EORTC study of the GIST, 1,000 mg per day was the maximally tolerated dose, with nausea, vomiting, fluid retention, and skin rashes being the dose-limiting toxicities.[53] The clinical recommend dose without significant side effect is 400-600mg/day for 18 months. When the drug was used in CML patients, the clinically safe plasma levels of imatinib mesylate were reported to be in the range of 0.17-5.68μM after treatment with 25-600 mg/day of imatinib mesylate.[54] According to the pharmacokinetics, median peak plasma concentrations at steady state are 5.4μM with administration of 400 mg/day orally and 7.8μM with administration of 600 mg/day (Table 5).[55,56]

This means that the therapeutic safe range in cellular level is less than 10 μM maximally. According to several studies, normal myeloid and erythroid colonies was reduced in CML samples using imatinib mesylate at concentrations of up to 10μM, whereas there was relatively little effect on normal cells.[57] Similar to the study demonstrated that the toxicity of imatinib mesylate was non-existent or limited in vitro[58] and animal studies[59] at concentrations about 10μM for non-transformed normal cells (c-Kit, PDGFR, and Abl are negative). Therefore, in this study the concentrations of imatinib mesylate were used in a range of 5-10μM.

Interestingly, this result demonstrated that synergized anti-tumor effect was observed in combination therapy with radiation and imatinib mesylate. Of course, additional toxic effects by radiation must be concerned in combination therapy. However, according to the previous study, the effects of ionizing radiation on normal hematopoietic cell colonies should not be altered significantly treated by co-treated imatinib mesylate in CML patients.[60] Furthermore, radiation dose range we used in this study was not significantly high dose clinically.

In this study, we did not investigate the combination therapy with imatinib mesylate and other cytotoxic drug (chemotherapy). Previously, many chemotherapy regimens were tried in combination therapy with imatinib mesylate in CML patients for the synergistic cytotoxic effect. For example, imatinib mesylate was used with 5-fluorouracil (5-FU) at concentrations that are achievable in patients within the therapeutic range in

CML (1μM).[61] By combination with 5-FU, cell growth was reduced significantly by an additional 20%. These data suggest that combination therapy with imatinib mesylate and other cytotoxic drugs like this need to be tested in future in vitro and vivo studies.

In conclusion, imatinib mesylate induces apoptosis in pancreatic cancer cell lines at intermediate concentrations. However, clinical application of monotherapy is doubtful in human because of possible severe toxicities. However, low-dose imatinib mesylate in combination with radiation is clinically applicable because radiation response is enhanced by imatinib mesylate and anti-tumor effect is synergized. Thus, these results demonstrated the potential role of low-dose imatinib mesylate in combination with radiation for the treatment of pancreatic cancers and suggest a promising therapeutic impact for imatinib mesylate with radiation in pancreatic cancer.

Table 4. Inhibition of protein kinases by imatinib mesylate

Enzyme	Substrate Phosphorylation IC_{50} (μM)	Cellular tyrosine Phosphorylation IC_{50} (μM)
p210Bcr-Abl	0.025	0.25
p185Bcr-Abl	0.025	0.25
TEL-Abl		0.35
PDGF-R a and b		0.1
TEL-PDGF-R		0.15
c-Kit		0.1
FLt-3		>10
c-Fms and v-Fms		>10
v-Src		>10
Jak-2	>100	>100
EGF-R	>100	>100
Insulin receptor		>100
IGF-IR		>100
FGF-R1	31.2	
VEGF-R2	10.7	
VEGF-R1	19.5	
c-Met	>100	

Reference; Druker BJ. Oncogene 2002.

Imatinib concentrations causing a 50% reduction in kinase activity (IC_{50}). PDGF-R, platelet-derived growth factor receptor; EGF-R, epidermal growth factor receptor; IGF-IR, insulin-like growth factor receptor I, FGF-R1, fibroblast growth factor receptor 1; VEGF-R, vascular endothelial growth factor receptor.

Table 5. Pharmacokinetic parameters in CML patients at steady state following once daily oral administration of imatinib mesylate

Dose (mg)	Subjects (*n*)	T*max* (h)	T1/2 (h)	AUC (0-∞) (μ g/h/ml)
50	2	4.8±4.6*	13.8±3.2	7.20±0.52
200	3	4.0±3.5	13.6±0.9	20.93±10.13
400	5	2.7±1.5	16.2±4.4	59.18±13.92
600	6	3.5±0.6	16.1±6.1	110.15±85.18

reference; Mauro MJ. Oncologist 2001
values listed as mean±SD

V. CONCLUSION

Pancreatic cancer cells express PDGFR and c-Kit. And, imatinib mesylate, a selective inhibitor of bcr-Abl, PDGF and c-Kit induces cytotoxicity in pancreatic cancer cell lines at relatively higher concentrations compared with other cell lines expressing c-Kit and PDGFR. The mechanism of cytotoxicity is produced via programmed cell death (apoptosis). In molecular level, this apoptotic process may be related to the casepase-dependent DNA fragmentation by inhibition of Bcl-2 through the inhibition of the *p*-PLCγ or PI3K/*p*-Akt pathway not MAPK cascade, which pathway is inhibited by the inhibition of *p*-c-Kit and *p*-PDGFR by imatinib mesylate. Apoptosis reactions may occur mainly early time (first 3 days), and apoptosis reactions may decrease with time. Cell cycle showed minimal change. Also, imatinib mesylate showed radiosensitization in pancreatic cancer.

Because imatinib mesylate demonstrated the growth inhibition of pancreatic cancer at e intermediate concentrations, imatinib mesylate mono-therapy is doubtful clinically. However, the characteristics of radiosensiti-zation in pancreatic cancer may suggest a potential role of low-dose imati-nib mesylate in combination with radiation for the treatment of pancreatic cancers.

In conclusion, this study may suggest a promising therapeutic impact for imatinib mesylate with radiation in pancreatic cancer clinically.

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Abstract (In Korean)

췌장암 세포 주에 있어서의 imatinib mesylate의 항암 효과 및 방사선 상승 효과

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정 혜 원

인체의 췌장암은 1년 생존율이 20% 미만인 예후가 불량한 암이다. 근치적 절제가 가능한 10-20% 내외에서도 2년 생존율이 10% 내외에 불과하며 대부분의 환자는 진단 후 6개월 내외에 사망에 이른다. 기존의 항암제나 방사선 치료 등은 췌장암에 있어서는 다른 암종에 비해 두드러지게 약제 저항성을 보이고 있어 그 효과가 미미하다. 비록 1999년 이후 gemcitabine이 췌장암에 보다 효과적인 항암 약제로 개발되어 사용되고 있으나 이외의 약제는 거의 효과가 없으며, 이 역시 생존율 향상에 큰 영향을 끼치지는 못하였다. 최근 들어 분자 생물학적 연구를 바탕으로 하여 암 발생 과정에서 관여하는 생장 호르몬 같은 단백질을 주 공격 목표로 하는 약물 개발이 활발히 진행되고 있는데, 이 중 하나가 protein tyrosine kinase로써 대표적인 약제가 현재 만성 골수성 백혈병에 효과적으로 쓰이고 있는 imatinib mesylate (STI-571, Gleevec®, Glivec®)이다. 이는 Bcr-Abl, PDGFR, c-Kit 수용체의 인산화를 선택적으로 억제하는 약물로 알려져 있는데, 따라서 c-Kit과 PDGFR 수용체 양성을 보이는 췌장암에서 향후 항암 효과를 기대해 볼 수 있는 항암제로 대두되고 있다. 따라서 본 연구는 췌장암 세포주에 있어서 imatinib mesylate의 항암 효과 및

그 기전을 살펴보고 임상적인 적용을 위한 연구를 실시하였다. 실험은 4개의 췌장암 세포주(BxPC-3, HPAC, MiaPaCa-2, Panc-1)과 c-Kit, PDGFR 음성인 정상 세포주인 NIH3T3 (음성 대조군)을 이용하여 실험을 진행하였다.

1. 췌장암 세포주는 세포주에 따라 차이를 보이기는 하나, 전반적으로 볼 때 imatinib mesylate에 대하여 약제 농도와 노출 시간에 비례하는 항암 효과를 나타내었다. 그러나 c-Kit과 PDGFR 수용체를 가지는 다른 세포주들에 비해 상대적으로 높은 IC_{50}값을 보였다.

2. 췌장암 세포들이 imatinib mesylate 에 대해 항암 효과를 보이는 것은 세포 증식의 억제에서 기인하기 보다 세포 사멸 유도에 기인되는 것으로 관찰되었다. (Annexin V/PI assay, DAPI 염색)

3. FACS를 이용한 세포 주기를 관찰한 결과 약물 노출 48시간 경과 후 약간의 S 상의 지연이 관찰되었으나 뚜렷하지 않았다. 그러나 시간이 지남에 따라 증가되는 세포 사멸 세포들을 관찰할 수 있었다. (Cell cycle analysis by FACS)

4. 세포 사멸에 관계되는 단백질을 약물 노출 72시간에 western blot으로 관찰한 결과 BCL-2가 감소하고 Bax가 증가하였다. Caspase 관련 신호 전달체계의 가장 마지막 단계인 caspase-3는 초기 6시간 내에 급격히 감소하였다가 시간이 경과함에 따라 서서히 본래의 수준 가까이 회복되는 것을 관찰할 수 있었다. 그러나 72시간까지 관찰한 결과 약물을 처리한 세포에 있어서 처리하지 않은 세포에 비해 감소되어 있음을 관찰할 수 있었다.

5. Western blot을 실시한 결과 imatinib mesylate 처리에 의해 c-Kit과 PDGFR

의 총량이 변하는 것이 아니라 인산화가 감소됨을 관찰하였다. 즉, 기능적인 면에서 억제가 됨을 알 수 있었다.

6. c-kit과 PDGFR관 관련된 세포 전달 단백질 중 생존과 분화, 증식에 관련되고 췌장암에서 발현하는 단백질, *p*-Akt, *p*-erk, *p*-PLCɣ의 발현이 imatinib mesylate에 영향을 받는지 western blot을 이용해 관찰한 결과 *p*-Akt과 *p*-PLCɣ은 감소되었으나 *p*-erk은 변화를 보이지 안았다.

7. 췌장암 세포주를 각각 5μM, 10μM, 20μM 농도의 imatinib mesylate에 1시간 정도 노출시킨 후 0, 1, 3, 6 Gy의 방사선에 노출시킨 후 14일 후에 증식 여부를 관찰한 결과 방사선에 대해 치료 상승효과를 관찰할 수 있었다. 이는 imatinib mesylate 노출 시간에 영향을 받는데, 노출 시간이 길수록 상승효과는 증가되어 2시간이 최대를 보이고 있으나 2시간 이후로는 오히려 효과가 감소되는 현상을 관찰하였다.

이상으로 췌장암에 있어 imatinib mesylate 의 방사선 상승 효과는 c-kit 수용체의 인산화 억제로 기인된 *p*-PLCɣ의 억제로 *p*-PLCɣ에 의해 발생된 radioprotective 효과를 억제함으로써 발생되는 것으로 추정하였다. c-Kit과 PDGFR 양성인 다른 세포주들에 비해 췌장암 세포주는 imatinib mesylate에 대해 상대적으로 미미한 항암 효과를 보이나 세포 사멸을 유도하고 있고 또한 radiosensitization의 효과를 지니고 있어 임상적으로 단독 사용은 무리가 있으나 병용치료에 이용될 때 기존의 치료에 저항을 보이는 췌장암 치료에 도움을 줄 수 있을 것으로 생각하였다.

핵심 되는 말: 췌장암, imatinib mesylate, 항암효과, radiosensitization