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TITLE: Apoptosis Induction by Targeting Interferon Gamma Receptor 2 (IFNgammaR2) in Prostate Cancer: Ligand (IFNgamma)-Independent Novel Function of IFNgammaR2 as a Bax Inhibitor

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14. ABSTRACT	n our preliminary st	udy, we found that I	FNyR2 has previou	sly unknown fi	unction as an inhibitor of Bax. Bax is		
a key mediator of apoptosis. We found that IFNvR2 is overexpressed in prostate cancer, and we hypothesize that abnormally							
high level of IFNy	R2 confers apopto	sis resistance of p	rostate cancer. In t	his project, w	e accomplished the following three		
tasks. Task1: Search for the binding domains of IFNyR2 and Bax. Our experiments suggest that Bax binding domain may							
exists in amino acid 301-308 of IFNyR2, and that IFNyR2 does not require the N-terminal 53 amino acids of Bax. Task2:							
Identification of IFNγR2 expressing PCa: We found that basal cell, but not luminal cell, of prostate tissue of prostate cancer							
patients expressed high level of IFNyR2. We also found that IFNyR2 expression level increases according to the progression							
of malignancy in c	cell lines (from and	arogen-dependent,	androgen-independ	ent, and bone	e metastatic). Furthermore, shRNA-		
mediated down regulation of IFNYK2 was able to Increase cell death of IFNYK2 expressing cancer cells both in cell culture and medica vanage and the mechanism of increased IENVR2 expression in PCa. We found that NEVR							
inhibitor suppressed IENvR2 expression, suggesting that hyper-activation of NEVR may be one of the mechanisms of IENvD2							
overexpression in PCa. These results support our hypothesis that IFNvR2 has an anti-apontotic activity in PCa and IFNvR2 is							
a promising future drug target to improve the efficiency of PCa treatment.							
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#### Introduction

Previously, we identified interferon  $\gamma$  receptor 2 (IFN $\gamma$ R2) as a Bax suppressor using yeast-based functional screening of Bax inhibiting proteins [1]. Bax is a key mediator of apoptosis which is essential for chemotherapy- and radiation-induced apoptosis of prostate cancer cells[2]. Previously, we reported that IFNyR2 levels were abnormally elevated in some of prostate cancer cell lines (LNCap and PC3) [1]. Short hairpin (sh) RNA-mediated knockdown of IFNyR2 was able to increase chemotherapy-induced apoptosis rate significantly in HeLa cells [1]as well as PC3 (a human prostate cancer cell line) cells (unpublished observation, manuscript in preparation), suggesting that IFNyR2 is a chemo-resistant factor in prostate cancer cells. Although IFNyR2 was previously known as a receptor of IFNy which is an anti-tumorigenic cytokine, our preliminary data suggest that IFNyR2 expresses its anti-apoptosis (anti-Bax) activity independent from IFNy and IFNy signaling[1]. Importantly, we found that IFNyR2 is expressed in mitochondrial membranes and endoplasmic reticulum (ER) membranes, but not on the plasma membranes of prostate cancer cells (unpublished observation, manuscript in preparation). Since we found that IFNyR2 can directly interact with Bax at least in vitro [1], we hypothesize that IFNyR2 confer apoptosis resistance of prostate cancer by directly binding and inhibiting Bax in intracellular membrane such as endoplasmic reticulum (ER) and mitochondria (Fig.1).

In this project, the following Tasks were accomplished and the detail experimental data are explained in *Results and Discussion* section.

**Task 1:** To determine the mechanism of Bax inhibition by IFNγR2 (to identify binding domains of Bax and IFNγR2), and to develop anti-IFNγR2 peptide that enhances Bax activation. (Months 1-24)

**Task 2**: To identify the subtype of prostate cancer that can be effectively treated by IFNγR2-targeting technologies (Months 13-36)

**Task 3**: Determination of the mechanism of abnormal expression of IFNγR2 in prostate cancer (Months 13-36)

#### **Results and Discussion**

#### Task 1a: Search for IFNyR2 binding domain of Bax

#### Accomplished experiments 1

We made a series of plasmids that express Bax mutant proteins lacking possible IFNyR2 binding domains. Bax mutant cDNAs were sub-cloned into Flag-tagged and GFP-tagged protein expression plasmids.

#### Strategy of making Bax mutants:

Human Bax protein has 198 amino acids. There are 9 alpha helices. The first N-terminal 53 amino acids before the 1<sup>st</sup> alpha helix is known to function as an auto-inhibitory domain. The 1<sup>st</sup> alpha helix is also known as BH3 domain that is essential to bind other Bcl-2 family proteins (including Bax itself). Alpha helices 5 and 6 creates channels in the membrane that are required for pore-forming activity of Bax, and this activity is implicated to be essential for cytochrome c release from mitochondria. Alpha helix 9 is a trans-membrane domain that is essential for mitochondrial membrane localization of Bax, and this domain is also essential for apoptosis induction. Each of these domains essential for Bax-mediated apoptosis was deleted, and fused with epitope tag (Flag- or GFP-tag) so that we can detect

expression of these Bax mutants by antibodies detecting epitope tags. Expression of these proteins in HEK293T cells have been confirmed (we will confirm the expression in PC3 soon).

List of newly made plasmid:

\*pCMV2B vector express Flag-tagged protein. pEGFP vector express EGFP-tagged protein. Bax full length cDNA has been already subcloned into these vectors.

pCMV2B-Bax  $\Delta$ N (N-terminal 53 amino acid was deleted) pCMV2B-Bax  $\Delta \alpha 1$  (a-helix 1 is deleted) pCMV2B-Bax  $\Delta \alpha$  5-6 (a5 and a6 are deleted) pCMV2B-Bax  $\Delta \alpha$  9 (a9 is deleted) pEGFP-Bax  $\Delta \alpha$  9 (a9 is deleted) pEGFP-Bax  $\Delta \alpha$  1 pEGFP-Bax  $\Delta \alpha$  5-6 pEGFP-Bax  $\Delta \alpha$  9

As explained from the next section, these plasmids were used for the search of IFN $\gamma$ R2 binding domain of Bax.

#### <u>Accomplished experiments 2</u> We found that the first 53 amino acid of Bax is not required for IFNyR2 to inhibit Bax.

The first N-terminal 53 amino acids is known to be auto-inhibitory domain that keeps Bax in the cytosol[3]. If this domain is cleaved, Bax is known to be hyperactive to induce apoptosis. The mechanism by which this N-terminal domain suppresses activation of Bax is not very well known.

We speculated that IFN $\gamma$ R2 binds to this N-terminal domain to stabilize inactive status of Bax. If it is the case, IFN $\gamma$ R2 will not be able to suppress apoptosis induced by Bax  $\Delta$ N (1-53 amino acids are deleted). To examine this speculation, we performed co-transfection of GFP- Bax  $\Delta$ N and Flag-tagged IFN $\gamma$ R2 in HEK293 cells (Fig.2).

The conclusion is that IFN $\gamma$ R2 (both full length and C-terminal 41 amino acids) was able to suppress Bax  $\Delta$ N –induced apoptosis. This result suggests that IFN $\gamma$ R2 interacts with unidentified domain in the region of amino acids 54-198 (next section explains the strategy to narrow down the binding domain within this region).

#### **Accomplished experiments 3**

# Co-immunoprecipitation (co-ip) binding experiments were performed to narrow down IFNγR2 binding site in Bax protein, however, we were not able to obtain clear conclusion due to the difficulties of the detection of IFNγR2 in Western blot of co-ip samples (IgG band disturbed the detection) :

Using GFP-Bax plasmids prepared in section 1, we performed co-immunoprecipitation (co-ip) experiments to identify essential domain for the binding of IFNyR2. IFNyR2 was expressed as Flag-tagged protein so that we can detect co-immunoprecipitated IFNyR2 by Flag-antibody. However, we faced a problem. Molecular Weight (MW) of Flag-IFNyR2 is approximately 52 kDa, and it is very close to 50 kDa IgG Heavy Chain. Unfortunately the 2<sup>nd</sup> antibody used to detect Flag-tagged IFNyR2 cross reacted with IgG of anti-GFP antibody that was used to pull down GFP-Bax-Flag-IFNyR2 complex, and IgG signal interfered the signals from 52 kDa IFNyR2. In general, to avoid this type of the problem, antibodies from different animal (e.g. the combination of rabbit IgG and mouse IgG) are

used for co-ip and Western blot detection. In our experiment, we used rabbit IgG for GFP antibody and mouse IgG for Flag-IFNyR2 detection, and the 2<sup>nd</sup> antibody to detect Flag-IFNyR2 was antimouse IgG, but not anti-rabbit IgG. But, still, there was a cross reactivity. To solve this problem, we made IFNyR2 antibody conjugated with HRP so that we can detect IFNyR2 directly without 2<sup>nd</sup> antibody. Theoretically, we will be able to detect Flag-IFNyR2 signal without the interference from IgG used for co-ip. Unfortunately, even after several months of attempts to improve the method (antibody concentration, incubation time, for example), we were not able to obtain convincing results even with our homemade IFNyR2 antibody conjugated with HRP. At present, we think that traditional binding experiment may not be useful to identify precise binding amino acid sequence in Bax. In addition to peptide-based inhibition of IFNyR2 activity, other strategies targeting IFNyR2 such as shRNA-mediated down regulation of IFNyR2 (as explained in Task 2 and 3) may be more promising strategy to develop IFNyR2-targeing therapy.

#### Task 1b: Search for Bax binding domain of IFNyR2

#### Accomplished experiments 4

### Bax binding domain of IFNγR2 is located in amino acids 296-337 (41 amino acids), and the minimum essential domain may exist in amino acids 301-308.

IFN $\gamma$ R2 has 337 amino acids. In our preliminary study shown in our proposal, we found that amino acids 296-337 of IFN $\gamma$ R2 (IFN $\gamma$ R2 (296-337)) are sufficient to bind Bax and inhibit Bax-induced apoptosis. In addition, we observed that expression of GFP-tagged IFN $\gamma$ R2 (296-337), but not IFN $\gamma$ R2 (1-295) suppressed Bax-overexpression-induced apoptosis [1]. These results suggest that Bax binding domain of IFN $\gamma$ R2 is located in 296-337 (41 amino acids).

Among 41 amino acids in IFNγR2 (296-337), there is an amino acid sequence of PILEADK (amino acids 301-308) which has a similarity to the Bax binding domain of Ku70 (Ku70 is an Bax inhibiting protein) that was discovered by PI's group[4-7]. Ku70's Bax binding domain is (V)PTLKEA. We examined whether this PILEADK contains Bax inhibiting activity by examining its effects on Bax-mediated cell death (Fig.3), and we observed that penta-peptides (PILEA and EALDK) designed from this sequence has a weak apoptosis inhibition activity. Ku70 derived Bax inhibiting peptide (VPTLK) was used as a positive control for Bax-mediated cell death inhibition. KLPVM is a negative control penta-peptide that does not have Bax inhibiting activity. In the experiment shown in Fig.3, we used Etoposide (10 uM 16hs and 24 hrs treatment)-induced apoptosis in HeLa cells that is known to be Bax-dependent cell death. All the peptides used in this experiments were tagged with fluorescent tag (Fam) so that peptide entry into the cells can be monitored and confirmed. All the peptides tagged with Fam were able to enter the cell in this experiment. Although further thorough study is clearly necessary to draw conclusion, these results suggest that Bax binding domain in IFNγR2 may exist in amino acids 301-308 of IFNγR2.

### Task 2: To identify the subtype of prostate cancer that can be effectively treated by IFN $\gamma$ R2-targeting

#### <u>Accomplished experiments 5</u> Immunohistochemistry of Tissue Microarray were performed.

We performed two different approaches to investigate IFN $\gamma$ R2 expression patters in prostate cancer. One is to examine the correlation between IFN $\gamma$ R2 expression and clinical outcome using patient specimen (please see Accomplished experiment 7), and another one is to utilize commercially available tissue microarray of human prostate cancer. the second strategy and examined 11 prostate cancer patient samples together with 8 normal prostate specimen as controls. The summary is presented in **Fig.4**.

We found that majority of progressed prostate cancer specimen showed elevated expression of IFN $\gamma$ R2, and these cells also express Bax. Some of advanced prostate cancer (such as No. 1-4, 9, 10 and 11 Fig.4) expresses high levels of IFN $\gamma$ 2 as well as Bax, but no detectable Bcl-2. In this type, prostate cancer cells may suppress Bax-induced apoptosis mainly by depending on IFN $\gamma$ 2, and thus IFN $\gamma$ R2 targeting therapy is expected to work well. In the case of prostate cancer expressing both IFN $\gamma$ R2 and Bcl2, combinational treatment of IFN $\gamma$ R2 inhibitor and Bcl2 inhibitor (such as ABT-263 derivatives) may be effective.

#### Accomplished experiments 6

### IFNγR2 expression patterns in prostate cancer were investigated by analyzing human patient database.

Using Oncomine database (publically available gene expression profile database of human cancer patients), we found that IFNyR2 expression levels of prostate cancer are significantly higher than normal prostate as we expected. To determine the significance of IFNyR2 elevation in apoptosis-resistance of prostate cancer, we also checked the expression levels of other well-known apoptosis inhibiting proteins such as BcI-2, BcI-XL, and McI-1. Results are presented in **Fig.5**. Very interestingly, only IFNyR2 (3.69 times increase, p<0.000115) and McI-1 (3.24 times increase, p<0.000319) showed significant increased expression in prostate cancer in comparison with normal prostate, but not BcI-2 and BcI-XL (BCLL1 in the figure). Expression of apoptosis inducer such as Bax and Bak did not show remarkable changes. These results suggest that IFNyR2 and McI1, but not BcI-2 and BcIXL, are the ideal targets to induce Bax/Bak-mediated apoptosis in prostate cancer. This information also suggests that McI1 inhibition may be also necessary to induce prostate cancer cell death when IFNyR2 inhibition is used to enhance Bax-induced apoptosis.

#### Accomplished experiments 7

### Significant IFNyR2 expression was detected in prostate basal cells (hyperplasia area) of patients who experienced recurrence after prostatectomy and radiation therapy.

In Figs. 6-7, representative results are shown. Fig.6 (A and B) show IFNvR2 expressing cells were clearly detected in prostates of patients with recurrence (in all 4 patients examined), but IFNyR2 expressing cells were NOT always found in prostates of patients who did not have recurrence (2 out of 3 patients did not have IFNyR2 positive cells) (Fig. 6C). Interestingly, IFNyR2 was detected in basal cells, but not in luminal cells. Furthermore, IFNyR2 is not expressed in all basal cells, but only in basal cells that show hyperplasia destructing the tube structure of prostate gland (Fig. 6A) or in basal cells in a few prostate glands that still maintains normal structure (Fig. 6B). We speculate that Fig.6B shows the emergence of tumor-initiating basal cells in particular area, and Fig. 6A shows the example of pre-tumorigenic growth of basal cells. In Fig. 7, other pictures of IFNyR2 positive prostate cells are displayed in the order of the progression of prostate cancer. Fig.7D shows the example of the destruction of luminal structure by abnormal growth of basal cells that express IFNyR2. Fig.7E is the results in metastatic prostate cancer section showing that majority of cancer cells express high level of IFNyR2. Interestingly, IFNyR2 was not detected in luminal cell type prostate cancer (see Fig. 6C) suggesting that IFNyR2 expression may decrease when basal cell differentiates into luminal cells. Since IFNyR2 protects prostate cancer cells from apoptosis, IFNyR2 (-) luminal cell type cancer cells are likely treatable (i.e. relatively easy to induce apoptosis) cancer and IFNyR2 (+) basal cells may represent apoptosis-resistant cancer initiating cells or cancer stem cells (Fig. 8.

#### **Accomplished experiments 8**

### *IFN*γ*R*2 *expression increases according to the progression of malignancy of prostate cancer cells.*

Please see Fig.9 We examined expression levels of IFNγR2 in widely used prostate cancer cell lines. These cell lines were originated from the same cell line to investigate the molecular mechanism of

progression of prostate cancer, i.e. from androgen-dependent state (LNCaP) to androgenindependent state (C4-2), and to bone metastatic state (C4-2B). Interestingly, IFNγR2 levels increased according to the progression of malignancy in these prostate cancer cells. It has been known that C4-2B has constitutive active androgen receptor (AR). Therefore, elevated activity of AR may contribute to the increased expression of IFNγR2.

Since C4-2B expresses high level of IFNyR2, we speculated that IFNyR2's role to suppress cell death is significant in this cell line. In fact, IFNyR2 knock down significantly slowed down the growth of C4-2B and induce spontaneous cell death as seen in Fig. 10 These results suggest that IFNyR2 has a significant role to suppress apoptosis in aggressive prostate cancer that shows androgen independency and metastatic activity.

**Task 3**: Determination of the mechanism of abnormal expression of IFNγR2 in prostate cancer (Months 13-36)

#### Accomplished experiments 9

### Effectiveness of IFNyR2 inhibition to promote prostate cancer cell death was examined using mouse xenograft experiments.

We prepared two cancer cell lines in which IFNyR2 was knocked down by shRNA. These cell lines are PC3 (human prostate cancer cell line) and A375 (human melanoma cell line). We examined A375 cell line, since we found that this cell line also expresses high levels of IFNyR2.

We injected one million cells of cancer cells to each nude mouse, and docetaxel (1mg/kg) and cisplatin (5mg/kg) were treated every week to mice. In PC3 cell line experiments, more than a half of docetaxel-treated mice (2-3 out of 4 mice) were dead within two weeks of treatment, therefore we were not able to obtain reportable result. In the case of cisplatin-treated A375 experiments, we were able to obtain preliminary results to determine the effects of IFNγR2 knockdown. IFNγR2 knockdown was able to slow down the growth of tumor (Fig.11 panel B) in comparison with control shRNA expressing cells (Fig.11 panel A). Tumor growth after cisplatin-treatment was also suppressed by shRNA-mediated IFNγR2 knockdown (Fig.11 panel D) in comparison with control (Fig.11 panel C), though whether cisplatin-induced cell death was "enhanced" is not yet clear, since IFNγR2 shRNA alone (without cisplatin) showed significant suppression of tumor growth (Fig.11 panel A vs B).

#### <u>Accomplished experiments 10</u> NFkB inhibitor (Parthenolide) suppressed IFNyR2 expression

To develop technologies targeting IFN $\gamma$ R2, we proposed to determine the effectiveness of currently available drugs that is predicted to decrease IFN $\gamma$ R2 expression in prostate cancer. Since previous studies have shown that NFkB is one of transcription factors that stimulate IFN $\gamma$ R2 gene expression [8], we proposed to determine the effects of NFkB inhibitor. Parthenolide is a plant-derived compound which is known to inhibit NFkB activity[9, 10]. In our preliminary study, we found that parthenolide was able to decrease IFN $\gamma$ R2 expression in PC3 prostate cancer cells. In this study, we examined another standard prostate cancer cells, LNCaP cell line (Fig.12. We found that IFN $\gamma$ R2 expression was suppressed by parthenolide (from 5 uM) within 1 day after the treatment (Fig. 12 shows the result of 1 day treatment). Importantly, Bax expression was not decreased by this treatment, suggesting that parthenolide can stimulate Bax-mediated cell death by decreasing Bax inhibitor, i.e. IFN $\gamma$ R2.

#### Methods:

#### Cell culture and plasmid transfection:

HEK293T cells and PC3 cells were obtained from ATCC, and these cells were cultured in DMEM containing 10%FCS and1% penicillin/streptomycin. For plasmid transfection to examine Bax inhibiting activity and Bax binding activity of IFN $\gamma$ R2, 2x10<sup>5</sup> cells/6 cm dish and 1x10<sup>6</sup> cells/10 cm dish, respectively, were used. Plasmid transfection were performed by using Superfect (Qiagen). For bax inhibiting activity, 0.5 ug Bax expressing plasmid and 2.0 ug IFN $\gamma$ R2 expressing plasmids were co-transfected to 6 cm dish culture. For Bax binding experiments (co-immunoprecipitation experiments), 1 ug of Bax expressing plasmids and 4 ug of IFN $\gamma$ R2 expressing plasmids were transfected to the cells cultured in 10 cm dish. 24 hrs after the transfection, cells were collected for apoptosis analysis or cell lysate preparation for binding experiments as described below.

#### Generation of Plasmid:

pCMV vector and pEGFP vectors were purchased from Clontech. Bax mutant cDNAs were generated by 2 step PCR methods, and these mutant cDNAs were subcloned into pCMV and pEGFP vectors.

#### Peptide Synthesis:

Peptides were purchased from Biopeptide (San Diego) by order made. Peptides were purified by HPLC and the purity is more than 99% so that possibly toxic chemicals were removed fro the peptide solution. Peptides were dissolved in PBS at the concentration of 100 mM, and 200 uM were added to the cell culture to examine whether the peptide can inhibit Bax-mediated cell death.

#### Cell Death Assay:

After 24 hrs of plasmid transfection, cells were re-suspended in HBSS (Hanks Buffered Saline Solution) at 4C, and Hochst 33258 (DNA staining fluorescent dye, purchased from Sigma) were added at 10 ug/ml concentration. Under fluorescent microscope, transfected cells were identified by GFP signal, and percentages of apoptotic cells showing apoptotic nuclear fragmentation or condensation among GFP positive cells were measured. Total 300 cells were counted in each sample.

#### Binding experiments:

Cells were lysed by lysis buffer (1% NP40 10 mM HEPES and 140 mM NaCl) containing protease inhibitor cocktail (purchased from Sigma). Cell lysates are adjusted to 2.5 ug/ul protein concentration by the lysis buffer. 200 ul of cell lysates were pre-cleaned by shepharose beads, and the cleaned lysates were subjected to co-immunoprecipitation using anti-GFP antibody. After 2 hrs of incubation with GFP antibody, immune-complex were pulled down by Protein A coated Sepharose beads (Pharmacia). After 3 times wash of the beads by lysis buffer, immunocomplex was eluted by 50ul of Lamli buffer for SDS-PAGE. Twenty ul of sample were applied to each lane of SDS-PAGE and Western blot was performed by using anti-flag antibody.

#### Cell culture and plasmid transfection:

HEK293T cells and PC3 cells were obtained from ATCC, and these cells were cultured in DMEM containing 10%FCS and1% penicillin/streptomycin. For plasmid transfection to examine Bax inhibiting activity and Bax binding activity of IFNγR2, 2x10<sup>5</sup> cells/6 cm dish and 1x10<sup>6</sup> cells/10 cm dish, respectively, were used. Plasmid transfection were performed by using Superfect (Qiagen). For bax inhibiting activity, 0.5 ug Bax expressing plasmid and 2.0 ug IFNγR2 expressing plasmids were co-transfected to 6 cm dish culture. For Bax binding experiments (co-immunoprecipitation experiments), 1 ug of Bax expressing plasmids and 4 ug of IFNγR2 expressing plasmids were transfected to the cells cultured in 10 cm dish. 24 hrs after the transfection, cells were collected for apoptosis analysis or cell lysate preparation for binding experiments as described below.

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#### shRNA-mediated down regulation of IFNyR2 in C4-2B cell line

IFNγR2 targeting shRNA was introduced into cells by using lentivirus transfection system (Thermo Fisher Scientific, USA). Control shRNA encodes shRNA against Green Fluorescent Protein (GFP) that does not exist in human cells. Cells successfully transfected by lentivirus were selected by puromycin, and cell lysates were collected to determine IFNγR2 protein expression.

#### Immunohistochemistry of human prostate cancer tissue microarray.

Human prostate cancer tissue microarray was purchased from BioMax (Maryland, USA). Immunohistochemistry of IFNyR2 was performed by the standard methods explained in detail in Abcam website (http://www.abcam.com/index.html?pageconfig=resource&rid=13046). Antibodies used in these experiments is: IFNyR2 (Abcam, #ab77246).

#### Cell culture and cell lysate preparation for Western blot

LNCaP, C4-2, and C4-2B cells were obtained from ATCC, and these cells were cultured in DMEM containing 10%FCS and1% penicillin/streptomycin. Cell lysates were prepared by solubilizing cells using 1% NP40 containing HEPES buffer. Insoluble fraction was removed by centrifuge separation (14k rpm for 20n min at 4C). For the analysis of protein expression, cell lysates containing 10 ug protein were used. SDS-PAGE was performed by using 4-20% gradient gel, and immuno-detection was performed by ECA Chemical luminescence detection kit (Amersham).

#### Immunohistochemistry of human prostate cancer tissue microarray.

Human prostate cancer tissue microarray was purchased from BioMax (Maryland, USA). Immunohistochemistry of IFNyR2, Bax, and Bcl-2 were performed by the standard methods explained in detail in Abcam website (http://www.abcam.com/index.html?pageconfig=resource&rid=13046). Antibodies used in these experiments are: IFNyR2 (Abcam, #ab77246), Bax (BD Pharmingen #554104), and Bcl-2 (BD Pharmingen #514202).

#### Definition of tumor grade

The Grade 1-3 in Pathology Diagnosis is equivalent to well-differentiated, moderately-differentiated or poorly differentiated, respectively, under microscope.

Grade 1 or well-differentiated: Cells appear normal and are not growing rapidly.

Grade2 or moderately-differentiated: Cells appear slightly different than normal.

Grade3 or poorly differentiated: Cells appear abnormal and tend to grow and spread more aggressively.

**Grade 4 or undifferentiated:** \*(for certain tumors), features are not significantly distinguishing to make it look any different from undifferentiated cancers which occur in other organs.

#### TNM grading:

#### T - Primary tumor

- Tx Primary tumor cannot be assessed
- T0 No evidence of primary tumor
- Tis Carcinoma in situ; intraepithelial or invasion of lamina propria
- T1 Tumor invades submucosa
- T2 Tumor invades muscularis propria

T3 - Tumor invades through musclaris propria into subserosa or into non-peritonealized pericolic or perirectal tissues

T4 - Tumor directly invades other organs or structures and/or perforate visceral peritoneum

#### N - Regional lymph nodes

- Nx Regional lymph nodes cannot be assessed
- N0 No Regional lymph node metastasis
- N1 Metastasis in 1 to 3 regional lymph nodes
- N2 Metastasis in 4 or more regional lymph nodes

#### M - Distant Metastasis

- Mx Distant metastasis cannot be assessed
- M0 No distant metastasis
- M1 Distant metastasis

#### Cell culture and cell lysate preparation for Western blot

PC3 and LNCaP cells were obtained from ATCC, and these cells were cultured in DMEM containing 10%FCS and1% penicillin/streptomycin. To determine the effects of parthenolide, cells were cultured in the presence of various concentration of parthenolide (5, 10, or 20 ug/ml) for 1 day. Cell lysates were prepared by solubilizing cell pellets using 1% NP40 containing HEPES buffer. Insoluble fraction was removed by centrifuge separation (14k rpm for 20n min at 4C). For the analysis of protein expression, cell lysates containing 10 ug protein were used. SDS-PAGE was performed by using 4-20% gradient gel, and immuno-detection was performed by ECA Chemical luminescence detection kit (Amersham).

#### Mouse xenograft experiments

One million cells of cancer cells (PC3 and A375) were subcutaneously injected to nude mice. One week later, docetaxel (1 mg/kg) or cisplatin (5 mg/kg) were administered (i.p. injection) once a week, for 4-5 weeks. When tumor size reaches 10% of mouse body, experiments were stopped, and mice were euthanized for tumor size analysis.

#### Key Research Accomplishment

- 1. Plasmids that express GFP- and Flag-tagged Bax mutant proteins were prepared.
- 2. We found that The N-terminus of Bax is not essential for IFNyR2 to inhibit Bax-induced apoptosis.

3. We confirmed that Bax binding domain of IFNyR2 is localized in the C-terminal 41 amino acids of IFNyR2 (IFNyR2 (263-337) was able to inhibit Bax-induced apoptosis).

4. Bax binding domain of IFNγR2 is located in amino acids 296-337 (41 amino acids), and the minimum essential domain may exist in amino acids 301-308.

5. Significant IFNγR2 expression was detected in prostate basal cells (hyperplasia area) of patients who experienced recurrence after prostatectomy and radiation therapy.

6. We found that IFNγR2 expression increases according to the progression of malignancy of prostate cancer cells.

7. We found that shRNA-mediated IFNγR2 inhibition was able to increase cancer cell death in mouse xenograft experiment.

8. NFkB inhibitor (Parthenolide) suppressed IFNyR2 expression

#### **Reportable Outcome**

We presented this study in the international meeting of interferon held in Melbourne, Australia, in November 2014. Actually, PI was invited as a selected speaker in this meeting. All the key research accomplishments are reportable results for the future publication. We are preparing an article to be submitted in 2017 that will report the effects of shRNA in C4-2B prostate cancer cells, mouse xengraft experiment result, IFNyR2's subcellular localization study, and the striking IFNyR2 staining pattern in prostate cancer tissue (only basal cell in particular area become IFNyR2 positive).

#### **References:**

- 1. Gomez, J.A., et al., *The C-terminus of interferon gamma receptor beta chain (IFNγammaR2) has antiapoptotic activity as a Bax inhibitor.* Cancer Biol Ther, 2009. **8**(18): p. 1771-86.
- 2. Korsmeyer, S.J., *BCL-2 gene family and the regulation of programmed cell death.* Cancer Res, 1999. **59**(7 Suppl): p. 1693s-1700s.
- 3. Suzuki, M., R.J. Youle, and N. Tjandra, *Structure of Bax: coregulation of dimer formation and intracellular localization.* Cell, 2000. **103**(4): p. 645-54.
- 4. Yoshida, T., et al., *Bax-inhibiting peptide derived from mouse and rat Ku70.* Biochem Biophys Res Commun, 2004. **321**(4): p. 961-6.
- 5. Gomez, J.A., et al., *Bax-inhibiting peptides derived from Ku70 and cell-penetrating pentapeptides.* Biochem Soc Trans, 2007. **35**(Pt 4): p. 797-801.
- 6. Gomez, J.A., et al., *Cell-Penetrating Penta-Peptides (CPP5s): Measurement of Cell Entry and Protein-Transduction Activity.* Pharmaceuticals (Basel), 2010. **3**(12): p. 3594-3613.
- 7. Gomez, J. and S. Matsuyama, *Cell-penetrating penta-peptides and Bax-inhibiting peptides: protocol for their application.* Methods Mol Biol, 2011. **683**: p. 465-71.
- Ebensperger, C., et al., Genomic organization and promoter analysis of the gene IFNγr2 encoding the second chain of the mouse interferon-gamma receptor. Scand J Immunol, 1996. 44(6): p. 599-606.
- 9. Shanmugam, R., et al., *A water-soluble parthenolide analogue suppresses in vivo prostate cancer growth by targeting NFkappaB and generating reactive oxygen species.* Prostate, 2011. **70**(10): p. 1074-86.
- 10. Sun, Y., et al., *The radiosensitization effect of parthenolide in prostate cancer cells is mediated by nuclear factor-kappaB inhibition and enhanced by the presence of PTEN.* Mol Cancer Ther, 2007. **6**(9): p. 2477-86.



**Fig.2.** The N-terninal 53 amino acids of Bax is not required for Bax inhibition by IFN $\gamma$ R2. Bax or Bax without 1-53 amino acids (Bax  $\Delta$ N) were expressed as GFP-fusion protein together with Flag-tagged IFN $\gamma$ R2 (full length) or –IFN $\gamma$ R2 (263-337). GFP vector or Flag-tagged control protein (firefly luciferase) were used as a negative control. IFN $\gamma$ R2 and IFN $\gamma$ R2 (263-337) were able to inhibit apoptosis induced by both Bax and Bax  $\Delta$ N, suggesting that the first 53 amino acids of Bax is not the target of IFN $\gamma$ R2 to inhibit Bax-induced apoptosis.



#### Fig.3. Peptides designed from IFNγR2 may have an anti-apoptotic activity.

HeLa cells were treated with etoposide (10 uM) (Eto) in the presence or absence of Fam (FITC fluorescence tag)-labeled peptides (200 uM). Cells were incubated for 16 or 24 hrs, and apoptosis was detected by Hoechst dye nuclear staining. Four types of peptides were tested. VPTLK: Bax inhibiting peptide (Bax inhibition has been confirmed previously) designed from Ku70 protein. KLPVM: Negative control peptide that does not have an activity to inhibit Bax. PILEA: Peptide designed from amino acids 301-305 of IFNγR2. EALDK: Peptide designed from amino acids 304-308 of IFNγR2. Peptides dissolved in DMSO were added to the culture medium so that the final concentration of DMSO will become 0.1 %. For negative control experiment, DMSO (0.1%) was used. Bck: Background cell death in complete negative control group (no DMSO, no drugs, no peptide).

A: Effects of peptides on etoposide-induced cell death are shown. B and C: Confirmation of the entry of Fam-tagged peptide (PILEA is shown as an example) in the cell (B: 10X objective lenses, C: 40X objective lenses)



**Fig.4** Increased expression of INFgR2 was observed in human prostate cancer tissue microarray. (Please see the definition of cancer progression grade (Grade) and metastasis activity (TNM) in the last page of the proposal)

No.	Position	Age	Grade	TNM	IFNyR2	Bax	Bcl-2
1	A1	69	2	T2N0M0	++	+	-
2	A2	69	2	T2N0M0	±	+	-
3	A3	76	2-3	T3N1M1b	±~++	+	+
4	A4	76	2-3	T3N1M1b	++	+	±
5	A5	69	2	T2N0M0	+	-	-
6	A6	69	2	T2N0M0	+	-	-
7	A7	76	2-3	T3N1M1b	±and++	-	-
8	A8	76	2-3	T3N1M1b	+	±	-and+
9	B1	72	3-4	T3N0M0	+	+	+(N)
10	B2	72	3-4	T3N0M0	+~±	+	+(N)
11	B3	60	4	T2N0M0	- ~±	+	+and-
12	B4	60	4	T2N0M0	±	±	+and-
13	B7	60	4	T2N0M0	±~+	-	+
14	B8	60	4	T2N0M0	±	±	-
15	B5	72	hyperplasia	T3N0M0	±~++	-and+	±
16	B6	72	hyperplasia	T3N0M0	++	-and+	+
17	C1	43	Normal	-	-	-	-
18	C2	43	Normal	-	-	-	-
19	C3	28	Normal	-	-	±	±
20	C4	28	Normal	-	±	±	±
21	C5	43	Normal	-	-	-	-
22	C6	43	Normal	-	-	-	-
23	C7	28	Normal	-	- ~ ±	-	±
24	C8	28	Normal	-	-~±	±	-

## **Fig.5** IFNgR2 expression is higher in prostate cancer than that in normal prostate



Comparison of Selected Genes in Singh Prostate Over-expression in Prostate Carcinoma vs. Normal log2 median-centered intensity

Rank	P-value	Fold Change	Gene		Reporter	Gene			
268	1.15E-4	3.69	IFNGR2		41140_at	IFNGR2			
430	3.19E-4	3.24	M CL1		33146_at	MCL1			
873	5.17E-4	1.22	BAK1		846_s_at	BAK1			
1648	0.008	1.67	BAX		1997_s_at	BAX			
2220	0.023	1.36	B CL2L1		1615_at	BCL2L1			
2749	0.053	1.64	XRCC6		32274_r_at	XRCC6			
4516	0.335	1.04	BCL2		1847_s_at	BCL2			
				1 2					
Legend				Singh Prostate	Singh Prostate				
1. Prostate Gland (50) 2. Prostate Carcinoma (52)			Carcinom	a (52) Cancer Cell 2002/03/01 102	samples				

Cancer Cell 2002/03/01102 samplesmRNA8,603 measured genesHuman Genome U95A-Av2 Array

Not measured

Note: Colors are z-score normalized to depict relative values within rows. They cannot be used to compare values between rows.

Most Expressed

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Least Expressed

Oncomine Source: https://www.oncomine.com/resource/main.html#a:1947;cso:sizeSmallLarge;cv:detail;d:258;dso:geneOverex;dt:predefinedClass;ec:[2];epv:150001.151078;et:over;f: 2506691;g:3460;p:200001343;pg:1;pvf:3483,5673,5699,5742,5744,15271,27047,39565,150004;scr:datasets;ss:analysis;th:g100.0,p0.050,fc2.0;v:10

Fig.6. Immunohistochemistry of IFNγR2 in human prostate. A and B: IFNγR2 staining (brown cells, stained by HRP (Horse Radish Peroxidase)-DAB (Diaminobenzidine)) is detected in basal cells, but not in luminal cells or other cell types in prostate. C: IFNγR2 was not detected in prostate cancer area. Note: A and B are results of prostates from patients with recurrence. C is from a patient with no recurrence.





IFNgR2 (No Recurrence)





Fig.7. Summary of immunohistochemistry experiments of IFNγR2 in prostates of patients with recurrence. From A to D, an emergence and growth of abnormal basal cells expressing IFNγR2 are shown. E shows IFNγR2 positive prostate cancer cells that showed bone metastasis.

Emergence of IFNgR2 (+) basal cells



Uncontrolled growth of IFNgR2(+) Basal cells





IFNgR2(+) Basal cells eliminate luminal cells

Metastatic/Apoptosis resistant prostate cancer?

#### <u>Fig.8</u> *IFNgR2 is expressed in a subgroup of basal cells showing hyperplasia*



Model of human prostate cancer initiation and propagation by distinct phenotypic cell populations (Modified from *Stoyanovaa et al. PNAS 2013*)

**Fig.9:** IFNγR2 expression levels become higher when prostate cancer cell line (LNCaP) acquire androgen-independency and bone metastasis capability



\*LNCaP has a Jak1 inactivating mutation, and thus IFN $\gamma$  does not activate canonical IFN $\gamma$  signaling pathway.

C4-2 and C4-2B cell lines were generated from LNCaP cell line, which is an androgendependent cell line. C4-2 is androgen-independent, and C4-2B is androgenindependent and bone metastatic cell lines. These 3 cell lines are used to study the mechanism of prostate cancer progression.

#### Fig.10 IFNgR2 shRNA suppressed C4-2B growth



C4-2B cells were treated by lentivirus encoding shRNA targeting IFN $\gamma$ R2 or no-targeting shRNA. Three days after the infection of lentivirus, cell culture images were taken, and the images are shown above. Cells treated by IFN $\gamma$ R2 targeting shRNA showed significant increase of spontenous cell death. Left bottom image of Western blot result shows the confirmation of the decrease of IFN $\gamma$ R2 by shRNA targeting IFN $\gamma$ R2. The decrease of IFN $\gamma$ R2 levels by shRNA was not dramatic as expected from the increased spontaneous cell death, however, it may be because we were able to analyze only survived cells in shRNA-treated cells (cells with dramatic decrease of IFN $\gamma$ R2 might have dead and disappeared). Please see more detail in Method section.



**Fig.11** IFNgR2 shRNA suppressed tumor growth and promoted cisplatin effects (Each line represents each mouse treated with cancer cell and drug (or PBS))

One million cells were injected to the subcutaneous tissue of nude mouse, and the growth of these cells were monitored by measuring the size of tumor formed under the skin of these mice. Before injection to the mice, cells were treated by shRNA targeting IFN $\gamma$ R2 (IFN $\gamma$ R2 sh-RNA) or no targeting shRNA. Mice were treated by cisplatin every week as described in Method section.

**Fig.12** Parthenlide treatment decreased IFNgR2 expression without changes in Bax expression



LNCaP cells were cultured (1 million cells/10 ml/10 cm dish) for 24 hrs in the presence of Parthenolide at various concentration (5, 10 , and 20 uM). Partenolide was added as DMSO solution at the final DMSO concentration of 0.1%. DMSO (0.1%) only was used as a control (DMSO). NT: no treatment, i.e. no DMSO and no Parthenolide. Expression levels of IFN $\gamma$ R2, Bax, and beta actin was analyzed by Western blot using 10 ug of total protein cell lysates in each lane. Please see detain in Method section.