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The Role of PTIP in Breast Cancer

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The Role of PTIP in Breast Cancer

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by

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Symbols and Nomenclatures

PTIP……..……Pax Trans-activation domain Interacting Protein

H3K4……..Histone 3 Lysine 4

RT-PCR…….Reverse Transcriptional-Polymerase Chain Reaction

MLL……..Mixed Lineage leukemia

DMEM……Dulbecco's Modified Eagle Medium

EDTA…….…Ethylenediaminetetraacetic Acid

DMSO……....Dimethyl Sulfoxide

dNTP…….…deoxyribonuleotide Triphosphate

TBST…….Tris-Buffered Saline and Tween 20

ECL.....…..Enzymatic Chemiluminescence

PBS……..……Phosphate Buffered Saline

DAPI…… 4',6-diamidino-2-phenylindole

LB Agar…….. Lysogeny Broth Agar

GFP……..……Green Fluorescent Protein

GAPDH…….……..glyceraldehyde-3-phosphate dehydrogenase

MTT…….….3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di- phenytetrazoliumromide

ABSTRACT

The Role of PTIP in Breast Cancer

In the U.S., breast cancer comprises about 30% of all cancer cases (excluding skin cancer) in women. Such a high incidence makes breast cancer a significant health concern, but our understanding of the molecular and cellular mechanisms of this disease is still limited. Growing evidence suggests that the development of human breast cancer may involve epigenetics, which attributes changes in phenotype to mechanisms other than changes in the DNA sequence itself. Histones as the chief proteins of chromatin work on gene expression, and methylation of histone 3 lysine 4 (H3K4) results in transcriptional activation. Lately, Paired box (Pax) trans-activation domain-interacting protein (PTIP), part of the H3K4 methyl-transferase complex, has been identified as a novel regulator of histone methylation. In this study, we hypothesize that PTIP plays a role in the epigenetic regulation of breast cancer cell growth. Two human breast cancer cell lines MCF-7 (adeno carcinoma) and T47D (ductal carcinoma) are used in this study. To test our hypothesis, we have first determined the PTIP mRNA expression level in MCF-7 and T47D cells by reverse transcription-polymerase chain reaction (RT-PCR) and the PTIP protein level by Western blot. We have also determined PTIP's sub-cellular localization in MCF-7 and T47D cells by immunofluorescence assay. In addition, we have knocked down and over-expressed PTIP. In other words, we reduced and increased the expression of PTIP in MCF-7 and T47D cells through siRNA (small interference RNA) and ectopic over-expression respectively. We then examined the effects of PTIP knockdown and PTIP over-expression on MCF-7 and T47D cell growth. As a result, we found that PTIP

knockdown in MCF-7 cells results in an increase of cell proliferation. The information collected from this study will allow us to comprehend PTIP's role in breast cancer cell growth and contribute to our understanding of the epigenetic basis of human breast cancer.

Key Words: breast cancer, PTIP, epigenetics, histone methylatio

Chapter One

Introduction

Breasts are located symmetrically in the left and right sides of the upper ventral region. They are composed of glands, vessels, adipose and fibrous tissues. For women, the breast is a secondary sex character and produces milk to children during lactation. Breast cancer is the most common cancer in women and it is caused by malignancy invasion that destroys the normal breast tissue. There are some factors affecting the occurrence of breast cancer, such as long term stimulus of estrogen, virus, heredity, nutrition, and psychological stress.

In the United States, cancer is a critical health issue that causes death in one of every four people. Breast cancer, as one of the four major cancers, accounts for about 34% of total cancers in women [1]. From 2010 to 2020, the largest increase of medical cost for women is predicted to be caused by breast cancer. The rate of cost increase would be around 32% [2]. For women between 20 and 59 years old, breast cancer is the leading cause in cancer death. In 2012, there are 226,870 new breast cancer cases, representing 29% of total new female cancer cases, predicted among women [1].

Breast cancer, taking up about 34% of total female cancers, is the most common cancer in the United States [3]. However, our understanding of breast cancer is still limited. Recently, it has been found that cancers are related to epigenetics, which attributes changes in phenotype to mechanisms other than changes in the DNA sequence itself, such as histone modification [4].

Histone modification plays various functions in regulating genome functions, such as, in DNA damage repair and gene transcription. Histone methylation is one of the histone modifications and is linked to transcriptional regulation. Methylation of specific lysine residues of histone, for example, lysine 4 on histone 3 (H3K4), is associated with transcriptional activation [5].

Paired box (Pax) trans-activation domain-interacting protein (PTIP), part of the H3K4 methyl-transferase complex, has been identified as a novel regulator of histone methylation [6]. In this study, hypothesize that PTIP plays a role in the epigenetic regulation of breast cancer cell growth. Two human breast cancer cell lines MCF-7 (adeno carcinoma) and T47D (ductal carcinoma) were used as the studying model.

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Objectives

1. Determine the gene expression of PTIP in breast cancer cell lines MCF-7 and T47D.
2. Determine the protein expression of PTIP in breast cancer cell lines MCF-7 and T47D.
3. Determine the sub-cellular localization of PTIP in breast cancer cell lines MCF-7 and T47D.
4. Determine the functions of PTIP in breast cancer cell lines MCF-7 and T47D.

Specific Aims

Specific Aim 1: Determine the gene expression of PTIP in breast cancer cells.

The RT-PCR technique was used to determine the gene expression of PTIP in MCF-7 and T47D breast cancer cells.

Specific Aim 2: Determine the protein expression of PTIP in breast cancer cells.

The protein expression of PTIP in MCF-7 and T47D cells was evaluated by Western blot.

Specific Aim 3: Determine the subcellular localization of PTIP in breast cancer cells.

The sub-cellular localization of PTIP in MCF-7 and T47D cells was examined by immunofluorescence.

Specific Aim 4: Determine PTIP function in breast cancer cells.

First, the protein level of PTIP in MCF-7 and T47D cells was knocked down by RNA interference (RNAi). Second, ectopic PTIP was overexpressed in MCF-7 and T47D cells. Third, we investigated the effects of PTIP knockdown and PTIP overexpression on cell proliferation.

Chapter Two

Review of Literature

Part I: Breast cancer and breast cancer cells

Part II: Epigenetic regulation and histone methylation

Part III: the PTIP gene

Part I: Breast cancer and breast cancer cells

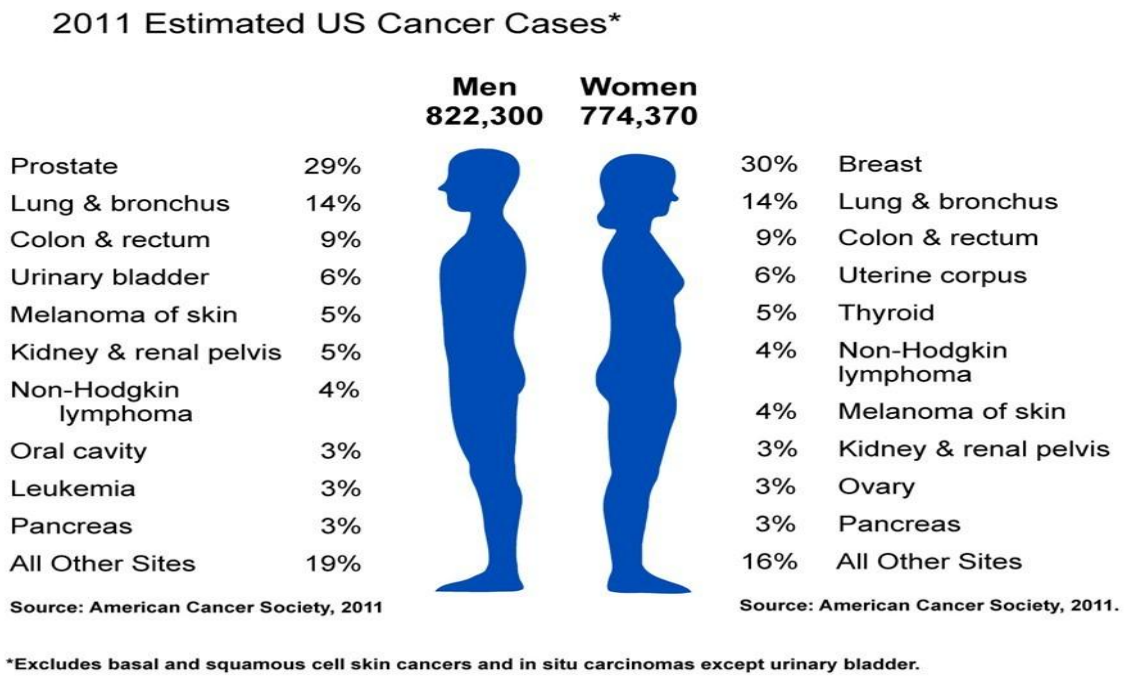

Figure 1. Estimated US cancer cases in 2011. http://www.cancer.org

In the U.S., as the most common cancer in women, breast cancer accounts for 30% or so of all female cancers [3]. It is estimated that there will be 226, 870 new breast cancer cases which make up 29% in 2012 [1]. In addition, the cancer treatment cost in

2010 was 12.57 billion dollars but in 2020 is predicted to be 157.77 billion dollars. The biggest increase, 32%, of medical spending was for breast cancer treatment for women [2]. However, our understanding of this disease is still limited. Public health data (PHD) pointed out that there were more than one million women who died from breast cancer each year [7]. It is significant to understand the cellular and molecular mechanisms of breast cancer so that we can develop better treatment for women with breast cancer.

The breast cancer cell lines are limited, and just a few of them are used for research, such as MCF-7 (adeno carcinoma) and T47D (ductal carcinoma). Also, a large percentage of available research reports on breast cancer have used the cell lines MCF-7 and T47D [8] [9]. Accordingly, we used MCF-7 and T47D in this study.

Part II: Epigenetic regulation and histone methylation

Epigenetics attributes changes in the phenotype to mechanisms other than changes in the DNA sequence itself, such as histone methylation and DNA methylation [10]. On a molecular level, epigenetics can activate expression of specific genes without changing the DNA structure. In addition, gene paramutation is an example of epigenetic inheritance [11].

Growing evidence shows that epigenetics is related to human cancers [12]. For example, regulating the tumor suppressor genes by histone modification influences the development of cancer [13].

Histones, or proteins in the nucleus, function on gene expression. Histones compact the DNA about 3-fold the remaining compaction is due to folding of chromatin [14]. There are two types of histones, core histones (H2A, H2B, H3 and H4) and linker

histones (H1 and H5) [15]. Several well-known histone modifications are lysine methylation (me), arginine methylation, lysine acetylation (ac) and Serine/Threonine/Tyrosine phosphorylation [16]. Histone lysine modifications, such as H3K4me1, H3K4me3, H3K9me1, H3K9ac, H3K14ac, H3K27me1, H3K79me1, H3K79me2, H3K79me3, H3K20me1, and H2BK5me1, can activate gene transcription. On the other hand, H3K9me2, H3K9me3, H3K27me2, H3K27me3, H3K79me3 and H2BK5me3 can repress gene transcription [17] [18] [19] [20] [21].

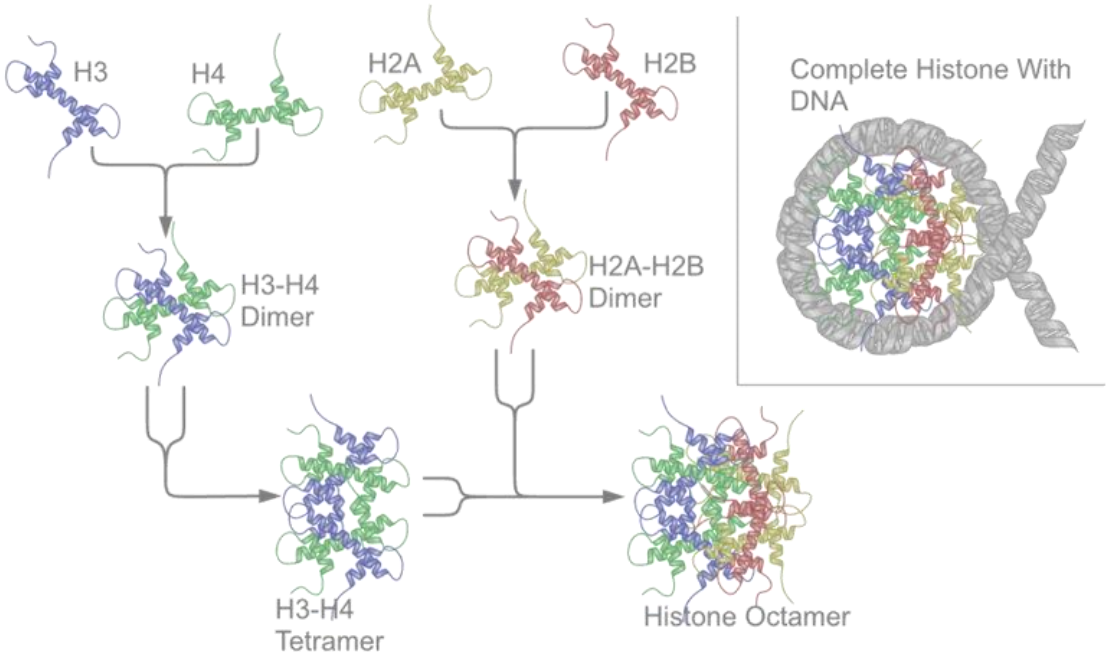

Figure 2. Histone and DNA. http://en.wikipedia.org/wiki/File:Nucleosome_structure.png

H3K4me, the example of histone methylation, plays a role in transcriptional activation [22]. Yeast SET1 and mammalian mixed lineage leukemia (MLL) can methylate H3K4. MLL, the gene related to aggressive acute leukemia (ALL) [23], can activate the transcription of oncogenes and consequently cause leukemias and cancers. [24] [25]. With this regard, H3K4me is associated with cancer development.

Part III: the PTIP gene

PTIP, Pax transactivation-domain interacting protein [26], is component of the histone methyltransferase (HMTase) complex which also contains MLL3 or MLL4 [27] [28] [29]. Recent studies have shown that PTIP is a novel regulator of H3K4 methylation [20] [31]. Additionally, because H3K4me can activate gene transcription, PTIP has been thought to be involved in transcriptional activation.

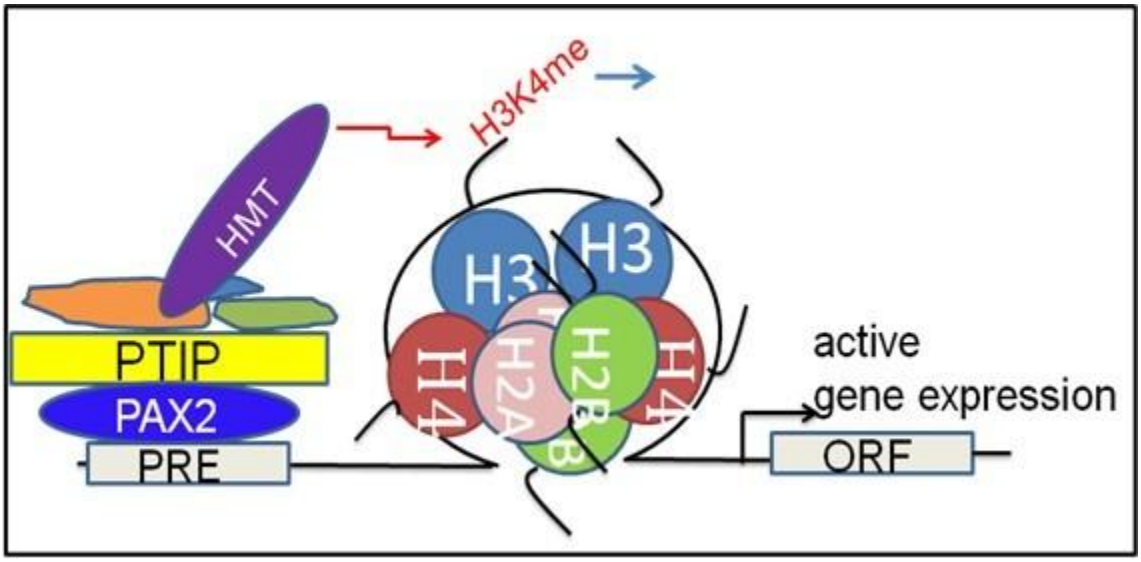

Figure 3. A hypothetical model of PTIP-mediated epigenetic regulation of breast cancer. HMT, Histone methyltransferase; H3K4me, histone 3 lysine 4 mono-, di-, or trimethylation; PAX2, paired homeobox 2; PRE, Pax2-response element; ORF, open reading frame.

Paired homeobox 2 (Pax2) engages PTIP to bind to the Pax2 response element (PRE). Subsequently, PTIP attracts the histone methyltransferase (HMT) complex which contains MLL (MLL3/MLL4). MLL methylates H3K4, and then H3K4me activates gene transcription. All in all, PTIP activates gene expression by epigenetic regulation. MLL is

known to activate oncogenes during cancer development. Thus, through functional association with MLL, PTIP may activate oncogenes in breast cancer cells.

Chapter Three

Methods and Materials

Cell culture (MCF7 and T47D)

Complete culture medium: Add 50ml of fetal bovine serum and 5ml of penicillin-streptomycin to 500ml of base medium.

Passage cells when they are 70-80% confluent of the plates.

1) Remove and discard culture medium.
2) Briefly rinse the cell layer with 5-10ml of PBS to remove all traces of serum which contains trypsin inhibitors.
3) Add 1.5ml of Trypsin-EDTA solution to the plate, and incubate the plate at 37℃ for 3-10 minutes to allow cells to detach. Do not agitate the cells by hitting or shaking the pate while waiting for the cells to detach.
4) Add 5 ml of DMEM and aspirate cells by gently pipetting, and then transfer cell suspension to a 15ml tube.
5) Centrifuge for 5 minutes at 1400rpm.
6) Discard supernatant, resuspend cells in 5ml of medium, and add appropriate aliquots of the cell suspension to a new culture plate. Add 10ml medium to a new plate before add the cell suspension.

Viable cell counting

1) Add 10μl of cell suspension and 10 λ of Trypan Blue to a 1.5ml tube and mix. Trypan Blue stains non-viable (dead) cells.

2) Place 10 λ of the mixture in both wells of a hemocytometer, make sure the entire surface of the rectangular grid of the hemocytometer is covered.

3) Count viable cells in central squares on both sides of the hemocytometer using a hand counter. If cells are on the border outlining each square, count only the cells on the top and left border of the square.

4) Calculate cell concentration:

# of cells / square $\times 2 \times 10^4$ = ? cells / ml

Thaw frozen cells

1) Thaw the vial by gentle agitation in a 37℃ water bath, keep the O-ring and cap out of the water to reduce the possibility of contamination. Thawing should be rapid, less than 2 minutes.

2) Remove the vial from the water bath as soon as the contents are thawed and decontaminate by spraying with 70% ethanol. Transfer the vial contents to a culture dish containing 10ml warm medium.

3) Let cells sit overnight at 37℃ in the incubator, and then change medium to get rid of DMSO.

Freeze cells

1) Harvest cells from plates with Trypsin-EDTA solution.

2) Resuspend cells in cryoprotectant medium (complete culture medium supplemented with 5% DMSO), final cell concentration is $1\sim3 \times 10^6$ / ml.

3) Add 1 ml cell suspension to each vial carefully.

4) Place vials at -20 ℃ for 2 hours, and then put vials into -80 ℃ freezer overnight.

5) The second day, transfer vials into a LN2 container for storage.

RNA Extraction from Cultured Cells

1) Remove medium from cultured cells.

2) Add TRI Reagent to homogenize cells, incubate the plate for 5 minutes at room temperature.

3) Add TRI Reagent 1ml to each 10cm culture dish, 0.5ml to each well of 6-well plate. Homogenized cells can be stored at -70 ℃, at least one month.

4) Add 200 λ of chloroform to 1ml of homogenized cells or 100 λ to 0.5ml of homogenized cells, vortex for 15 seconds, incubate the mixture for 10 minutes at room temperature.

5) Centrifuge for 10 minutes at 12,000rpm at 4 ℃.

6) Transfer the aqueous phase to a fresh tube carefully.

7) Add 500 λ of 2-propanol for original 1ml of homogenized cells or 250 λ for original 0.5ml of homogenized cells, vortex for 10 seconds, incubate for 10 minutes at room temperature.

8) Centrifuge for 10 minutes at 12,000rpm at 4 ℃.

9) Discard supernatant carefully by pipetting.

10) Wash the pelleted RNA with 500 λ of 70% ethanol in nuclease-free water.

11) Air dry RNA pellet for 3-10 minutes.

12) Dissolve RNA in 10-30 λ of nuclease-free water for 5 minutes at room temperature, and then put on ice.

13) Determine RNA concentration.

RNA Reverse Transcription (RT) with Advantage RT-for-PCR Kit

1) Make a mixture as follows:

RNA	1 μg
Random primer	1 λ
Nuclease-free water	? λ
Total	13.5 λ

2) Mix and spin down once.

3) Heat for 2 minutes at 70 ℃.

4) Quench rapidly on ice.

5) Add components as follows:

5 × reaction buffer	4 λ
dNTP mix (10mM each)	1 λ
MMLV reverse transcriptase	1 λ
Nuclease-free water	0.5 λ
Total	6.5 λ

6) Mix and spin down, incubate for two hour at 42 ℃.

7) Spin down the contents of the tube.

8) Dilute cDNA to a final volume of 100 λ by adding 80 λ nuclease-free water.

9) Vortex and spin down.

10) Use 3 μl of diluted cDNA for each 30-μl PCR reaction:

10 × reaction buffer	3 λ
dNTP mix (10mM each)	0.6 λ
Taq	0.6 λ
Primer	0.6 λ
DNA sample	3 λ
Nuclease-free water	22.2 λ
Total	30 λ

Protein Extraction from Cultured Cells

1) Remove cultured medium, add 5ml PBS to rinse the cells.
2) Add 1.5ml Trypsin-EDTA solution to the plate and place it for 5 minutes at 37 ℃.
3) Add 5ml medium, remove the cells to a 15ml tube, spin down for 5 minutes.
4) Resuspend cells in 5ml cold PBS, centrifuge for 5 minutes at 1400rpm at room temperature.
5) Remove PBS carefully by pipetting.
6) Add the IP lysate buffer 90 λ and proteinase inhibitor 10 λ, mix with pipette, on ice.
7) Transfer all mixture to a 1.5ml tube, incubate for 30 minutes on ice.
8) Centrifuge for 10 minutes at 12,000 rpm at 4 ℃.
9) Transfer supernatant (proteins) to a new 1.5ml tube, and keep the tube on ice.
10) Determine protein concentration.
11) Stored at -20 ℃.

Western blot

1) Add 100μg=0.1mg protein sample and 2λ 6×R buffer to a 500λ tube.
2) Heat tubes at 100℃ for 3 minutes.
3) Spin down once.
4) Load 10 λ protein marker and protein samples into the gel. Add 2-3 λ 6×R buffer to each blank wells.
5) Add running buffer (1×SDS), run at 80V for 10 minutes, then at 100V for 1 hour.
6) Add transfer buffer to 3 boxes. Put 2 pieces of sponge and 2 pieces of blot paper in a box. Put 1 membrane in a small box (can't touch the membrane by hands).
7) Remove the gel from glass plates and place the gel in transfer buffer.
8) Wash membrane with methanol one time (20 seconds) and D-water two times (shake 2 minutes for the second time).
9) Make a sandwich on the transfer machine of sponge, blot paper, membrane, gel, blot paper, sponge, make sure to add transfer buffer between each component of the sandwich and roll out any air bubbles at each step.
10) Add transfer buffer to a transfer box, run at 100V for 65 minutes.
11) Shake the membrane in blocking solution (0.75g 0.5% milk in 20ml 1×TBST) for 1 hour.
12) Discard blocking buffer, then wash the membrane with 1×TBST one time.
13) Add 1.5λ primary antibody and 3000 λ 1×TBST, shake for 1 hour.
14) Wash the membrane with TBST 4×5 minutes.
15) Add 0.6λ secondary antibody and 4000 λ 1×TBST, shake for 1 hour.

16) Wash the membrane with TBST 4×5 minutes.

17) Develop picture using ECL color solution.

Immunofluorescence

1) Culture cells in glass coverslips and place coverslips in 3.5cm Petri dish.

2) Wash coverslips with PBS one or two times.

3) Fix cells with 1 ml 2% formaldehyde for 15 minutes (Don't shake).

4) Wash coverslips with PBS 2×5 minutes.

5) Optional: add 0.1% Trition X-100 to break cell membrane for 5 minutes.

6) Wash coverslips with PBS 2×5 minutes.

7) Block nonspecific binding by incubating 1 ml blocking solutions for 30 minutes (0.3g BAS in 10ml PBS).

8) Put a piece of parafilm on a plate, also put a piece of kimwipe with water near parafilm in order to keep moist.

9) Add primary antibody and incubate 1 hour (antibody:1×TBST=1:200).

10) Wash coverslips with PBS 3×5 minutes.

11) Add secondary antibody and incubate 1 hour (antibody:1×TBST=1:500).

12) Wash coverslips with PBS and DAPI (5λ) 1 time, with PBS 2×15 minutes.

13) Add coverslips to slides and seal with nail polish.

14) Observe with fluorescent microscope.

Amplification of the flag-mPTIP plasmid and transform the plasmids into the competent cells

1) Add 8g LB Agar to 200ml D-water and auto-clave.

2) Add 10ul ampicillin (50μg/ml, Amp) in LB Agar solution.

3) Add 100 λ E.coli competent cells and 2 λ flag-mPTIP plasmid to each tube. Incubate on ice for 30 minutes. Then incubate in 42 ℃ water bath for 30-40 seconds. Lastly, incubate on ice for 5 minutes.

4) Add 500 λ S.O.C medium to each tube.

5) Shake at 37 ℃ for 1 hour.

6) Spread 100 λ competent cells on each Amp LB Agar plate.

7) Invert the plate and place in incubator at 37 ℃ overnight.

8) Add 5ml LB Broth and 1.25 λ Amp (200μg/ml) to each 13ml tube. Shake overnight.

9) Transfer 1.5ml LB Broth to 1.5ml tube. Centrifuge the tubes at 12,000rmp for 5 minutes.

10) Remove the supernatant and then add 1.5ml LB Broth to the tube.

11) Centrifuge the tubes and remove the supernatant.

12) Repeat the step 10 and 11.

13) Add PBS to wash the cells.

14) Centrifuge and remove the supernatant.

15) Plasmid extraction was done by a Qiagen kit.

Transfer the flag-mPTIP plasmids to cultured cells (overexpression)

1) Prepare 2×10^5-6×10^5 adherent cells/well (6-wells plate).

2) Remove the old medium. Add 1.5ml DMEM medium to each well.

3) Prepare 2 tubes: add 4 λ DNA (the flag-mPTIP plasmid) and 250 λ DMEM medium to tube 1; add 10 λ transfer reagent (lipofectamine 2000) and 250 λ DMEM medium to tube 2 (The volume is for each well).

4) Combine tube 1 and 2, then incubate for 20 minutes at room temperature.

5) Add 514 λ of the mixed solution to each well.

6) Culture cells for 2 days. After that, Western blot or MTT assay was carried out.

Transfer siRNA-PTIP to cultured cells (knockdown)

1) Prepare 2×10^5-6×10^5 adherent cells/well (24-wells plate).

2) Remove the old medium. Add 1.5ml serum-free DMEM medium to each well.

3) Prepare 2 tubes: add 10λ siRNA-PTIP and 190λ serum-free DMEM medium to tube 1; add 5λ transfer reagent and 195λ serum-free DMEM medium to tube 2 (The volume is for each well).

4) Combine tube 1 and 2, then incubate for 20 minutes at room temperature.

5) Add 400 λ the mixed solution to each well.

6) Culture cells for 2 days. After that, Western blot or MTT assay was performed.

MTT assay (overexpression or knockdown)

1) After overexpression or knockdown as described above, the cells are ready for further experiment.

2) Remove old medium, and add 2ml MTT solution to each well (24-wells plate).

3) Incubate at 37℃ for 3 hours.

4) Remove MTT solution and add 1ml SMDO solution to each well.

5) Shake for 5 minutes.

6) Determine the cell proliferation by a plate reader machine (BIO-RAD) on day 1, day 2 and day 3.

Chapter Four

Results

1. The PTIP gene is expressed in MCF-7 and T47D breast cancer cells.

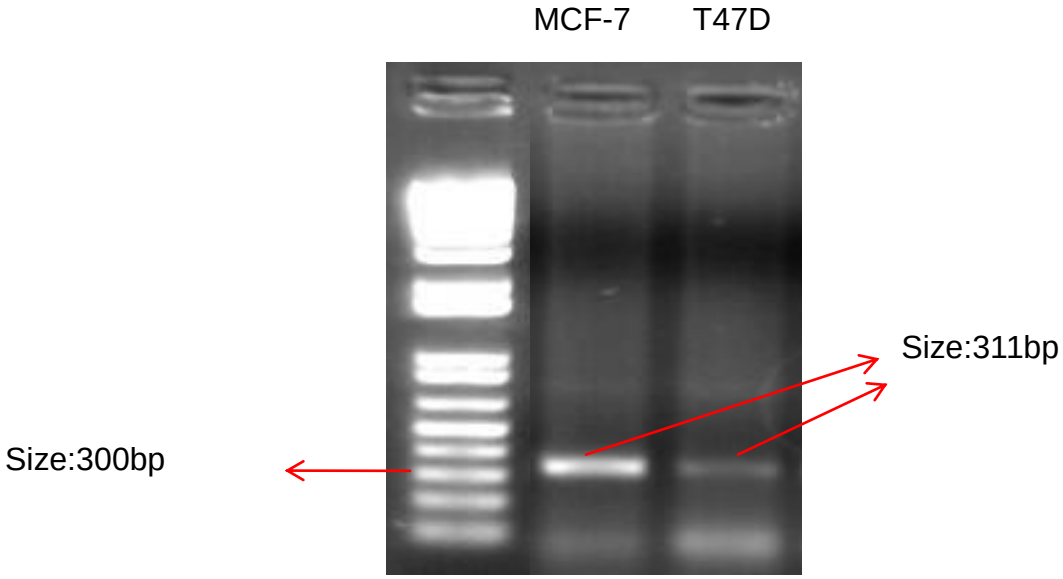

Figure 4. Gene expression of PTIP in MCF-7 and T47D human breast cancer cells

The gene expression of PTIP in MCF-7 and T47D was determined by RT-PCR. The expected size of the PCR product is 311bp. The result indicates that PTIP is expressed in these two human breast cancer cell lines.

2. The PTIP protein is expressed in MCF-7 and T47D breast cancer cells.

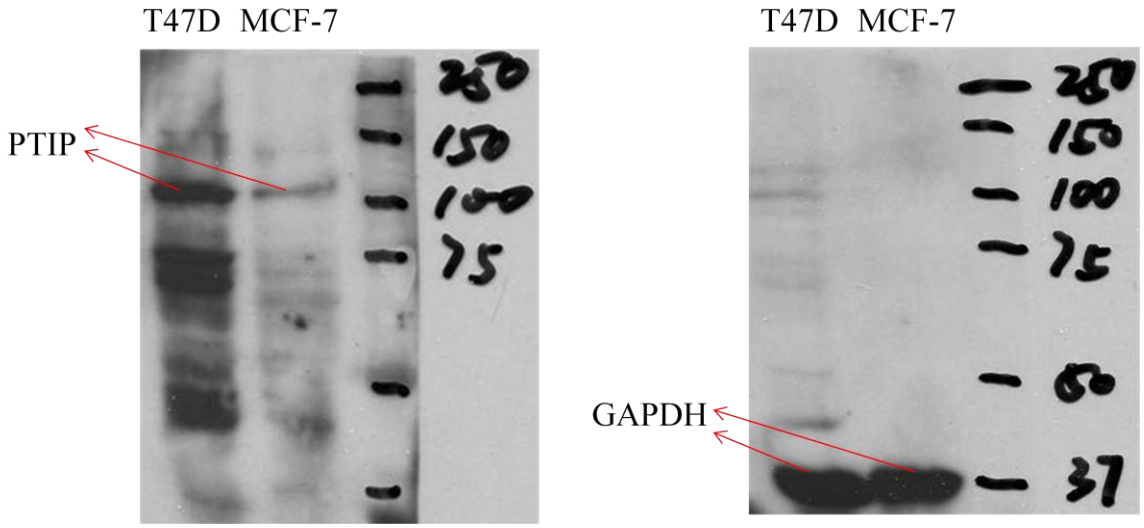

Figure 5. Left, protein expression of PTIP in MCF-7 and T47D human breast cancer cells; right, protein expression of GAPDH in MCF-7 and T47D human breast cancer cells.

The protein expression of PTIP in MCF-7 and T47D was determined by Western Blot. We ordered anti-PTIP from Sigma and got the size of PTIP protein was 120KD. Our result reveals that PTIP is expressed in these two human breast cancer cell lines on the protein level.

In addition, the protein of GAPDH was examined by Western Blot in order to serve as the loading control. The expression level of GAPDH in MCF-7 human breast cancer cells is almost the same as that in T47D human breast cancer cells. The protein molecular weight of GAPDH is about 37KD.

3. PTIP is localized in the nucleus of MCF-7 and T47D breast cancer cells.

MCF-7

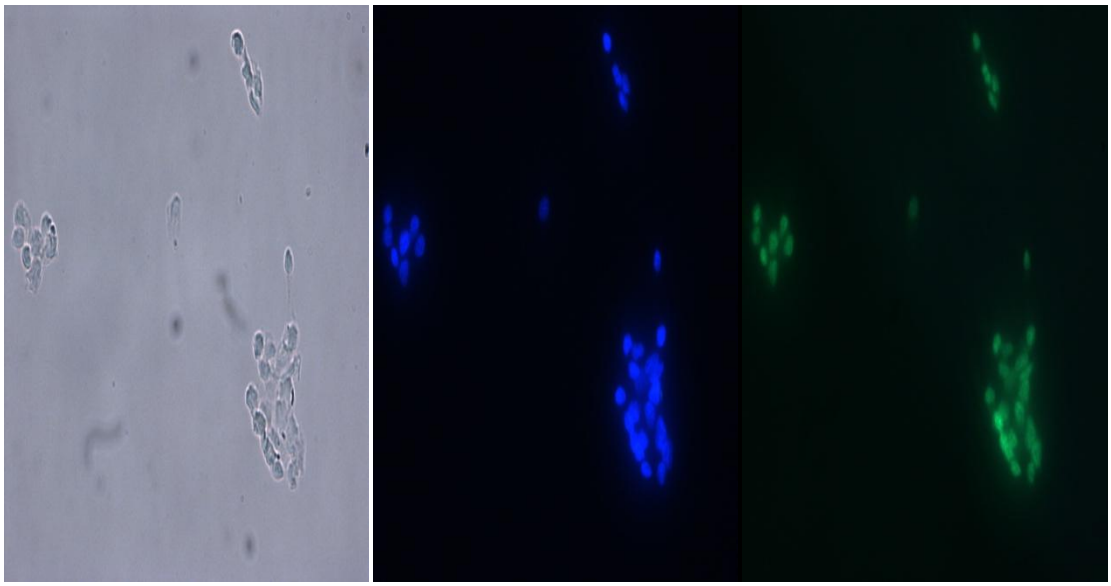

Figure 6-1. Subcellular localization of PTIP in MCF-7 human breast cancer cells by immunofluorescent assay. Magnification: 1220×20.

Left panel: phase contrast; middle panel: DAPI staining (blue); right panel: PTIP staining (green).

T47D

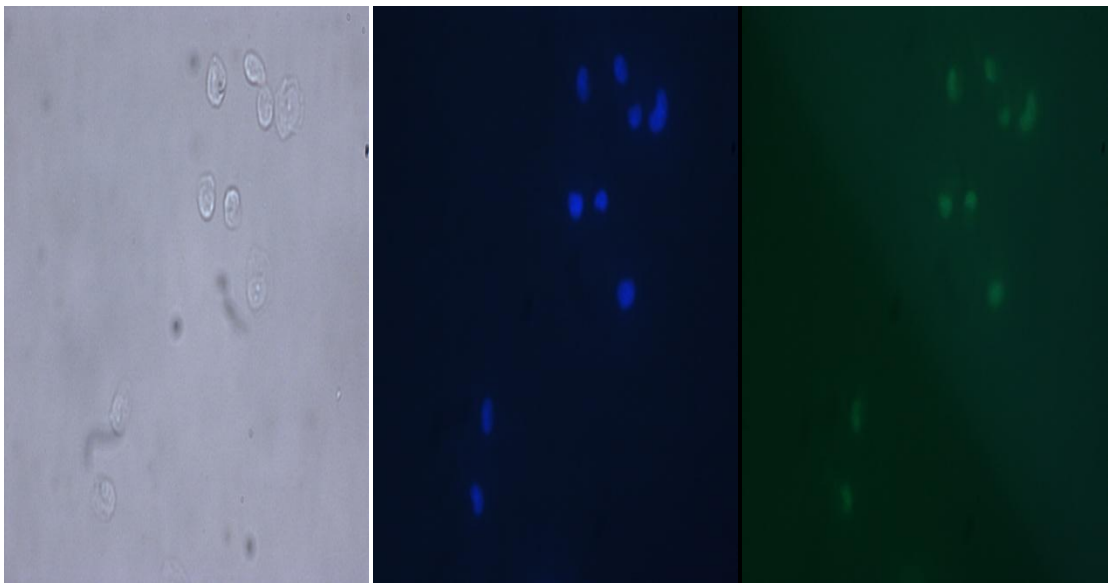

Figure 6-2. Subcellular localization of PTIP in T47D human breast cancer cells by immunofluorescent assay. Magnification: 1220×20.

Left panel: phase contrast; middle panel: DAPI staining (blue); right panel: PTIP staining (green).

The phase contrast images reveal cellular morphology. The blue images show the location of nuclei and the green pictures disclose the location of PTIP. After merging the phase contrast, blue and green images together, we determine that the subcellular localization of PTIP in MCF-7 and T47D human breast cancer cells is in the nucleus, not in the cytoplasm. The result suggests that PITP might function in the nucleus to regulate gene expression. Furthermore, the green signal in MCF-7 cells is much stronger than that in T47D cells, suggesting that PTIP is expressed at a higher level in MCF-7 cells than in T47D cells.

4. Amplification of the flag-mPTIP plasmid in E. coli

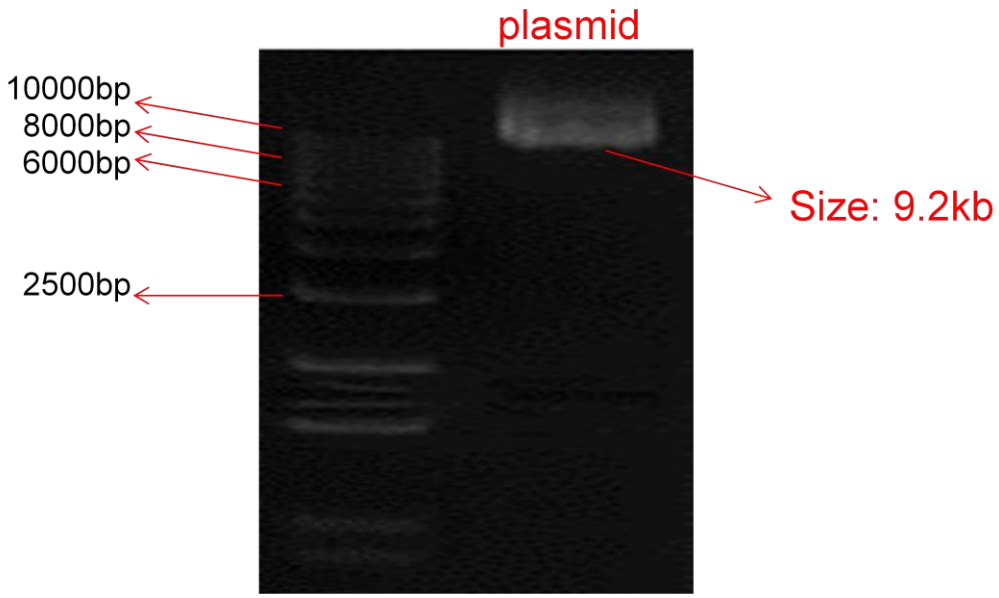

Figure 7. Gel electrophoriesis of the flag-mPTIP plasmid.

E.coli was transformed by the flag-mPTIP plasmid. Subsequently, a single colony was picked up for plasmid amplification and purification. Gel electrophoresis was used to detect the purified DNA. We observed a single band of 9.2 kb, which is the expected size of the flag-mPTIP plasmid.

5. GFP plasmid DNA transfection

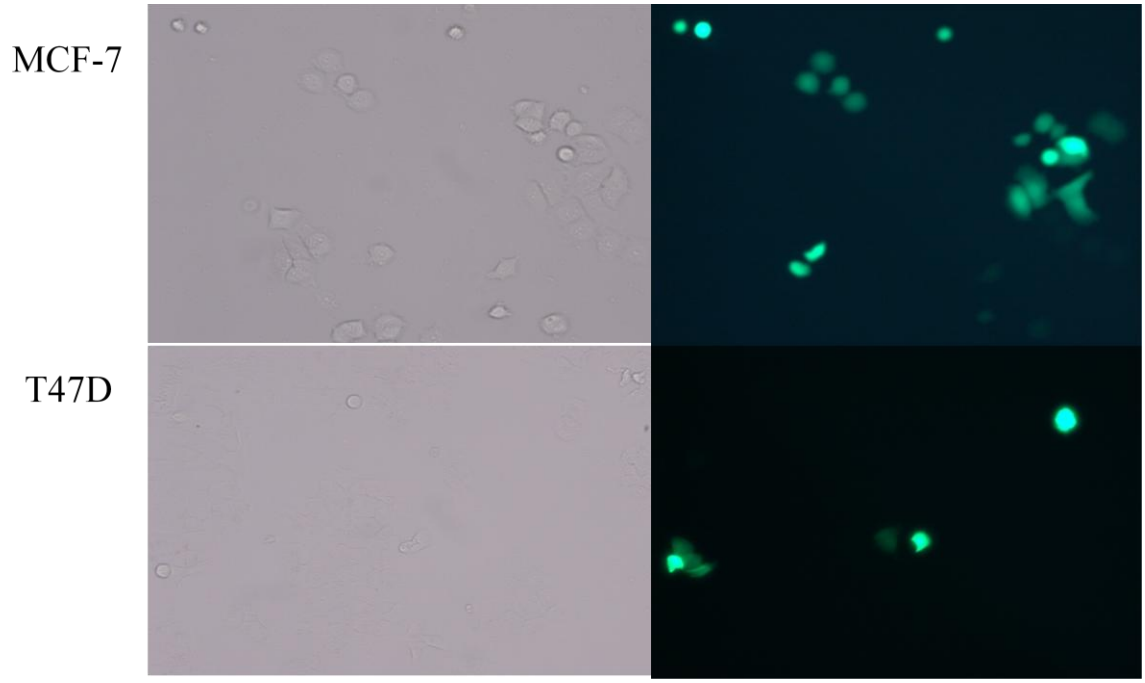

Figure 8. Transfection of the GFP plasmid in MCF-7 and T47D human breast cancer cells. Magnification: 1220×40.

Because GFP is easy to be transferred into mammalian cells and also is convenient to be monitored by a fluorescent microscope, we used GFP to practice the transfection skill.

The top row represents the breast cancer cell line MCF-7 and the bottom row represents the breast cancer cell line T47D. The gray pictures (fluorescence) show cells morphology. The green pictures (fluorescence) illustrate GFP signal.

On the top row of MCF-7 cells, for example, there are two cells at the left-top corner of the gray picture and two green dots at the left-top corner of the green picture. The transfection ratio for MCF-7 cells is more than 90%. The bottom row is T47D cells. The cell morphology is not very clear, but we still can see a larger cell at the right-top

corner and a large green dot at the same location in the green picture. The transfection ratio for T47D cells is about 70%.

6. Western blot of ectopically expressed flag-mPTIP

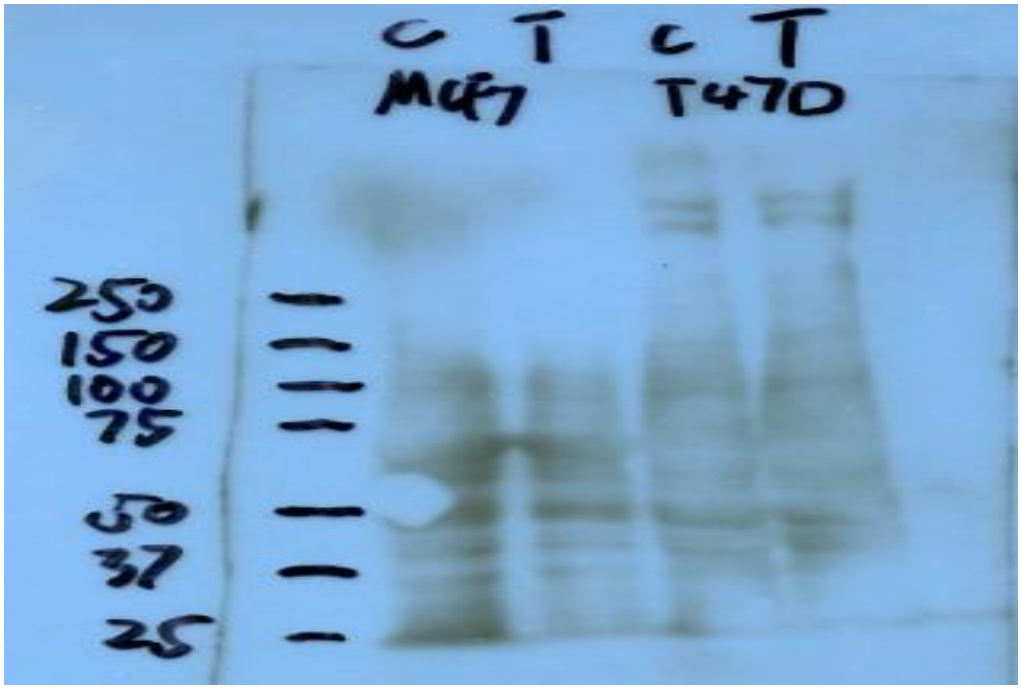

Figure 9. Protein expression of the flag-mPTIP plasmid in MCF-7 and T47D breast cancer cells. C, control without the flag-mPTIP plasmid; T, transfection with the flag-mPTIP plasmid.

After transfection, cell lysates were prepared for Western blot. We did not detect a specific band of flag-mPTIP (the expected size is about 120kD). Instead, many nonspecific bands were noticed.

7. The protein expression of PTIP in MCF-7 human breast cancer cells (control/knockdown)

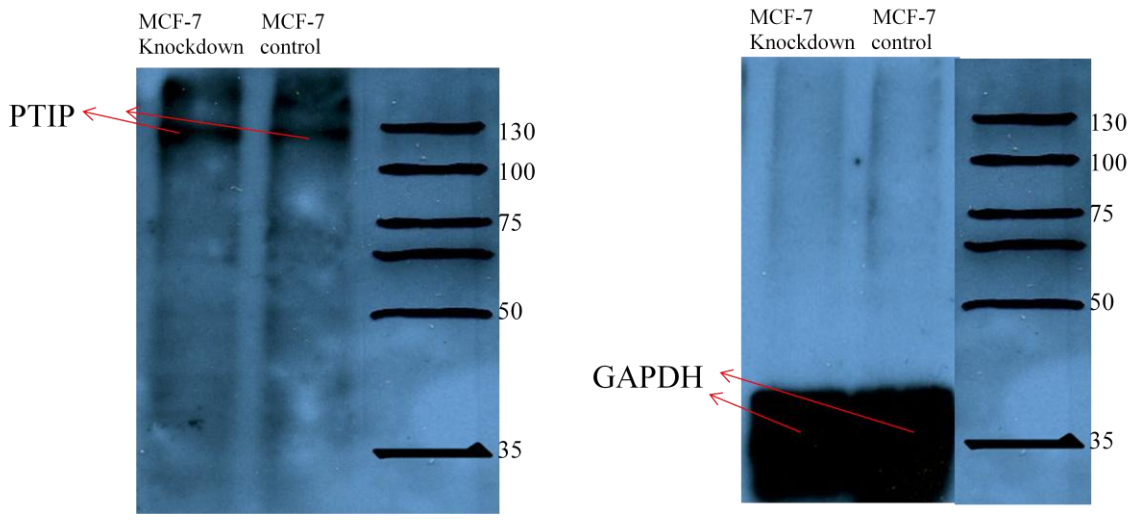

Figure 10. Left, Protein expression of PTIP in MCF-7 human breast cancer cells; right, protein expression of GAPDH in MCF-7 human breast cancer cells (knockdown/control).

Small interfering RNA (siRNA) binds to its target mRNA and stops translation. Thus, siRNA is a powerful approach to knock down protein expression of a specific gene. We used this approach in an attempt to knock down PTIP in MCF-7 cells and T47D cells.

The protein expression of PTIP control and PTIP knockdown in MCF-7 was determined by Western blot (Figure 10, left panel). PTIP (about 120KD) was detected in both PTIP control and knockdown samples.

The protein of GAPDH was examined in order to work as the loading control (Figure 10, right panel). The expression of GAPDH in PTIP control cells is almost the same as that in PTIP knockdown cells. The protein molecular weight of GAPDH is about 37KD.

PTIP knockdown	55.74%	PTIP control	44.26%
GAPDH (PTIP knockdown cells)	51.80%	GAPDH (PTIP control cells)	48.20%
Effect (knockdown)	1.076	Effect (control)	0.918
Effect=PTIP/GAPDH			

Table 1. Western blot quantification by Image J.

By using the software ImageJ to quantify Western blot results, we found that the protein expression of PTIP in the knockdown cells is higher than that in the control cells.

8. MTT cell proliferation assay (PTIP knockdown)

Day 1				**Average**					**Average**	**Effect**
MCF-7 control	1.329	0.806	0.699	0.945	MCF-7 knockdown	1.488	0.695	0.516	0.900	0.952
T47D control	2.282	1.716	2.255	2.084	T47D knockdown	2.044	2.532	2.463	2.346	1.126
Day 2										
MCF-7 control	1.089	1.34	1.499	1.309	MCF-7 knockdown	1.277	1.482	1.209	1.323	1.010
T47D control	2.513	2.014	1.945	2.157	T47D knockdown	1.637	1.039	1.898	1.525	0.707
Day 3										
MCF-7 control	0.147	0.141	0.06	0.116	MCF-7 knockdown	2.233	0.527	0.826	1.195	10.305
T47D control	1.711	0.967	2.168	1.615	T47D knockdown	2.098	1.596	2.348	2.014	1.247

Effect=average knockdown/average control

Table 2. MTT assay for PTIP knockdown in MCF-7 and T47D human breast cancer cells.

MTT produces the purple color in living cells. MTT assay determine the cell proliferation due to measuring the absorbance of purple solution at the wavelength 530nm.

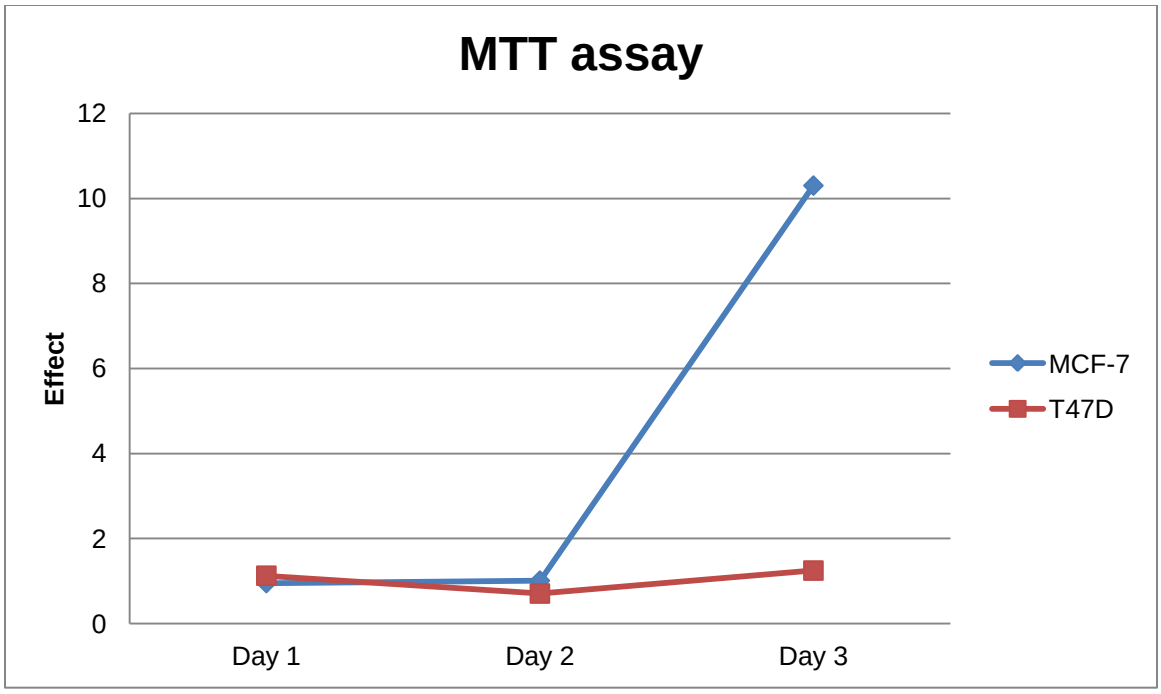

Figure 11. MTT assay for PTIP knockdown in MCF-7 and T47D human breast cancer cells.

After transferring siRNA-PTIP to MCF-7 and T47D human breast cancer cells, we determined the cell proliferation of these two cell lines by MTT assay on DAY 1, DAY 2 and DAY 3. As shown in Table 1 and Figure 11, the cell proliferation rate of T47D didn't show much variance, however, the cell proliferation rate of knockdown MCF-7 cells increased relative to normal MCF-7 cells after DAY 2.

Chapter Five

Conclusions

1. PTIP is expressed in human breast cancer cell lines MCF-7 and T47D on both RNA level and protein levels. The protein molecular weight of PTIP is about 120KD.
2. The subcellular localization of PTIP in MCF-7 and T47D human breast cancer cells is nucleus.
3. The GFP plasmid can be transferred into MCF-7 and T47D cells at a ratio of 90% and 70% respectively.
4. The flag-mPTIP plasmid is not suitable for direct transfection in MCF-7 and T47D cells.
5. PTIP knockdown through siRNA results in an increased rate of cell proliferation of knockdown MCF-7 cells relative to normal MCF-7 cells.
6. Taken together, PTIP might play a suppressor role in breast cancer.

Chapter Six

Discussion and Future Study

Discussion

During this research process, there are some unclear results the need to be determined in the future.

The result of ectopic PTIP overexpression in human breast cancer cell lines MCF-7and T47D didn't meet our expectation. Maybe the size of the flag-mPTIP plasmid is too large (9.2kb) to be directly transferred into breast cancer cells.

The cell proliferation rate of T47D didn't show much variance; however, the cell proliferation rate of knockdown MCF-7 cells increased relative to normal MCF-7 cells after DAY 2. So PTIP may not work in all human breast cancer cell lines.

Future study

In this research, we used breast cancer cell lines MCF-7 and T47D. In future studies, other breast cancer cell lines, such as MDA-MB-231, BT-474 and SK-BR-3, can be tested. In addition, the primary breast cell line MCF-10 can be utilized as the control cell line. Immunohistochemistry assay of PTIP in human breast cancer tissues and normal breast tissues would be a good experiment to do.

PTIP has been shown to be capable of regulating histone methylation. Therefore, whether PTIP regulates histone methylation in human breast cancer cells remains an interesting question to be examined.

Finding a way to transfer such a large plasmid, flag-mPTIP, into human breast cancer cells is a significant assignment for determining the function of PTIP in breast cancer. The virus-based transfection may be an alternative approach in future studies.

PTIP is related to histone methylation, so we can use Western blot to determine the protein expression of histone methylation, such as H3K4me, after PTIP knockdown and overexpression.

Apply qRT-PCR to qualify the gene PTIP in order to compare the gene expression of PTIP in various human breast cancer cells.

The technique microarray can analyze expression level of thousand genes. So we can use it to compare gene expression changing between PTIP control and PTIP knockdown, or PTIP control and PTIP overexpression.

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Appendix

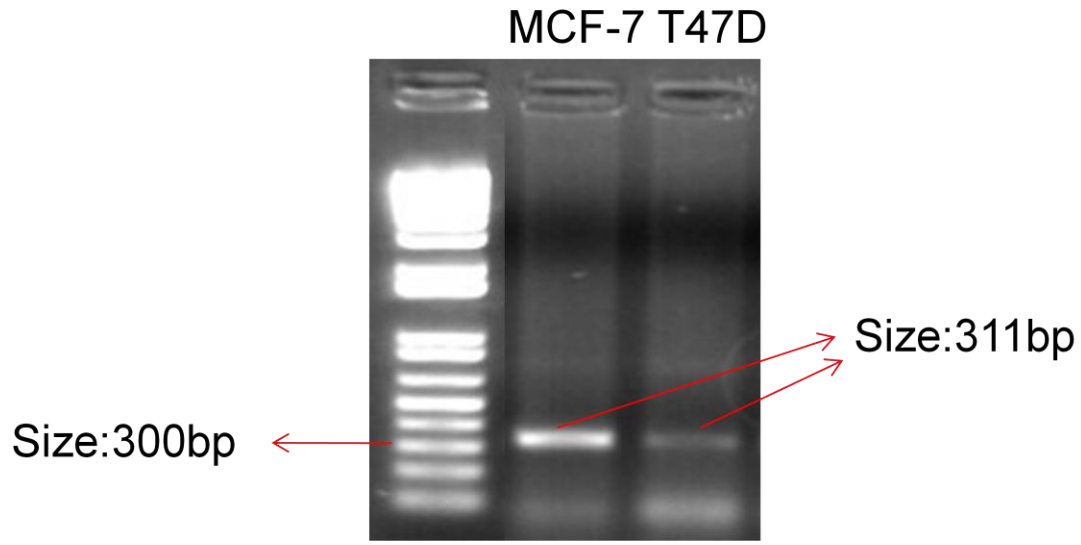

Figure 4. Gene expression of PTIP in MCF-7 and T47D human breast cancer cells

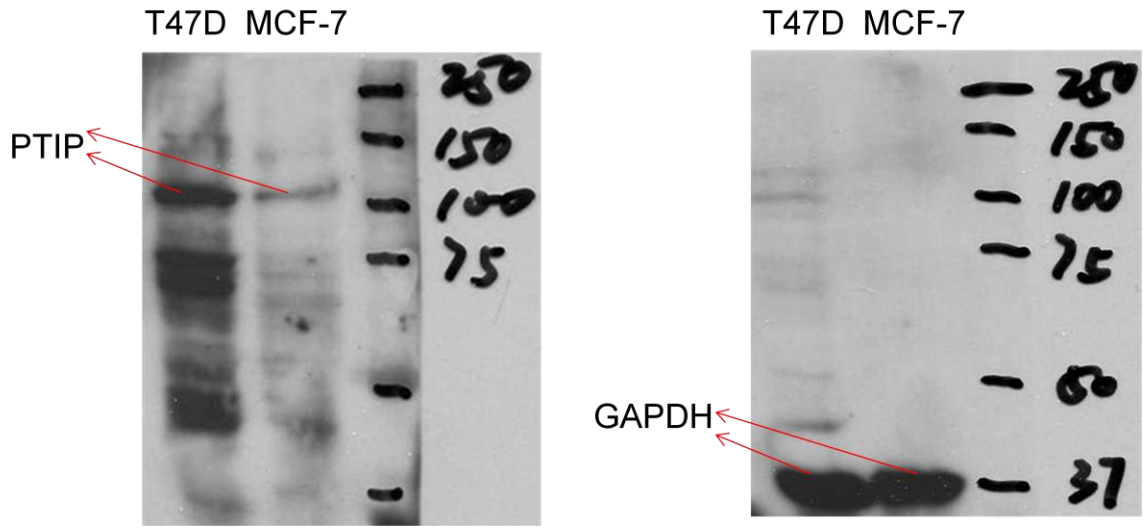

Figure 5. Left, protein expression of PTIP in MCF-7 and T47D human breast cancer cells; right, protein expression of GAPDH in MCF-7 and T47D human breast cancer cells.

MCF-7

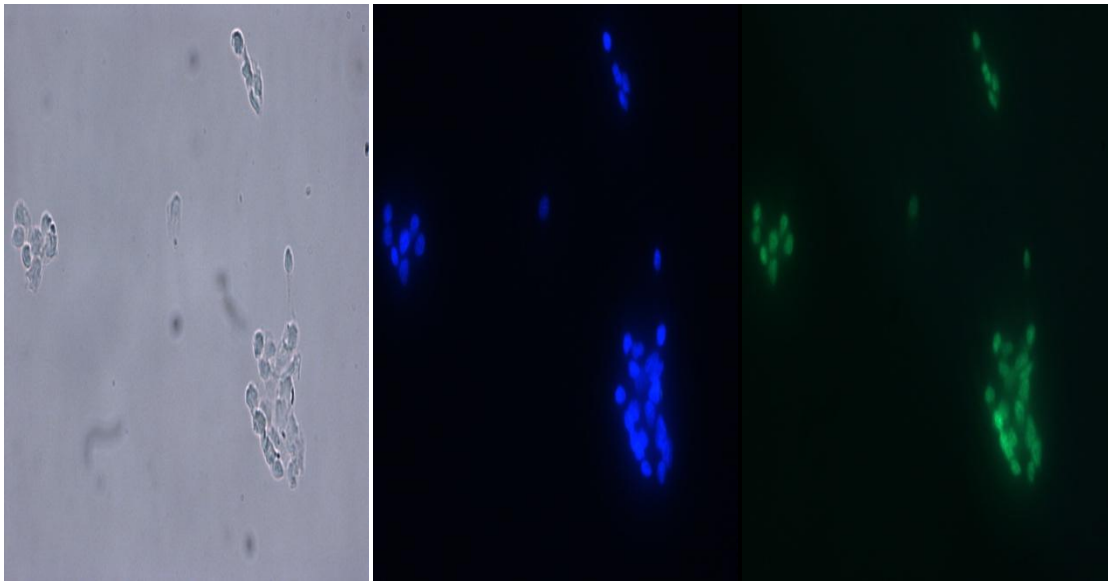

Figure 6-1. Subcellular localization of PTIP in MCF-7 human breast cancer cells by immunofluorescent assay

Left panel: phase contrast; middle panel: DAPI staining (blue); right panel: PTIP staining (green).

T47D

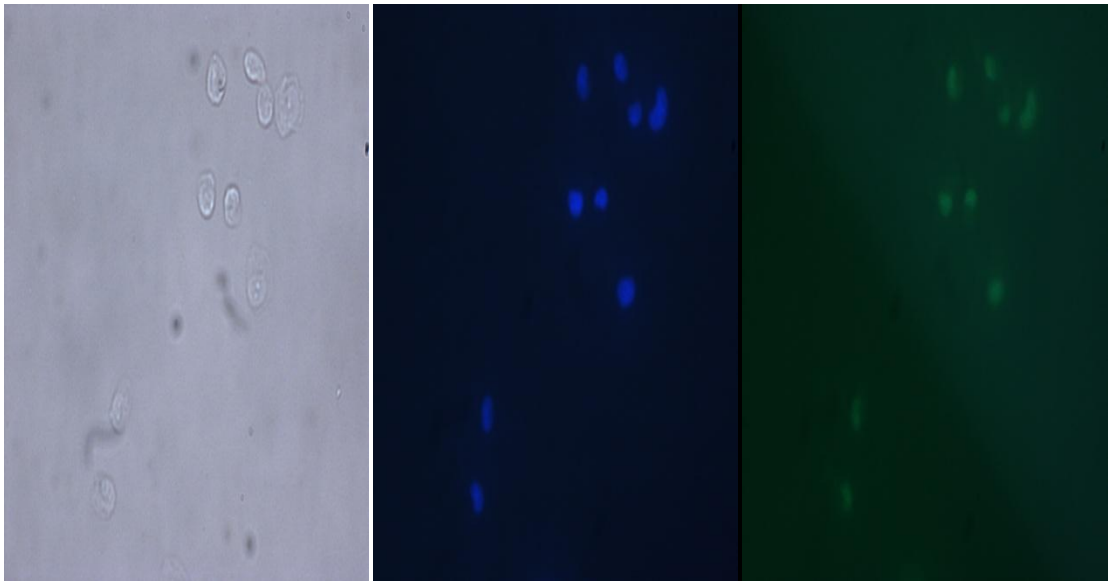

Figure 6-2. Subcellular localization of PTIP in T47D human breast cancer cells by immunofluorescent assay.

Left panel: phase contrast; middle panel: DAPI staining (blue); right panel: PTIP staining (green).

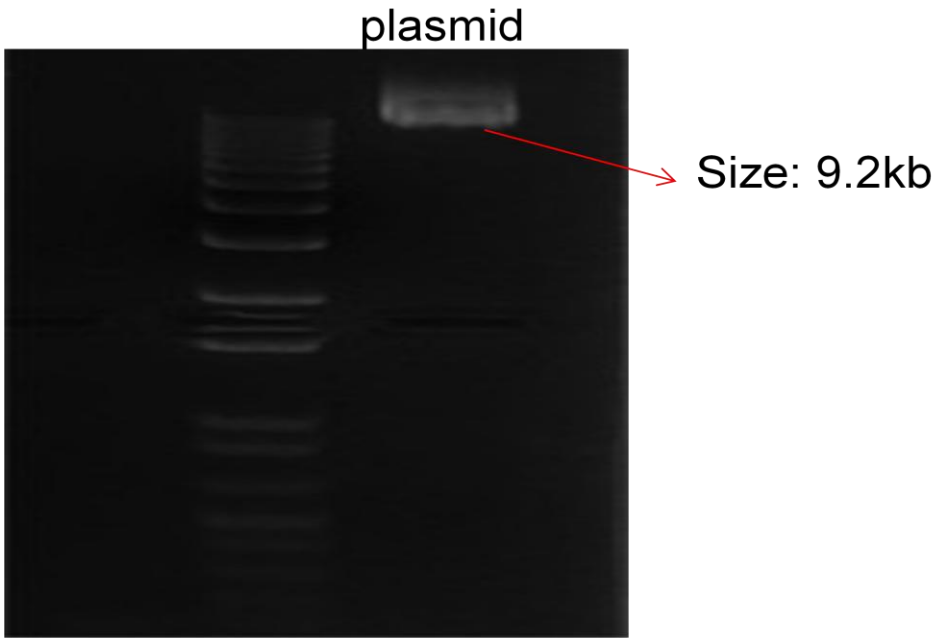

Figure 7. Gel electrophoresis of the flag-mPTIP plasmid.

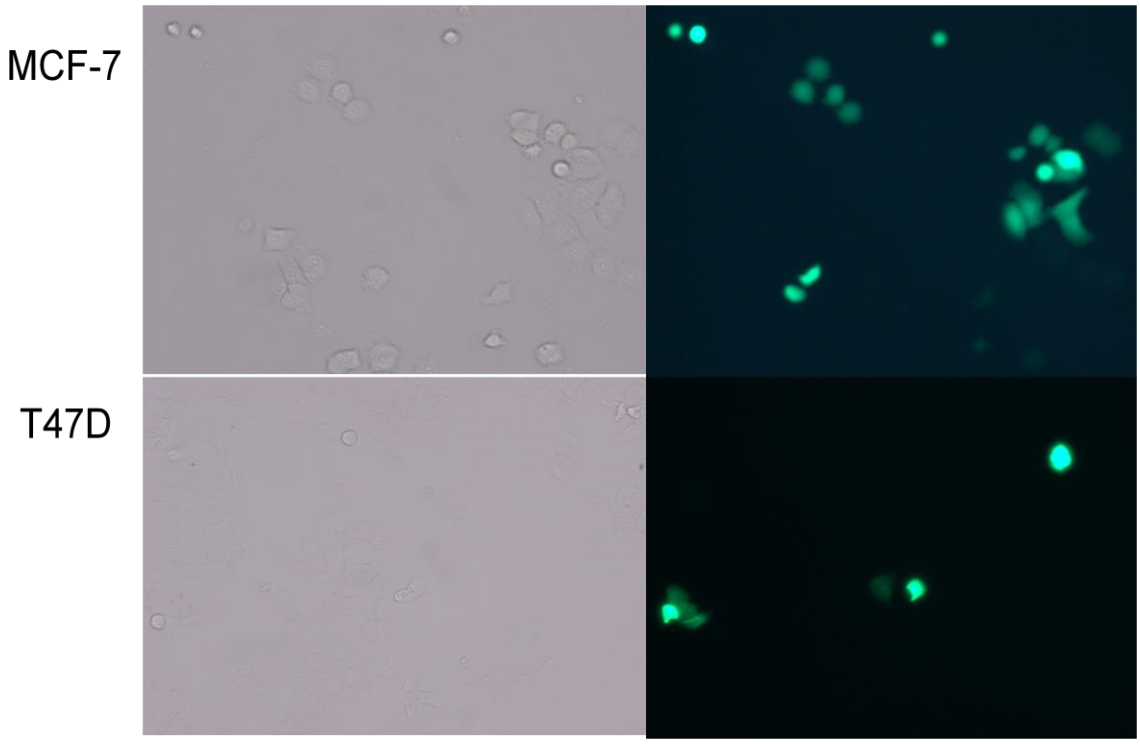

Figure 8. Transfection of the GFP plasmid in MCF-7 and T47D human breast cancer cells.

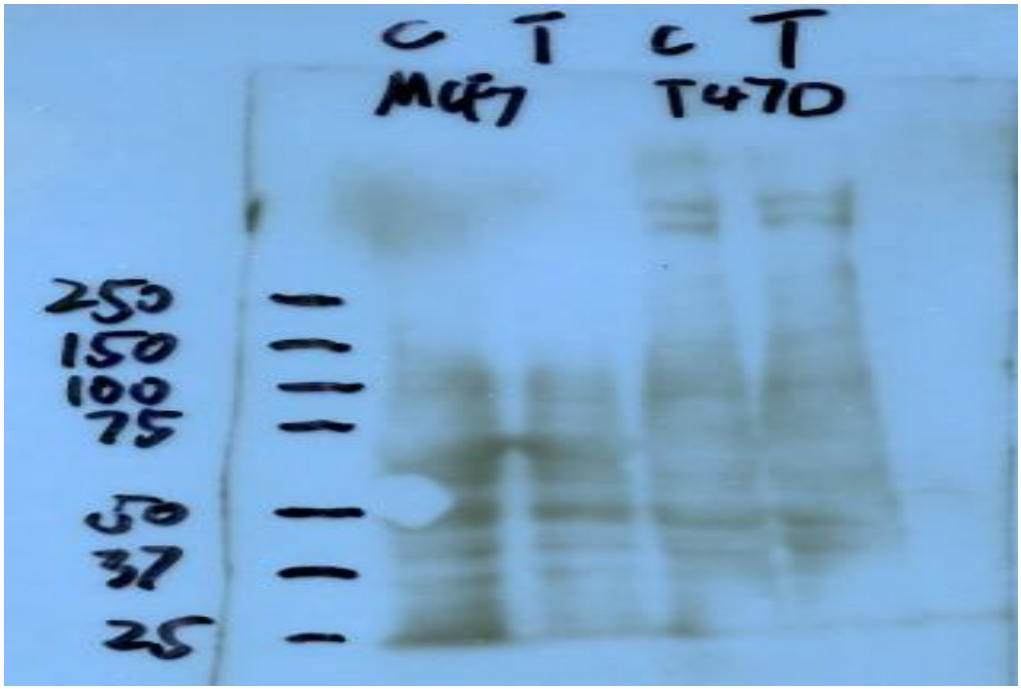

Figure 9. Protein expression of the flag-mPTIP plasmid in MCF-7 and T47D breast cancer cells. C, control without the flag-mPTIP plasmid; T, transfection with the flag-mPTIP plasmid.

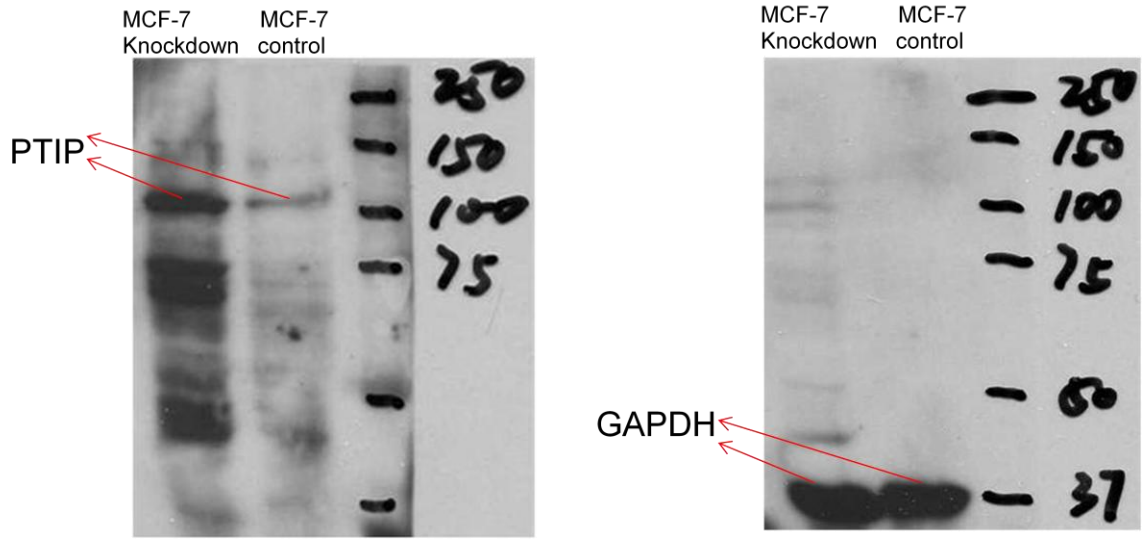

Figure 10. Left, Protein expression of PTIP in MCF-7 human breast cancer cells; right, protein expression of GAPDH in MCF-7 human breast cancer cells (knockdown/control).

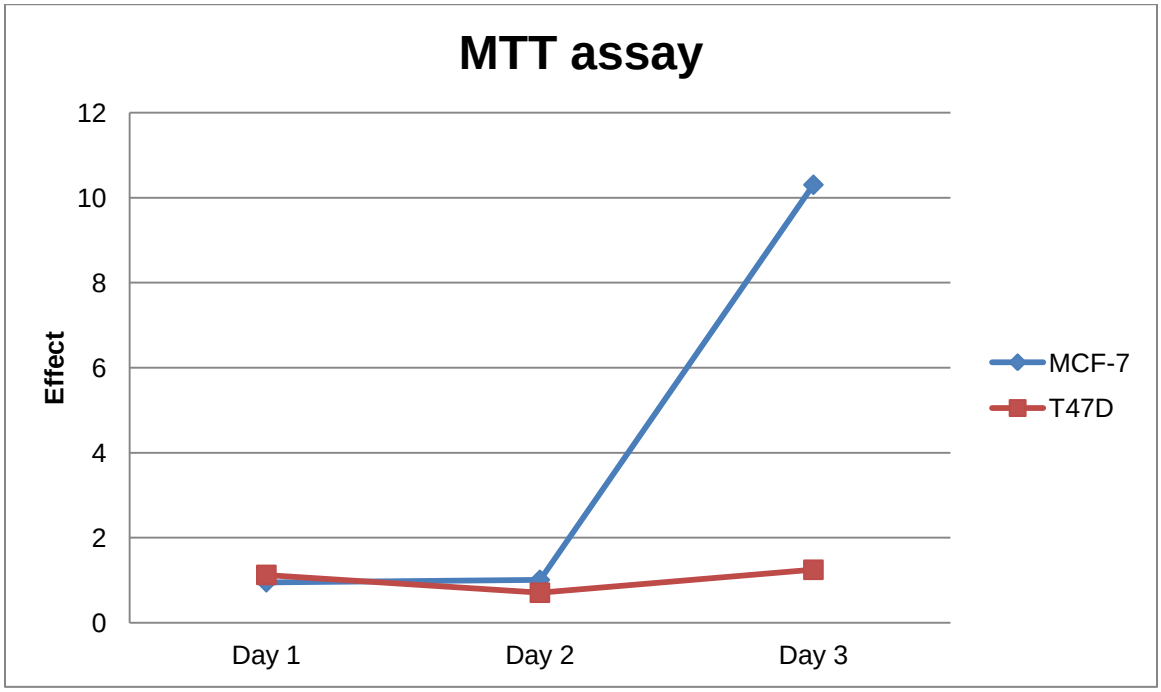

Figure 11. MTT assay for PTIP knockdown in MCF-7 and T47D human breast cancer cells.

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01/2010-Present Master of Science in Biology

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Research Experience

01/2010-Present Research Project: The Role of PTIP in Breast Cancer

01/2010-12/2010 Research Assistant in Dr. Guozhang Zhu's lab, Marshall University

02/2008-06/2008 Graduate Thesis: Preparation and Characterization of Anodic Aluminum Oxide membrane

10/2007-02/2008 Publication: Li, Y., Tian, XD., Ouyang, GQ., Niu, LN., Treating NH_3-N Containing Wastewater with Microwave Irradiation. J. Technological Development of Enterprise, 2008, 27(2): 21-24

11/2007-02/2008 Graduate Project: Design of an alcohol factory with annual producing capacity of 30,000 tons consumable alcohol

09/2007-11/2007 Team work: Design of 200 cubic meters alcohol fermenter and cooling equipment

Internship with Research

- **06/2007-08/2007 Served as an intern in Yanjing Brewery CO., LTD. (Hunan Province, China)**
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01/2011-05/2012 Teaching Assistant in Biology Department, Marshall University

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