의학석사학위논문

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허샘의 의학석사 학위논문을 인준함

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위 원 장 _____ (인)

부위원장 _____ (인)

위 원 _____ (인)

Abstract

PPM1H Regulates the Response to Paclitaxel in Triple Negative Breast Cancer.

Saem Hur

Interdisciplinary Program in Cancer Biology

Seoul National University

College of Medicine

There has been an increasing interest in patient-derived tumor xenograft (PDX) models for cancer research. Since these models mostly retain histological and genetic characteristics of patients, they are also useful for studies that elucidates the cancer cell survival during cytotoxic chemotherapy. Paclitaxel is one of the well-known chemotherapeutic agents for breast cancer patients, but most advanced cases often develop resistance to the paclitaxel during the treatment. To understand the molecular adaptation of cancer cells during the paclitaxel treatment, we performed *in vivo* paclitaxel experiment using

a patient-derived xenograft model. We established two triple negative breast cancer (TNBC) PDX models separately derived from single patient's primary and recurrent tumor. For each TNBC PDX model, ten mice were intraperitoneally (IP) injected with either PBS or paclitaxel (15mg/kg) for 4 weeks. Both PDX models showed statistically significant tumor growth inhibition after paclitaxel treatment and we obtained transcriptome and exome sequencing data from both groups. Transcriptome data identified thirteen genes that were commonly upregulated after paclitaxel treatment in both PDX models ($p \leq 0.05$, 2-fold ≥ 0.5) and four genes (CXCL10, FMO2, PPM1H and RNF150) showed differential expression with the adjusted p value of less than 0.1. Whole-exome sequencing data were used to identify the fluctuations of somatic mutations in paclitaxel-treated tumors. Interestingly, PPM1H, one of the genes that were upregulated in transcriptome data analysis, had changes in the prevalence of somatic mutations in paclitaxel-treated tumors. We further observed that the paclitaxel treatment increased PPM1H gene expression levels in both TNBC PDX tumors and breast cancer cells. PPM1H gene expression was also upregulated by short-term *in vitro* paclitaxel treatment using

various breast cancer cell lines. We established a breast cancer cell line (MDA-MB-231) that stably overexpressed PPM1H and tested various phenotypic aspects. MDA-MB-231 cells overexpressing PPM1H had no substantial effects on cell proliferation, migration, and invasion, but were more sensitive to paclitaxel in both 2D and 3D cell cultures. Increased PPM1H expression levels also elevated tumor suppressor p27 protein levels. Our data demonstrate that breast cancer cells undergo diverse genomic changes during paclitaxel treatment and PPM1H may regulate the paclitaxel sensitivity in breast cancer cells. Further studies are needed to clarify the mechanistic pathways of PPM1H.

Keywords: breast cancer; patient-derived tumor xenograft (PDX); PPM1H; p27

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List of Figures and Tables

List of abbreviation

FACs	Fluorescence-activated cell sorting
qRT-PCR	Quantitative real-time polymerase chain reaction
FPKM	Fragments Per Kilobase of transcript per Million mapped reads
DEG	Differentially expressed gene

Contents

Introduction

Breast cancer is the most common cancer in women worldwide and is the primary cause of cancer-induced mortality [1]. It accounts for approximately 23% of all cancer cases and 14% of cancer deaths [2].

Although various therapeutic approaches in diagnosing and treating breast cancer have developed, several important clinical and scientific problems are still unresolved. These are related to prevention, diagnosis, tumor progression and recurrence, treatment, and therapeutic resistance [3]. Understanding all these problems is complicated since breast cancer is a highly heterogeneous at both the molecular and clinical level [4,5].

Breast cancer is composed of distinct subtypes associated with different clinical outcomes such as luminal A, luminal B, HER2 over-expression, basal and normal-like tumors [6]. These five intrinsic subtypes can be also categorized into immunohistochemistry (IHC) defined subtypes: hormone receptor-positive (ER+ or PR+), HER2 positive (ER-, PR-, and HER2+), and triple-negative breast cancer (TNBC) (ER-, PR-, and HER-) [6,7]. Compared to other subtypes of breast cancer, triple negative breast cancers (TNBCs) are the most aggressive and the most difficult breast cancer subgroup to treat due to its unresponsiveness to current clinical targeted therapies, high rate

of recurrence, and poor prognosis [8,9]. Thus, it is critical to detect therapeutic targets and develop more effective medicine for the treatment of TNBCs [9].

Traditionally, cell line derived xenograft models have been widely used for cancer research. However, using these models results in alterations in biological properties of original tumor such as genetic information, growth and invasion properties and loss of specific cell populations [10]. In recent years, there has been renewed interest in the development of patient-derived tumor xenografts (PDX) models for cancer research. Since PDX models are developed by the immediate transfer of fresh tumor tissue from patients into immunosuppressed mice, they mostly retain the principal characteristics of donor tumors such as molecular diversity, cellular heterogeneity, and histology seen in patient tumors [11]. They are models that allow us to more effectively evaluate drug delivery, therapeutic response and biomarker expression of cancer cells [10,11,12].

Paclitaxel is a well-known breast cancer chemotherapeutic agent that has been successfully used in the clinical treatment [13]. It acts by blocking mitosis by promoting and stabilizing microtubule formation [14]. In the most advanced cases, however, tumor resistance to paclitaxel limits the effectiveness of current cancer therapies. Since toxicity to normal tissues restricts the amount of drug that can be systemically applied to and the

effective amount of drug that can reach the tumor is limited, therapeutic resistance is a major clinical barrier [14].

In this study, we hypothesized that there is a gene controls the response to paclitaxel in triple negative breast cancer. Through data analysis based on exome and transcriptome sequencing of TNBC PDX models, PPM1H was selected to be the best candidate gene for this study. It has been reported previously that PPM1H has a role in trastuzumab resistance by reduction in protein levels of the tumor suppressor p27 [15]. We performed *in-vitro* and *in-vivo* studies to investigate the possible role of PPM1H in paclitaxel treatment of triple negative breast cancer.

Materials and Methods

In vivo xenograft and paclitaxel experiments

In vivo paclitaxel experiment using a patient-derived xenograft model was performed (Figure 1). We established two TNBC PDX models separately derived from single patient's primary and recurrent tumor. For each TNBC PDX model, control (n=5) and paclitaxel (n=5) mice were intraperitoneally (IP) injected with either PBS or paclitaxel (15mg/kg) for 4 weeks. With remaining TNBC PDX tumors, transcriptome and exome sequencing data were analyzed.

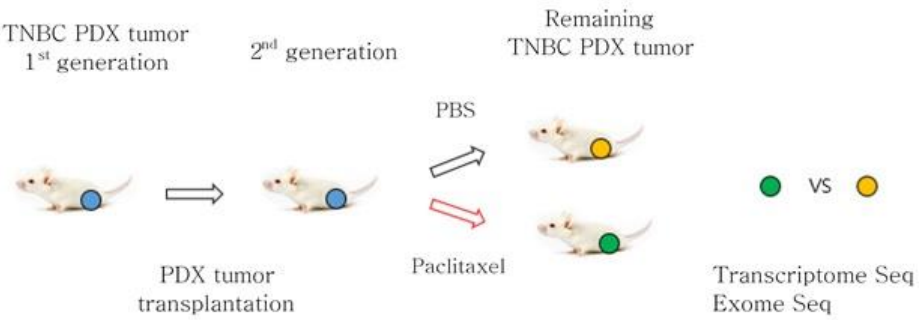


Figure 1. The scheme of the experimental design. We performed in vivo paclitaxel experiment using a patient-derived xenograft model.

Cell culture

MDA-MB- 231 cells were cultured in Dulbecco's Modified Eagle's Medium with 10% FBS, 1% penicillin/streptomycin. PPM1H overexpressed MDA-MB-231 cells were cultured in Dulbecco's Modified Eagle's Medium with 10% FBS, 1% penicillin/streptomycin, and 0.05 ug/ml puromycin. Cells were sub-cultured every 3 to 4 days.

Quantitative real-time PCR

RNAs were extracted from cells lysated by TRIzol (Favorgen, Taiwan). Prime Script 1st strand cDNA Synthesis Kit (Takara, Japan) was used for reverse transcription of RNA, and then cDNA was amplified by using Power SYBR® Green PCR Master Mix (Applied Biosystems). The sequence of the primer used for PPM1H is forward 5'-CCAATTTCATGGGCGGCATC-3' and reverse 5'- TCCAC CTCGTCCTGAGACAG-3'. The sequence of the primer used for MDR1 is forward 5'- CCCATCATTG CAATAGCAGG - 3' and reverse 5'- GTTCAAACTTCTGCTC CTGA - 3'.

Cloning of human PPM1H cDNA

Human PPM1H cDNA was amplified by RT-PCR from SK-BR-3 human breast cancer cell lines were obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea) with the primers 5'-

GCTAGCGCCACCATGCTCACTCGAGTGAAATCT and 5'-CCGG AATTCCGGTCATGACAGCTTGTTTCCAT GT. The fragment of the PCR product was ligated at the *NheI* and *EcoRI* sites of Pgem-T Easy vector (Promega, CA, USA). The resulting sequence was inserted into pCDH-CMV-MCS-EF1-RFP-T2A-Puro (System Biosciences, CA, USA).

Western blot and antibodies

Proteins were harvested with RIPA buffer (Thermo scientific, Palm Springs, CA, USA), protease&phosphatase inhibitor and 0.5M EDTA solution. Protein concentration was measured by BCA assay kit (Thermo scientific, Palm Springs, CA, USA). Cell lysates were loaded onto 10% gels and transferred to a PVDF membrane. The membrane was blocked with 5% skim milk and incubated with primary antibody overnight at 4℃. The peroxidaseconjugated secondary antibody was used for detection. Bands were detected by LAS. Antibodies against PPM1H, p-27 and phospho p-27 were purchased from Abcam (Cambridge, MA, USA).

Cell proliferation assay

Cells were seeded in triplicate into 96-well plates at a density of 3,000 cells per well. Proliferation assays were conducted by using CellTiter Glo Luminescent Cell Viability Assay

kit (Promega, Madison, USA) or MTT solution following the manufacturer's protocol.

Migration and invasion assay

For migration assay, 2 x 10^4 cells were seeded in an insert (8μm pore size) with serum free media. Media with 10% FBS was added in lower chambers. Cells were incubated for 24 hours and fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. For invasion assay 9.8 μg/ml matrigel was added to an insert before seeding cells. Experiments were duplicated.

Soft-agar colony formation assay

3,000 cells per well were seeded in triplicate into 6-well plates. Paclitaxel (Merck, Darmstadt, Germany) was treated every 4 days. After 2 weeks, colonies were fixed in 4% paraformaldehyde and stained with 0.1% crystal violet.

3D culture and drug sensitivity test

Cells were seeded in triplicate into 3D 96-well plates at a density of 3,000 cells per well. Matrigel was added two days after cells were seeded. Cells were treated with 5 μM of each breast cancer chemotherapy drug. 72 hours after drug treatment, cell viability was measured by celltiter glo-3D.

Immunohistochemistry

Immunohistochemistry was performed with the immunohistoche- mistry staining kit (Dako, CA, USA). Tissue sections were deparaffinized in xylene, hydrated in phosphate buffered saline, and blocked with normal goat serum. Slides were incubated with primary antibody (1:500) at 4 ℃ for overnight. The next day, the tissue sections were incubated with biotinylated anti-rabbit antibody, followed by exposure to preformed avidin/biotinylated peroxidase solution. Sections were then developed with diaminobenzidine and hydrogen peroxide, which produces a brown precipitate. Sections were counterstained with hematoxylin, dehydrated, and mounted.

Whole Exome Sequencing and RNA-seq data analysis

We carried out whole exome sequencing on 11 samples (X61 case – 3 vehicle and 2 paclitaxel, X110 case – 3 vehicle and 3 paclitaxel). Exome enrichment was performed using the Agilent SureSelect Human All Exon V5 kit. Sequencing was performed on an Illumina HiSeq 2500 platform. To analyze patient derived xenograft (PDX) sample, we used paired-end reads aligned to the human reference after mapping to the reference combined human GRCh37 with mouse mm10 genome versions using Burrows-Wheeler Aligner (version 0.7.10) [16]. PCR duplicates were removed using Picard (version 1.124) (http://broadinstitute.git

hub.io/ picard/). Read realignment around insertion/deletions and base quality score recalibration steps were performed by GATK packages (version 3.2.2) [17]. Somatic single nucleotide variants were called by Mutect (version 1.1.7) [18]. Small insertions and deletions were detected by GATK IndelgenotyperV2. Variants were annotated using Annovar software [19]. We filtered out synonymous variants and non-coding variants among called variants. We further excluded variants with a variant allele frequency greater than 1% in the 1000 Genomes Project (1000g, http://www.1000genomes.org/), the Exome Sequencing Project (ESP, http://evs.gs.washington.edu/EVS/) and the Exome Aggregation Consortium (ExAC, http://exac.broadin stitute.org) databases.

RNAseq libraries were constructed with a TruSeq RNA Kit (Illumina, San Diego,CA, USA), according to the manufacturer's instructions. The cDNA fragments were sequenced on the Illumina HiSeq platform to generate 101-bp paired-end reads. Sequencing reads from the PDX sample were aligned to the reference as in the whole exome sequencing analysis by using STAR aligner (STAR version 2.4.1d) [20]. The transcript abundance was estimated using HTSeq-count [21], according to the Ensemble transcript annotation (GRCh37.75). Read counts from HTSeq-count were converted to a fragments per kilobase per million mapped reads (FPKM) value using the rpkm function from the

edgeR package [22]. The R package DESeq2 [23] was used to perform the differential gene expression analysis. We defined DEGs by P value ≤ 0.05 and log2 (fold-change) ≥ 0.5. Expression profiles were clustered and visualized using Cluster3.0 (log transformed, mean-centered, uncentered correlation, average linkage) and JAVA TreeView (version 1.1.6).

Results

Triple negative breast cancer PDX models derived from single patient's primary and recurrent tumor were both sensitive to paclitaxel treatment.

To understand the molecular adaptation of cancer cells during the paclitaxel treatment, we performed in vivo paclitaxel experiment using a patient-derived xenograft model. We established two TNBC PDX models, X61 and X110, separately derived from single patient's primary and recurrent tumors, respectively (Figure 2). For each TNBC PDX model, control group (n=5) and paclitaxel group (n=5) were intraperitoneally (IP) injected with either PBS or paclitaxel (15mg/kg) for 4 weeks. The patient's primary and recurrent tumor were both sensitive to paclitaxel treatment.

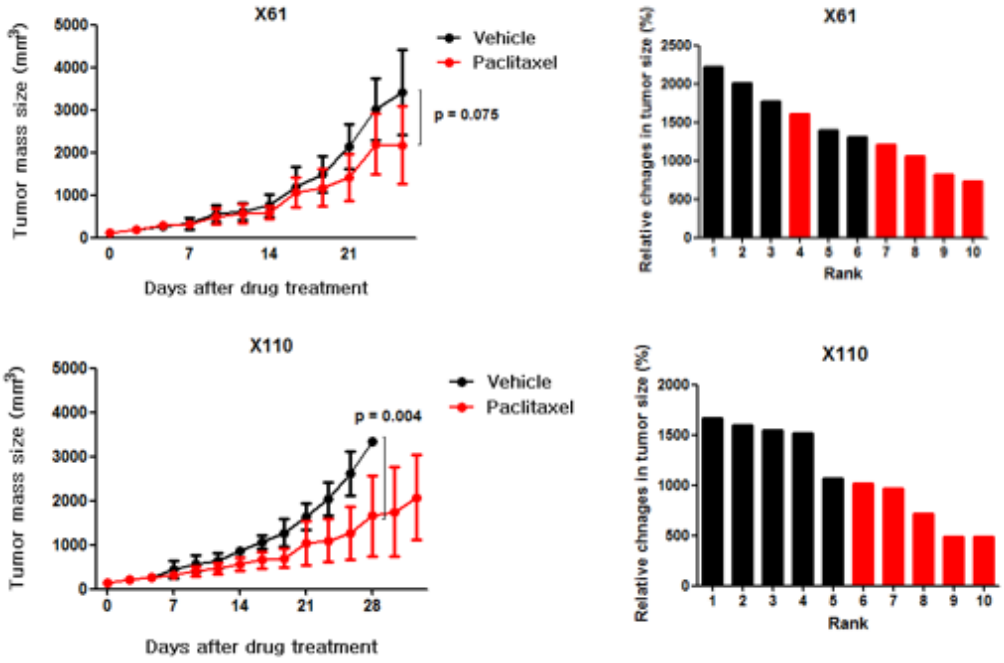
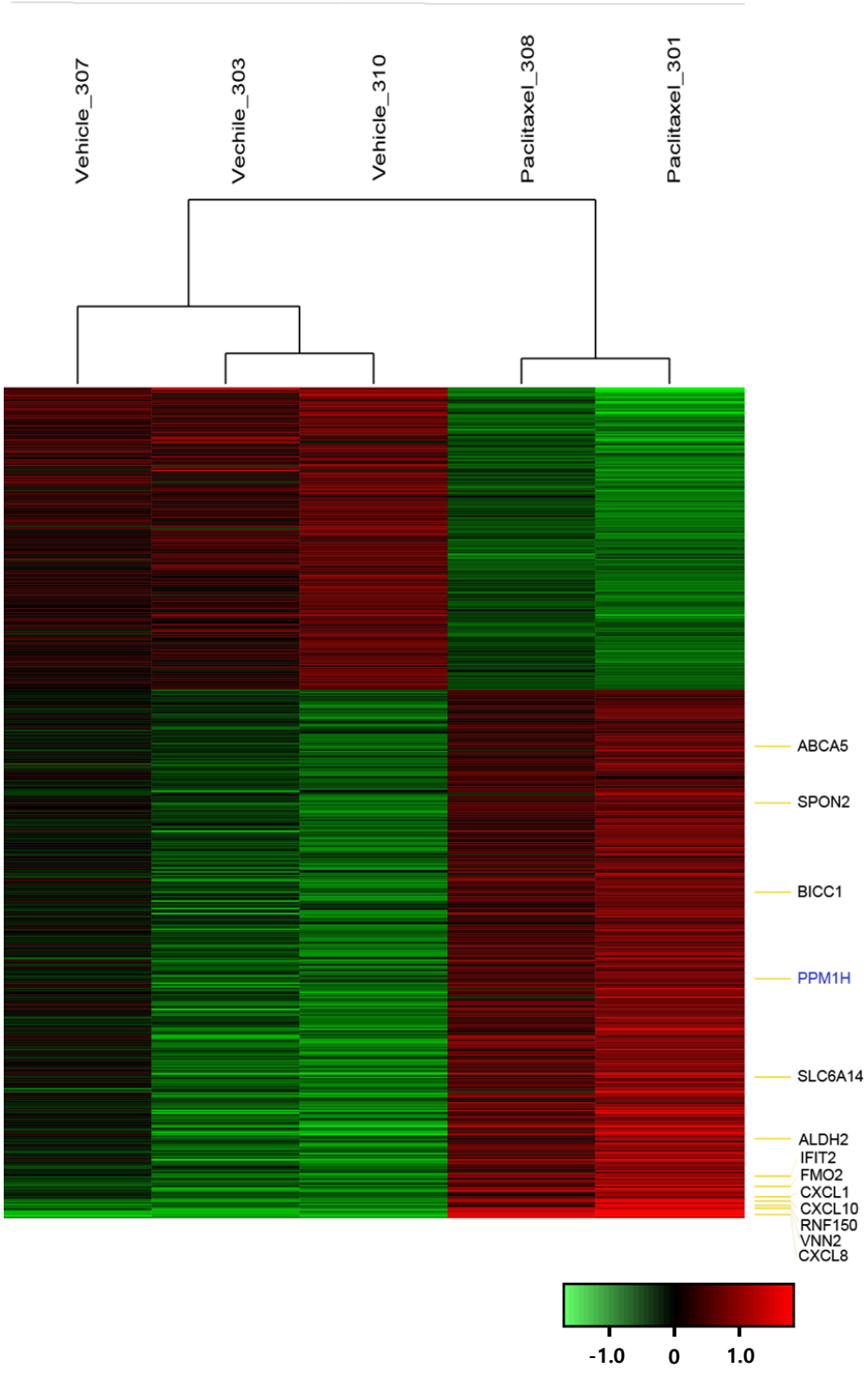


Figure 2. TNBC PDX in vivo paclitaxel experiment. PDX models were established from single patient's primary and recurrent tumor. X110 is a recurred case of X61. Both models were sensitive to paclitaxel treatment (mean ± S.D).

DEG analysis of transcriptome sequencing data showed 13 genes that were upregulated in both PDX models of paclitaxel group.

We selected three of five tumors for each control and paclitaxel group and then obtained transcriptome and exome sequencing data. Based on transcriptome data, we found differentially expressed genes between control and paclitaxel group for X61 and X110 (Figure 3). Among these genes, we found 13 genes that were upregulated in paclitaxel group for both X61 and X110 PDX models ($p \leq 0.05$, 2-fold ≥ 0.5) (Table 1). In addition, we found FPKM (Fragments Per Kilobase of transcript per Million mapped reads) expression for these 13 genes (Figure 4) in X61 and X110. In paclitaxel group for both cases, the expression of the CXC chemokines, immune-response related genes such as CXCL1 and CXCL10, were highly upregulated, but the rest of the genes had similar expression levels (Table 2).

X61


Vehicle_307
Vechile_303
Vehicle_310
Paclitaxel_308
Paclitaxel_301
ABCA5
SPON2
BICC1
PPM1H
SLC6A14
ALDH2
IFIT2
FMO2
CXCL1
CXCL10
RNF150
VNN2
CXCL8
-1.0
0
1.0

X110

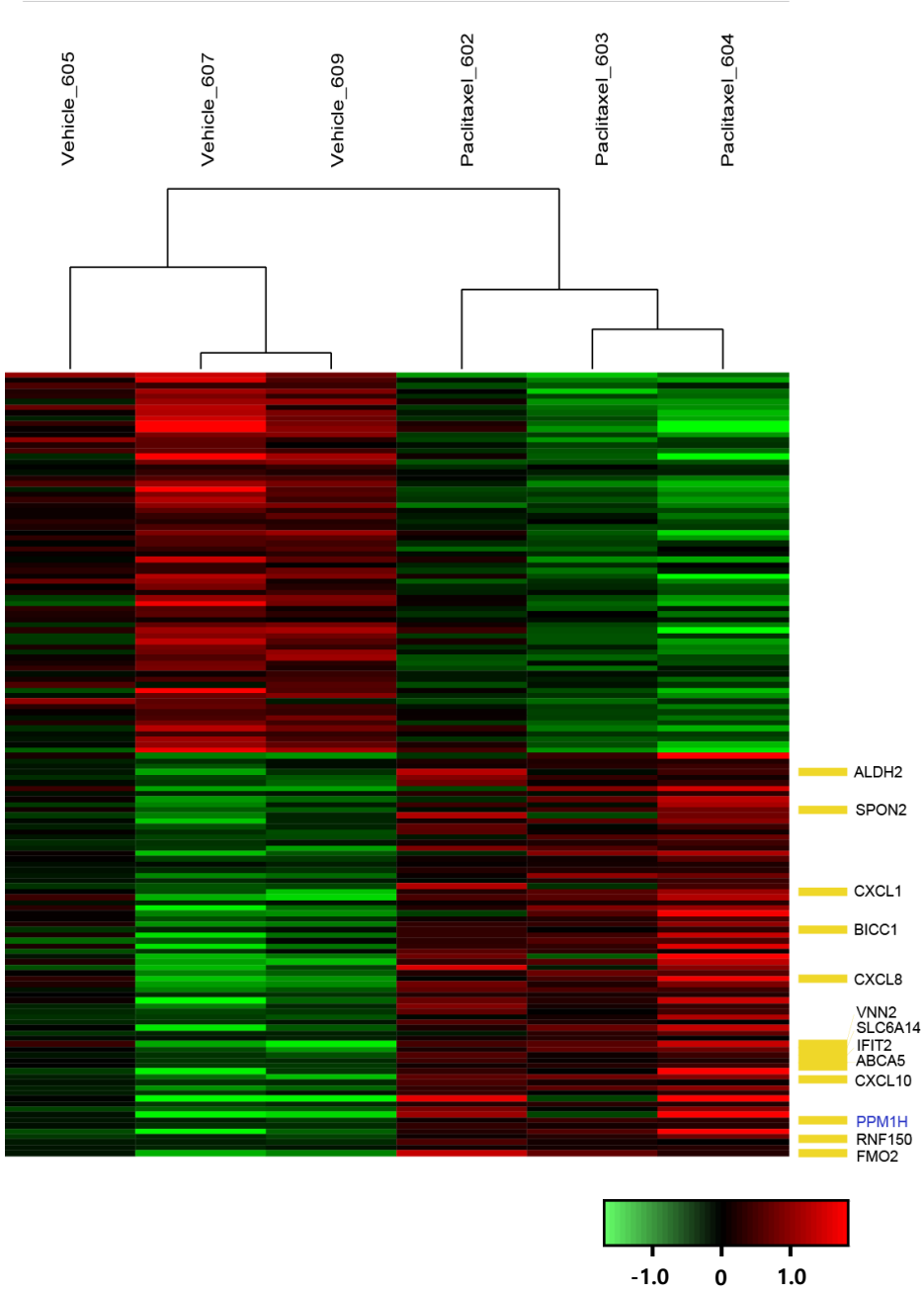


Figure 3. Heat map showing differentially expressed genes (DEGs) between vehicle and paclitaxel group of X61 and X110.

Table 1. DEG analysis on transcriptome data with 13 genes upregulated in both PDX paclitaxel groups.

	X61		X110	
	log2 Fold Change	p-value	log2 Fold Change	p-value
ABCA5	0.51	0.038901	0.66	0.000519
BICC1	0.60	0.006475	0.57	0.00249
CXCL1	1.40	2.27E-15	0.54	0.001308
CXCL10	1.43	1.19E-19	0.69	1.28E-05
CXCL8	1.99	2.06E-22	0.60	0.002253
ALDH2	1.14	3.94E-06	0.51	0.005225
FMO2	1.27	6.20E-07	0.85	6.44E-06
IFIT2	1.19	1.18E-05	0.65	0.000753
PPM1H	0.67	0.000688	0.80	5.06E-05
RNF150	1.50	1.37E-08	0.82	3.20E-05
SLC6A14	0.78	0.000448	0.64	0.00058
SPON2	0.52	0.029616	0.52	0.004594
VNN2	1.71	7.84E-17	0.63	0.000938

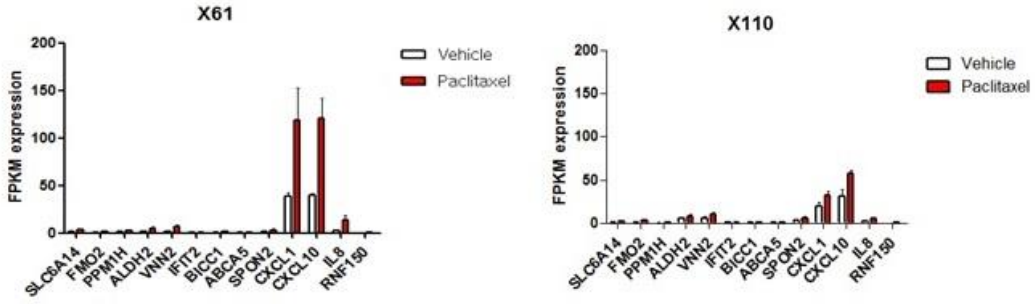


Figure 4. Paclitaxel up DEG 13 genes in FPKM expression.

Based on transcriptome data, FPKM (Fragments Per Kilobase of transcript per Million mapped reads) expression for 13 genes that were upregulated in paclitaxel group for both X61 and X110.

Table 2. FPKM expression for paclitaxel up DEG 13 genes

	X61					X110					
	vehicle	vehicle	vehicle	paclitaxel	paclitaxel	vehicle	vehicle	vehicle	paclitaxel	paclitaxel	paclitaxel
	X160706.303	X160706.307	X160706.310	X160706.308	x6116070 6.301	x110160630.605	x110160630.607	x1101606 30.609	x1101606 30.602	x1101606 30.603	x1101606 30.604
ABCA5	0.22	0.13	0.17	0.30	0.28	0.32	0.15	0.19	0.56	0.36	0.43
ALDH2	1.82	1.33	1.12	2.70	5.88	5.75	3.57	5.29	10.85	5.97	7.47
BICC1	1.55	1.03	0.90	1.59	2.38	0.24	0.29	0.36	0.62	0.43	0.61
CXCL1	40.66	34.83	41.14	94.40	142.64	24.09	19.31	13.81	26.65	30.91	37.31
CXCL10	37.77	40.74	41.69	105.39	135.75	38.72	31.09	24.62	54.48	57.70	60.74
CXCL8	3.01	2.24	2.71	10.71	17.20	3.80	1.49	1.84	3.23	4.11	7.28
FMO2	0.68	0.52	0.29	1.06	2.51	1.90	0.91	1.16	4.28	3.01	2.40
IFIT2	0.37	0.28	0.17	0.64	1.62	0.49	0.38	0.27	0.96	0.64	0.85
PPM1H	2.06	1.35	1.82	3.33	2.75	0.19	0.16	0.07	0.30	0.47	0.41
RNF150	0.16	0.09	0.06	0.36	0.73	0.14	0.13	0.09	0.49	0.29	0.22
SLC6A14	2.44	1.71	1.25	4.22	2.94	1.43	1.00	0.62	1.61	2.14	2.24
SPON2	1.41	2.00	1.69	1.47	4.16	3.48	2.70	3.76	7.46	3.31	6.12
VNN2	1.50	1.50	1.95	5.82	8.09	8.52	4.16	3.24	8.84	9.35	13.09

Whole-exome sequencing data identified mutational profiles of common and specific cancer genes

Exome sequencing data was also analyzed to identify any somatic mutations (Figure 5). There were common somatic mutations found between control and paclitaxel group of X61 and X110 PDX models. In control and paclitaxel treated PDX tumors, we also found cancer related genes that had various somatic mutations. Especially PPM1H, one of the genes that were upregulated in transcriptome data analysis, had double mutations in amino acids at K120E, S97N sites (red arrow in Figure 5). Through Polyphen2 analysis, we found that amino acid alteration at S97N site has possible damage on the structure and function of a human protein. Based on transcriptome and whole-exome sequencing data, PPM1H was selected as a candidate gene for further study.

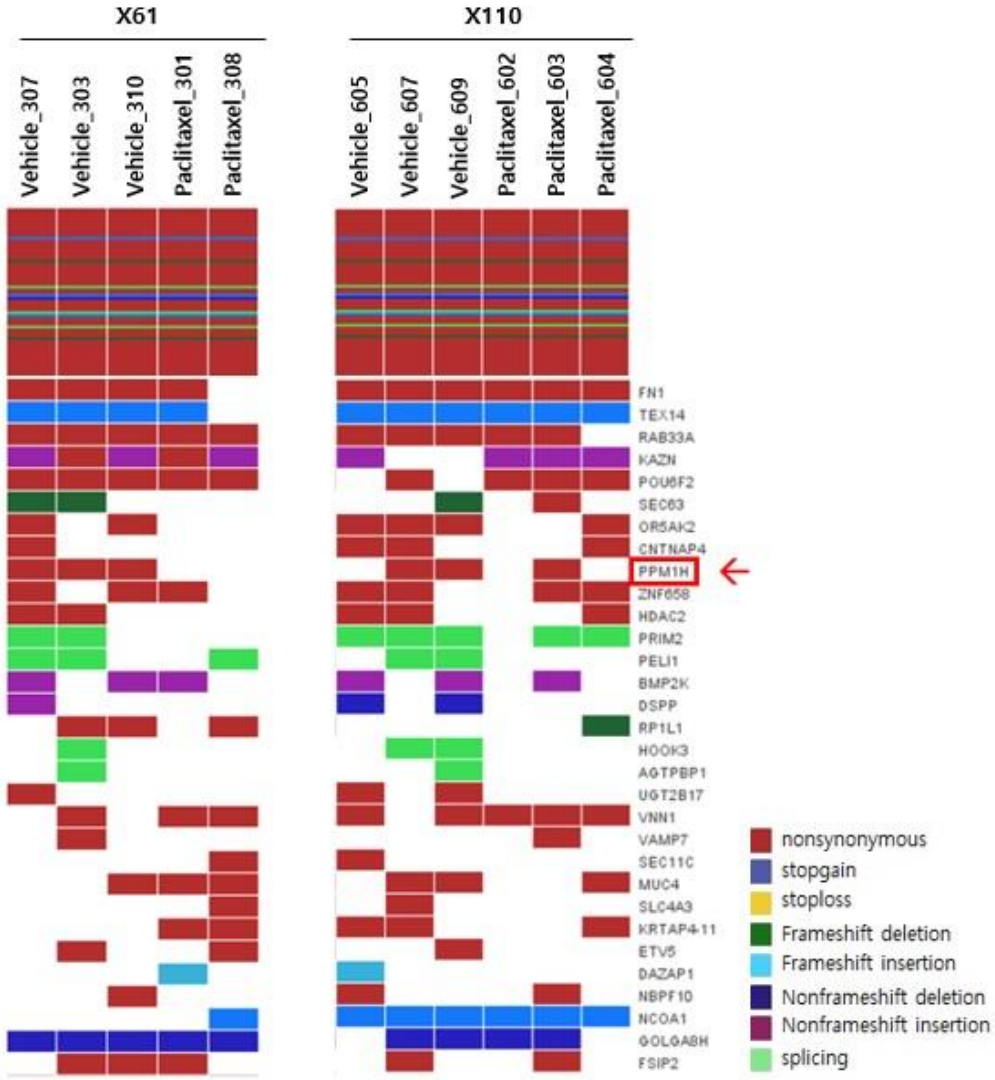


Figure 5. Identification of somatic mutations of PDX models using whole-exome sequencing. Whole-exome sequencing data identified mutational profiles of common and specific cancer genes.

PPM1H overexpressing triple negative breast cancer cell was established.

To validate the functions of PPM1H genes in vitro, we established PPM1H overexpressed triple negative breast cancer cell MDA-MB-231 using a lentiviral vector. We first determined PPM1H mRNA expression level in breast cancer cell lines by quantitative PCR (Figure 6a). PPM1H expression level was higher in ER+ and HER2+ cell lines (ZR-751 and SKBR3) than in TNBC cell lines. To incorporate TNBC PDX models into in vitro system, we chose TNBC cell line MDA-MB-231 to establish PPM1H overexpressing cell. PPM1H overexpression vector was established by lentiviral transduction into MDA-MB-231 cell (Figure 6b). PPM1H gene overexpression was verified by qPCR and western blot (Figure 6c, 6d). Control and PPM1H overexpressed MDA-MB-231 cells had no morphological differences (Figure 6e).

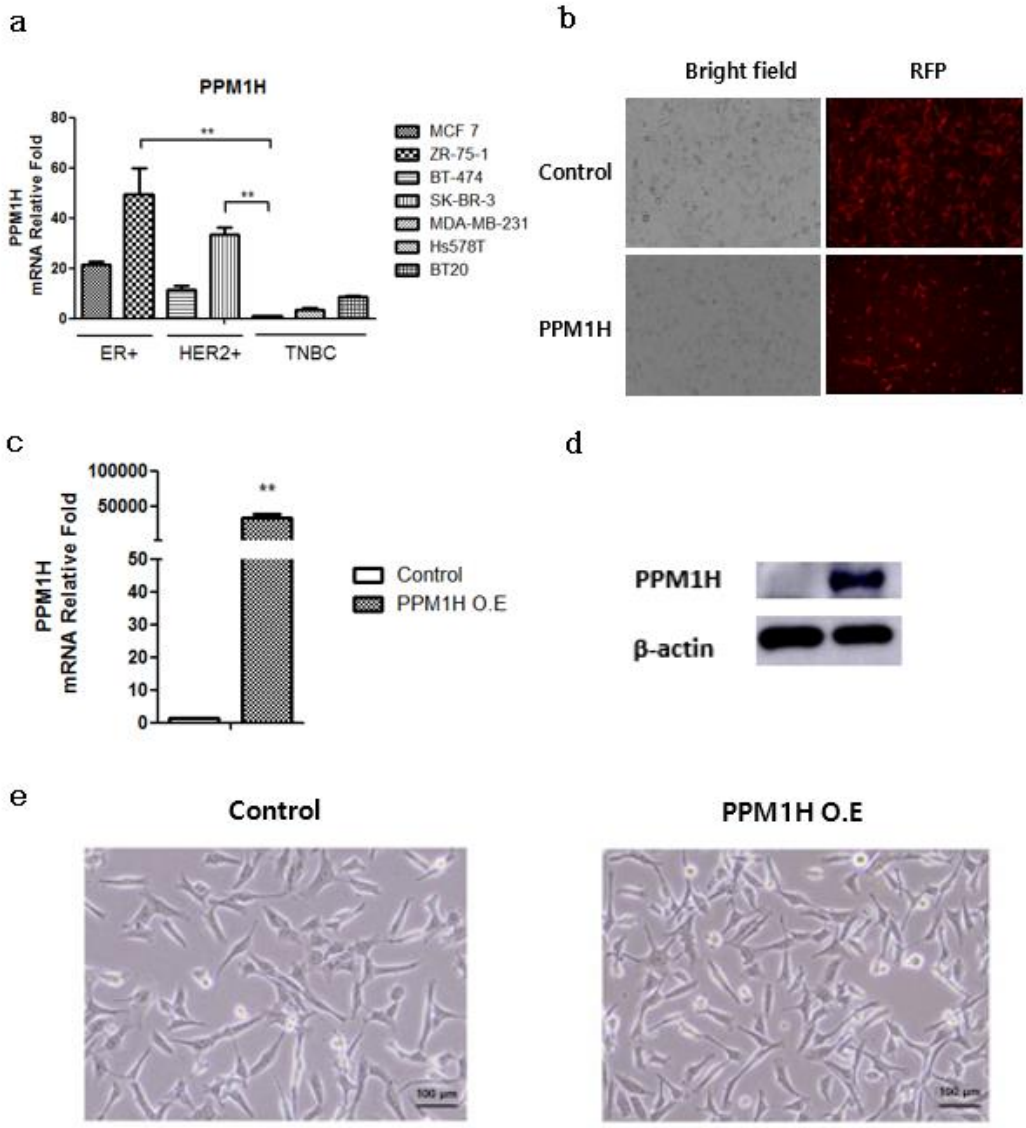


Figure 6. PPM1H overexpressed breast cancer MDA-MB-231 cell was established. PPM1H mRNA level in breast cancer cell lines was determined by qPCR (a). Infection of MDA-MB-231 cells with RFP tagged lentivirus (b). Gene overexpression was verified by qPCR and western blot (c, d). Cell morphology of control and PPM1H overexpressed MDA-MB-231 cells in 100x magnitude (e).

Paclitaxel treatment increased PPM1H gene expression level in both TNBC PDX tumors and MDA-MB-231 breast cancer cells.

PPM1H mRNA levels were increased in MDA-MB-231 cells after 24 and 48 hours of paclitaxel treatment (Figure 6a). PPM1H protein levels were also increased after 24 and 48 hours of paclitaxel treatment (Figure 6b). In control MDA-MB-231 cells, PPM1H protein levels were too low to determine its expression levels.

To examine the similar result in TNBC PDX tumors, immunohistochemistry staining with PPM1H antibody was performed on control and paclitaxel treated X61 and X110 PDX tumors (Figure 6c). We found that paclitaxel treated groups had significantly higher PPM1H gene expression levels. Moreover, immunohistochemistry staining with PPM1H antibody was performed on additional TNBC PDX cases, MX130 and MX158. Similarly, we found that paclitaxel treated groups had higher PPM1H gene expression levels (Figure 6d).

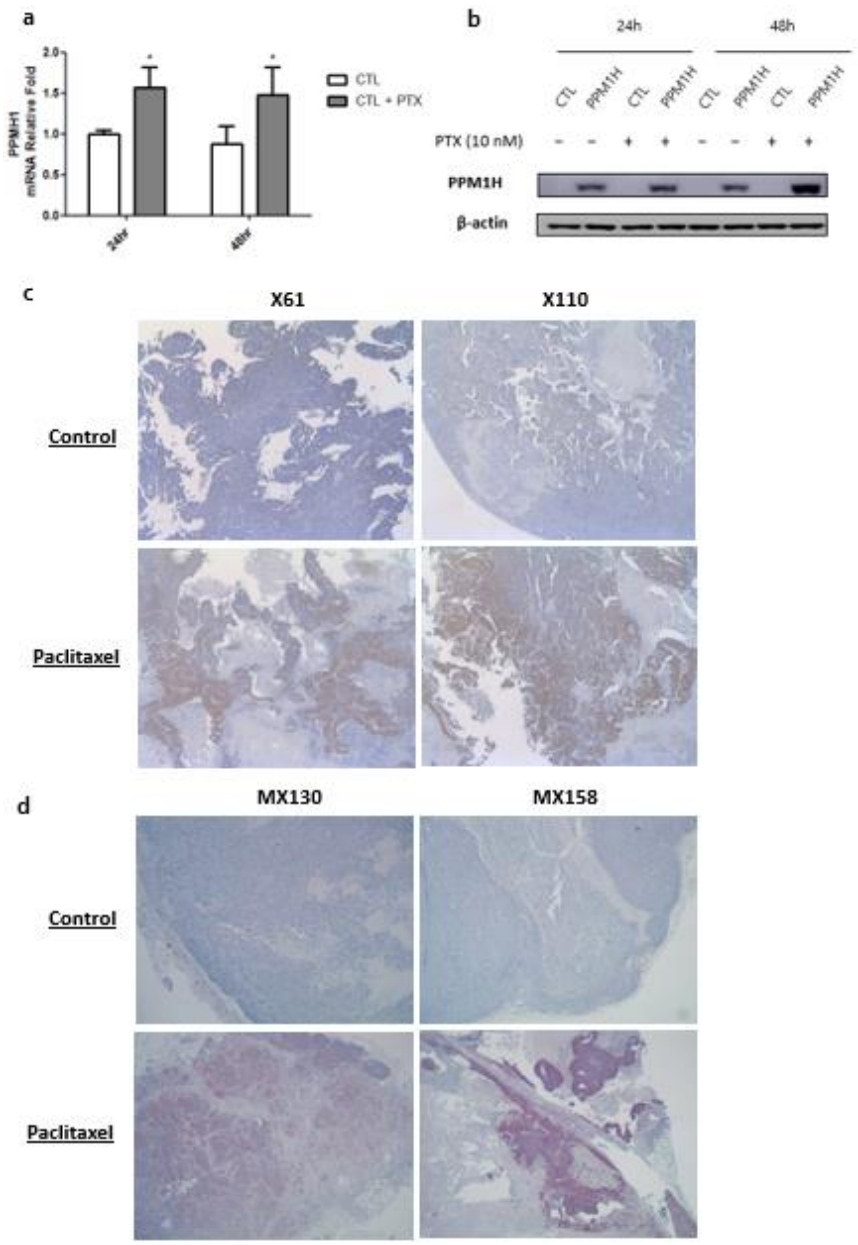


Figure 7. PPM1H gene expression level after paclitaxel treatment.

Quantitative PCR with MDA-MB-231 cell (a); western blot (b) Immunohistochemistry of X61 and X110 TNBC PDX tumors (c); Immunohistochemistry of MX130 and MX158 TNBC PDX cases for control and paclitaxel group (d).

PPM1H overexpressed MDA-MB-231 cells had no effects on cell proliferation, migration and invasion, but were more sensitive to paclitaxel in 2D cell cultures.

To validate the function of PPM1H, in vitro experiments were performed. Cell proliferation rates were measured by celltiter-glo luminescent cell viability assay, but there were no significant changes in between control and PPM1H overexpressed cells (Figure 8a). Also, cell migration and invasion assays were measured by transwell 24-well plates, but PPM1H overexpressed cell had no effects on both cell migration and invasion (Figure 8b and 8c). To determine sensitivity to paclitaxel, control and PPM1H overexpressed cell was treated with paclitaxel for 72 hours and cell viability was measured by MTT assay (Figure 8d). The results showed that PPM1H overexpressed cells were more sensitive to paclitaxel in 2D cell cultures.

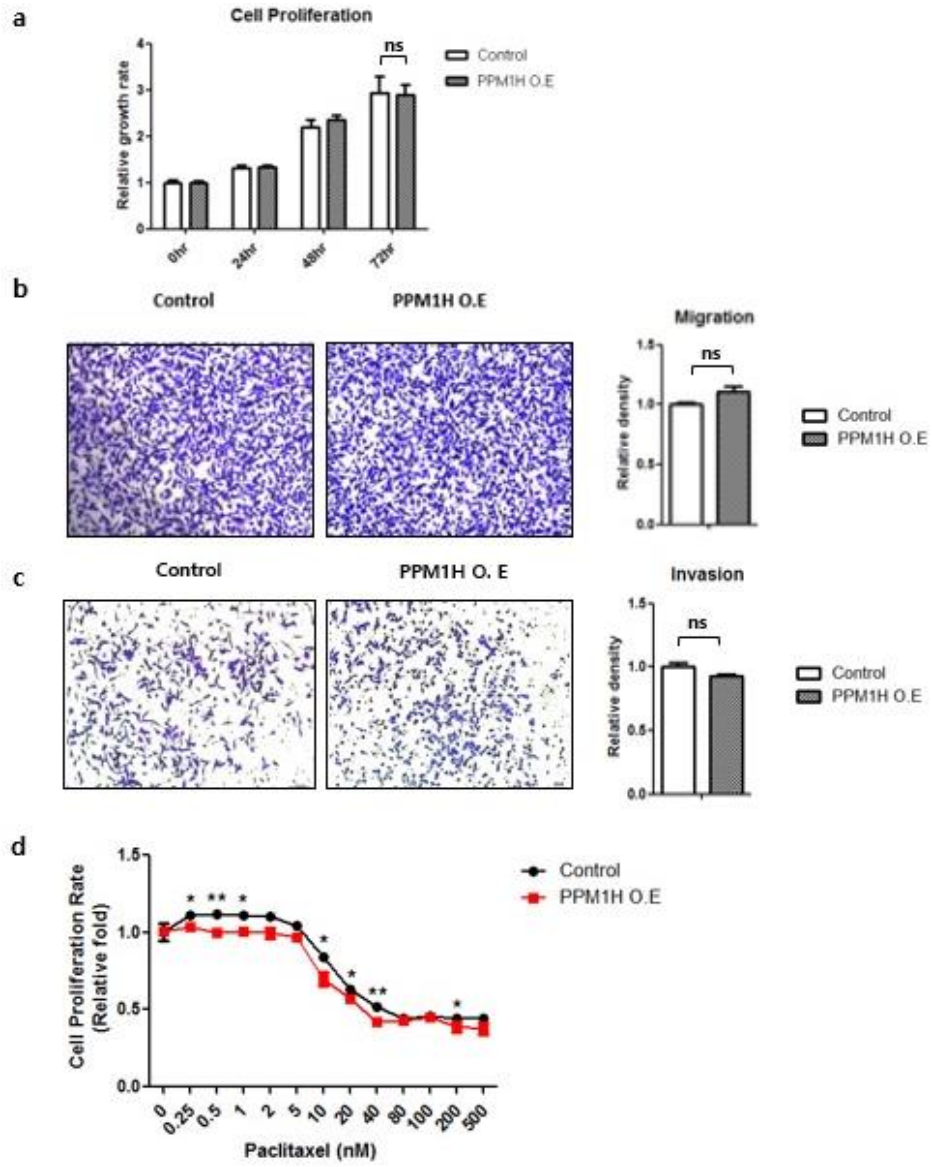


Figure 8. Cell proliferation, migration, invasion had no effects, but sensitive to paclitaxel in 2D cultures. All experiments were conducted in triplicate in three independent experiments (mean ± S.D). Cell proliferation assay by celltiter-glo luminescent cell viability assay kit (a). Migration and invasion assay (b, c). After 72h of paclitaxel treatment, cell proliferation rates were measured by MTT assay (d).

PPM1H overexpressed MDA-MB-231 cells were more sensitive to paclitaxel in 3D cell cultures.

Soft agar colony formation assay was performed to determine paclitaxel sensitivity for control and PPM1H overexpressed MDA-MB-231 cells (Figure 9a). After day 15, cells were fixed and stained with crystal violet. Then colonies were counted by image J. PPM1H overexpressed cells were more sensitive to paclitaxel (≥ 40nM). We also performed 3D cell culture in matrigel (9b). PPM1H overexpressed cells resulted in fewer number of spheroids that were smaller in size, compared to control cells. Then tested paclitaxel sensitivities for both control and PPM1H overexpressed cells (9c). After 72 hours of paclitaxel treatment, we found that PPM1H overexpressed cells were more sensitive to paclitaxel (≥ 0.1 μM). Then we evaluated the effects of breast cancer chemotherapeutic drugs on 3D cultured control and PPM1H overexpressed MDA-MB-231 cells (Figure 9d). Table 3 shows the list of drugs that were used. Among these breast cancer chemotherapeutic drugs, PPM1H overexpressed cells were more sensitive to paclitaxel than control cells. However, PPM1H overexpressed cells were resistant to some protein kinase

inhibitors such as Crisotinib, Everolimus, AZD6482, and estrogen receptor down regulator Fulvestrant.

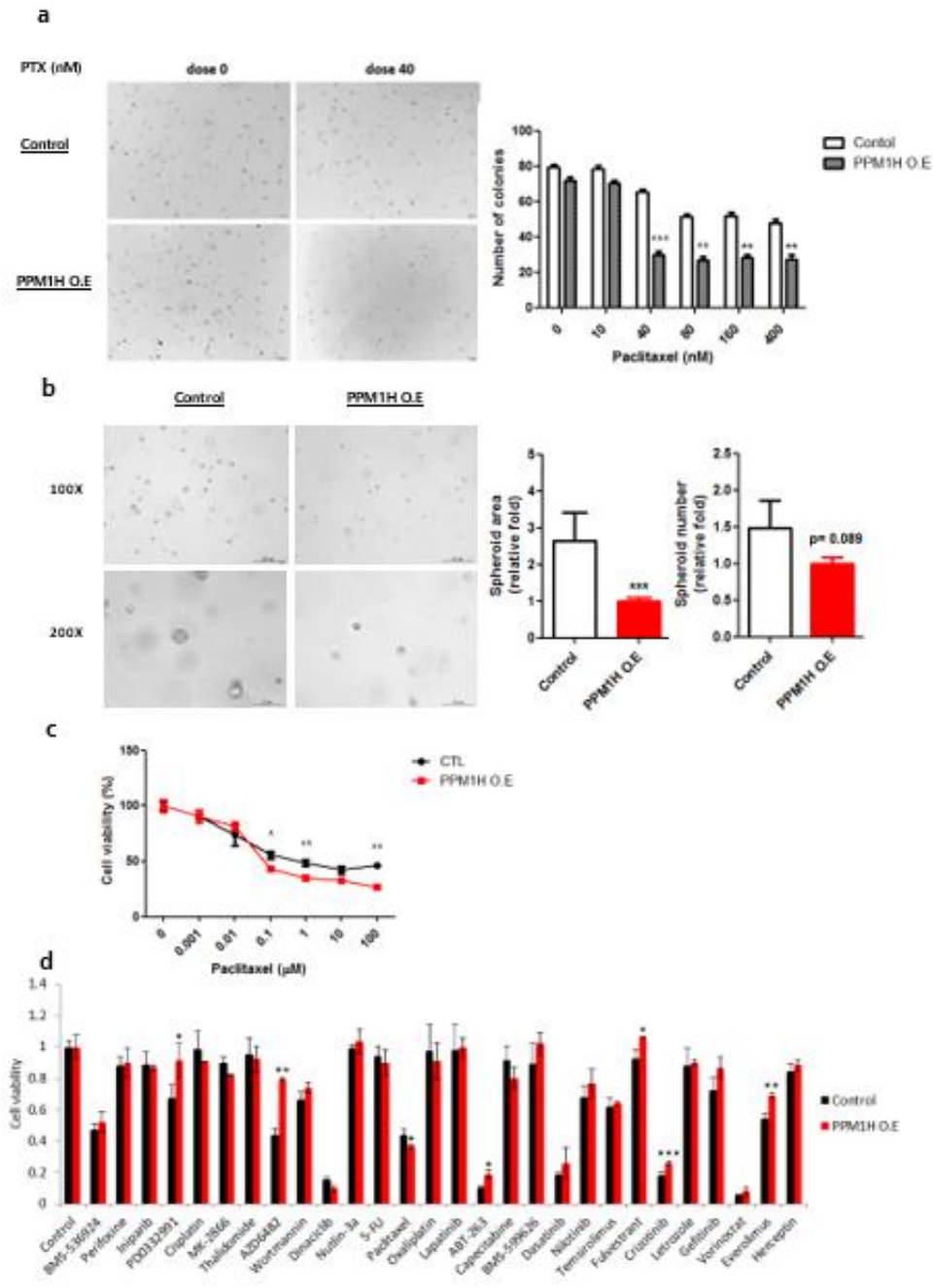


Figure 9. Paclitaxel sensitivity observed in 3D cell culture assays. Soft agar colony formation assay (a); 3D culture in matrigel (b); sensitivity to paclitaxel in 3D culture (c); drugs screening after 3D culture in Matrigel (d).

Table 3. List of breast cancer chemotherapy drugs

Drugs	Function	Relative Difference (median ± S.D)	P-value
BMS-536924	IGF-1R inhibitor	0.048 ± 0.040	0.3628
Perifosine	Akt inhibitor	0.031 ± 0.042	0.5027
Iniparib	PARP inhibitor	-0.013 ± 0.136	0.5160
PD0332991	CDK inhibitor	0.327 ± 0.105	0.0467*
Dinaciclib	CDK inhibitor	-0.040 ± 0.025	0.0701
AZD6482	PI3K inhibitor	0.372 ± 0.033	0.0028**
Wortmannin	PI3K inhibitor	0.061 ± 0.045	0.1063
Nutlin-3a	Mdm2 inhibitor	0.063 ± 0.050	0.2197
ABT-263	Bcl-2 inhibitor	0.102 ± 0.031	0.0322*
Dasatinib	BCR/ABL inhibitor	0.116 ± 0.063	0.1337
Nilotinib	Bcr-Abl inhibitor	0.005 ± 0.135	0.5260
Temsirolimus	Mtor inhibitor	-0.015 ± 0.047	0.8196
Everolimus	Mtor inhibitor	0.144 ± 0.012	0.0021**
Crizotinib	ALK and ROS1 inhibitor	0.081 ± 0.003	0.0004***
Gefitinib	EGFR inhibitor	0.239 ± 0.108	0.1056
Lapatinib	HER2, EGFR inhibitor	0.093 ± 0.142	0.4223
Herceptin	HER2 inhibitor	-0.022 ± 0.083	0.7100
BMS-599626	HER2 inhibitor	0.176 ± 0.079	0.0652
Fulvestrant	ER downregulator	0.163 ± 0.039	0.0224*
MK-2866	Androgen receptor modulators	-0.069 ± 0.068	0.4357
Capecitabine	Antimetabolite	-0.050 ± 0.090	0.2808
5-FU	Antimetabolite	-0.033 ± 0.021	0.0733
Cisplatin	Alkylating agent	-0.044 ± 0.168	0.8996
Oxaliplatin	Alkylating agent	0.027 ± 0.208	0.7668
Paclitaxel	Antimicrotubule agent	-0.089 ± 0.031	0.0384*
Letrozole	Aromatase inhibitor	0.038 ± 0.160	0.3636
Thalidomide	Immunomodulatory agent	0.047 ± 0.108	0.9563
Vorinostat	Histone deacetylase inhibitor	0.004 ± 0.025	0.3576

PPM1H increased p27 protein levels.

PPM1H protein levels have increased after 24 and 48 hours of paclitaxel treatment (10 nM). PPM1H has been identified as phosphatase impacting p27 stability by dephosphorylation at Thr-187. To determine the relationship between PPM1H and p27 during paclitaxel treatment, PPM1H and p27 protein levels were measure by western blot (Figure 10). Interestingly, p27 protein levels were elevated as PPM1H protein levels increased by paclitaxel treatment.

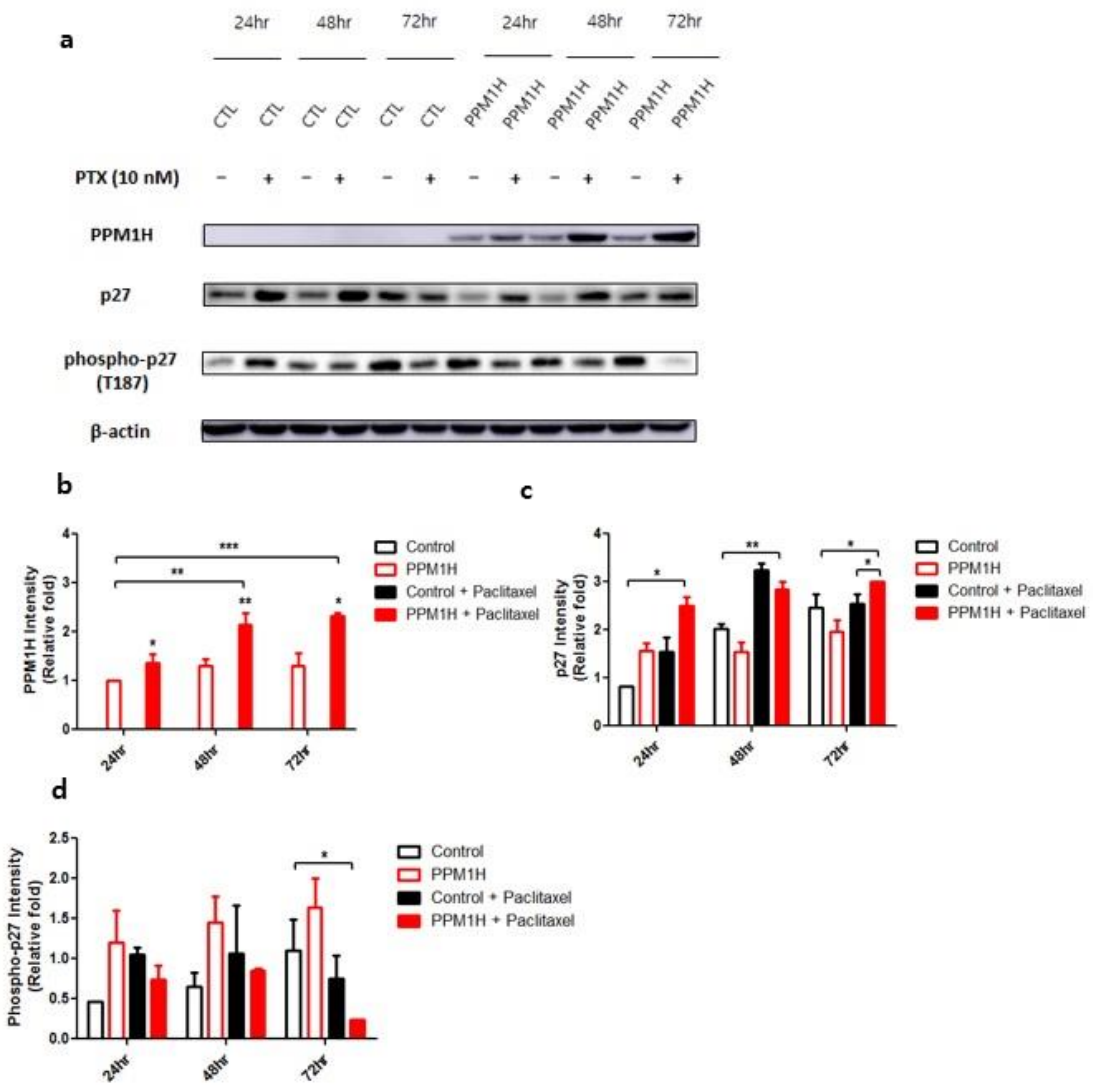


Figure 10. Paclitaxel treatment increased PPM1H expression level and PPM1H increased p27 protein levels. Western blot (a), western blot quantification (b, c, d).

PPM1H gene expression levels in breast tumor tissues were higher than that in normal breast tissues.

To compare PPM1H gene expression levels in patient-derived xenograft (PDX) model to the actual patient tumors, with patients' normal and tumor breast tissues, we determined PPM1H expression levels in FPKM expression (Figure 11). We found that breast tumor tissues had higher PPM1H expression levels compared to normal breast tissues.

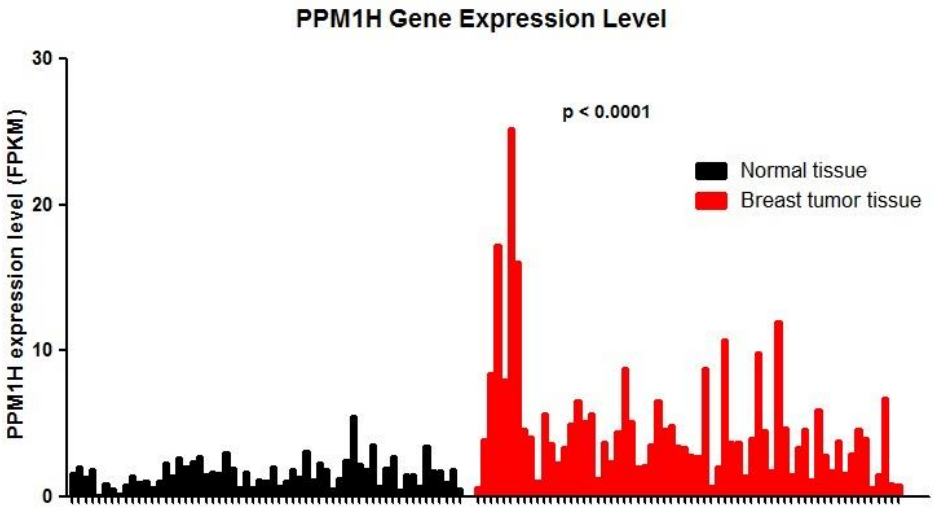


Figure 11. PPM1H gene expression level in normal and breast tumor tissues.

PPM1H reduced multidrug resistance protein 1 (MDR1) gene expression level.

To determine the relationship between PPM1H and MDR1 gene, we found MDR1 gene expression levels for control and PPM1H overexpressed cells by using quantitative real time PCR. Compare to control cells, PPM1H overexpressed cells showed lower MDR1 gene expression levels (Figure 12). Similar gene expression patterns were found even after paclitaxel treatments.

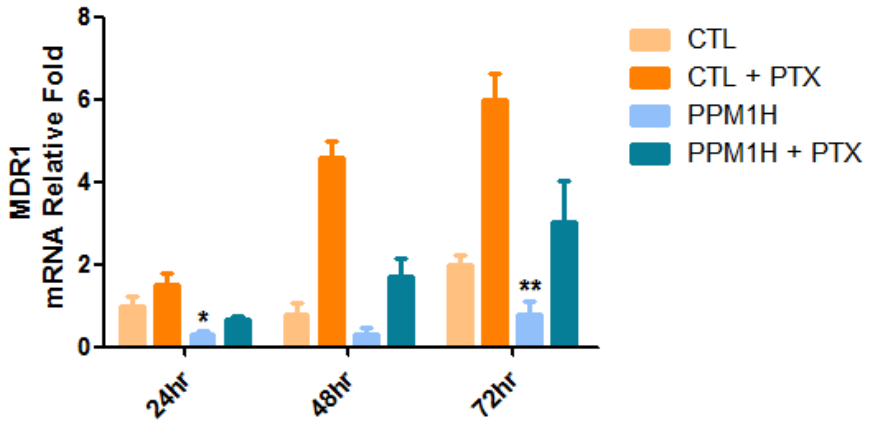
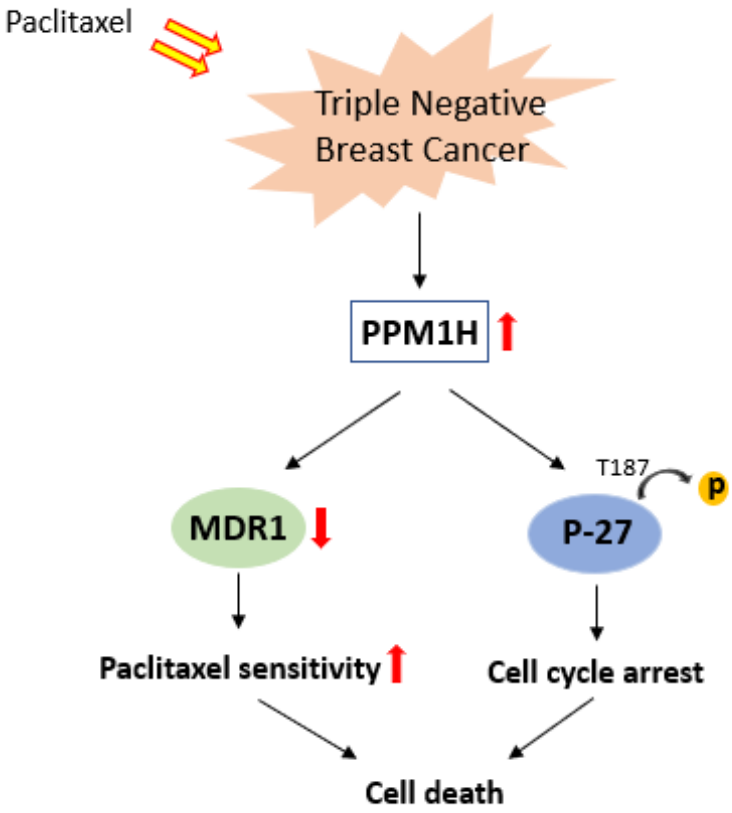


Figure 12. MDR1 gene expression levels in control and PPM1H overexpressed MDA-MB-231 cells.


Paclitaxel
Triple Negative
Breast Cancer
PPM1H
MDR1
T187
P
P-27
Paclitaxel sensitivity
Cell cycle arrest
Cell death

Discussion

In this study, we have shown that there is a gene controls the response to paclitaxel in triple negative breast cancer. Paclitaxel treatment increased gene expression levels of PPM1H in both TNBC PDX and breast cancer cells. Increased PPM1H expression levels elevated cell cycle inhibitor p27 protein levels but decreased multiple drug resistance 1 (MDR1) gene expression levels. These observations suggest that PPM1H might have a possible role reducing breast cancer cell survival during paclitaxel treatment by increasing cell cycle arrest and reducing paclitaxel efflux. Furthermore, we found that PPM1H overexpressed triple negative breast cancer cells were more sensitive to paclitaxel in 2D and 3D cell cultures. Our results demonstrate that PPM1H plays an important role as a novel therapeutic target in breast cancer chemotherapy by increasing cell sensitivity to paclitaxel.

Paclitaxel is a natural compound isolated from the bark of the *Taxus baccata* plant and is one of the well-known chemotherapeutic agents for triple negative breast cancer patients. Paclitaxel could bind to the β-tubulin subunit of microtubules, thus stabilizing it and arresting dividing cancer cells in the M phase of the cell cycle, causing cell death. Paclitaxel has been effectively used in the clinical treatment for more than two decades, which considerably improved the outcomes of breast cancer patients [13].

Compared to other breast cancer subtypes, triple negative breast cancers (TNBCs) are the most aggressive subtype with its high rate of recurrence and poor prognosis. TNBCs often develop resistance to the paclitaxel during the treatment [24]. Resistance to paclitaxel can be either intrinsic or acquired. Intrinsic resistance defines resistance-mediating factors pre-exist in the tumor cells that cause the therapy ineffective prior to drug treatment. Acquired drug resistance can develop during treatment of tumor. Tumors were initially sensitive, but drug resistance can be triggered by mutations arise during treatment as well as through various other adaptive responses, such as increased expression of the therapeutic target and activation of different signaling pathways [14]. Moreover, recent studies have been suggested that tumors can contain a high degree of molecular heterogeneity, thus drug resistance can arise through survival of resistant minor subpopulation cells that were present in the original tumor [14]. Recent studies have been focused on the mechanisms of paclitaxel resistance that they are related to tubulin mutations, MDR1 overexpression, and chromosomal instability [25, 26, 27, 28].

Lee-Hoeflich, ST et al. [14] have previously shown that PPM1H, a protein phosphatase Mg2+/Mn2+ dependent 1H, dephosphorylates p27 at threonine 187, thus removing a signal for proteasomal degradation. The study suggests that PPM1H has a

role in trastuzumab resistance by reduction in protein levels of the tumor suppressor p27. Based on this study, we have also demonstrated that PPM1H increases p27 protein levels, but still need to confirm that PPM1H dephosphorylates p27 at threonine 187.

Moreover, there have been several researches on the effect of PPM1H in other cancer cells, such as pancreatic cancer and colon adenocarcinoma. Both mRNA and protein levels of PPM1H in all the examined pancreatic cancer cell lines were lower than that of normal pancreatic ductal epithelial cells [29]. Silencing PPM1H gene on malignant phenotype of human pancreatic cancer cell line BxPC-3 induced EMT. Also silencing PPM1H gene increased cell proliferation and invasion, but inhibited BxPC-3 cells apoptosis. However, PPM1H expression level was upregulated in colon adenocarcinomas compared with normal colon tissues [30]. Higher PPM1H expression level in colon cancer cell lines relative to normal colon cell lines was also found. Through co-immunoprecipitation coupled with mass spectrometry analysis, they found CSE1L as a PPM1H interacting protein. The study suggested that PPM1H regulates cell cycle and proliferation of cancer cells possibly through dephosphorylation of CSE1L [30]. PPM1H expression also reduced BMP signaling, while loss of PPM1H activity significantly increases BMP-dependent gene regulation and mesenchymal differentiation [31].

Cytoplasmic phosphatase PPM1H avoids excessive BMP signaling through dephosphorylation of phospho-Smad 1/5/8 in cytoplasm.

Our study has some important limitations. The first limitation is the small number of the TNBC tumors were used for transcriptome and exome sequencing data analysis. We used only two paclitaxel sensitive TNBC PDX models. It might be necessary to study PPM1H expression levels from additional PDX cases, also from paclitaxel non-sensitive cases. Second limitation is the lack of comparative data in other breast cancer subtypes such as luminal or HER2 subtype. Other breast cancer subtypes may have different PPM1H expression levels. Further studies are needed to find whether the effects of paclitaxel on PPM1H gene expression levels are not only limited to MDA-MB-231 cells but also to other triple negative breast cancer cells. Also, it is necessary to establish PPM1H overexpressing cells in different TNBC cell lines whether they have similar paclitaxel sensitivities. Additionally, animal experiment using PPM1H overexpressed MDA-MB-231 is needed to determine the effect of PPM1H on paclitaxel in vivo. There are still limitations to fully understand the relationship between paclitaxel and PPM1H involving p27 and MDR1. Therefore, further studies are needed to elucidate these mechanisms.

In conclusion, *in vivo* and *in vitro* paclitaxel experiment demonstrated the elevation of gene expression levels in PPM1H

in both TNBC PDX and breast cancer cells. Increased PPM1H also increased tumor suppressor p27 but decreased MDR1 gene expressions. Since paclitaxel and p27 are both cell cycle inhibitors, they might induce cancer cell death through synergistic effects of stopping cell cycle progression. Our data suggest that PPM1H could be a target gene that may regulate response to paclitaxel in triple negative breast cancer. Therefore, PPM1H may be a novel target of anticancer drugs for breast cancer.

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국문초록

최근 암 연구를 위한 환자 유래 종양 이종 이식 (PDX) 모델에 대한 관심이 증가하고 있다. 이 모델은 대부분 환자의 조직학적 및 유전적 특성을 유지하기 때문에 세포 독성 화학 요법 중 암 세포 생존을 밝혀주는 연구에 유용하다. 파클리탁셀은 유방암 환자에게 잘 알려진 화학 요법 제제 중 하나이지만 대부분의 이미 진행이 많이 된 환자들은 치료 중 파클리탁셀에 내성을 보이는 경우가 많다. 파클리탁셀 치료 중 암세포의 분자 적응을 이해하기 위해 우리는 환자 유래 이종 이식 모델을 사용하여 생체 내 파클리탁셀 실험을 수행했다. 우리는 단발성 환자의 원발성 및 재발성 종양에서 분리하여 얻은 두 개의 TNBC PDX 모델을 확립했습니다. 각각의 TNBC PDX 모델들은, 대조군 (n = 5)과 파클리탁셀 (n = 5) 마우스를 PBS 또는 파클리탁셀 (15mg / kg)로 4 주간 복강 내 주사 하였다. 두 PDX 모델 모두 파클리탁셀 치료에 민감했다. 전사체와 유전체 시퀀싱 데이터는 대조군과 파클리탁셀 군의 5 종 중 3 종을 선택하여. 전사체 분석결과 파클리탁셀 군에서 13 가지 유전자가 상향 조절되었다 ($p \leq 0.05$, 2-fold ≥ 0.5). 이 유전자들 중, CXCL10, FMO2, PPM1H 그리고 RNF150 을 선정했다 (adjusted $p \leq 0.1$). 유전체 시퀀싱 분석 결과 공통되는 그리고 특정 암 유전자의 변이가 확인 되었다. 특히 전사체 데이터 분석에서 상향 조절 된 유전자 중 하나 인 PPM1H 는 K120E, S97N 부위의 아미노산에서 이중 돌연변이를 보였다. 흥미롭게도, 파클리탁셀 치료는 TNBC PDX 종양 및 유방암 세포 모두에서 PPM1H 유전자 발현 수준을 증가시켰다. PPM1H 유전자 발현은 유방암 세포주를 이용한 단기간의 시험 관내

파클리탁셀 치료로 증가되었다. PPM1H 가 과발현 된 MDA-MB-231 세포는 세포 증식, 이동 및 침윤에 실질적인 영향을 미치지 않았지만 2D 및 3D 세포 배양에서 파클리탁셀에 보다 민감했다. 증가 된 PPM1H 발현 수준은 또한 종양 억제 p27 단백질 수준을 상승시켰다. 본 연구에서는 유방암 세포의 유전자가 파클리탁셀 치료시 다양하게 변하고, 유전자 PPM1H 가 유방암 세포의 파클리탁셀 감수성을 조절한다는 것을 보여 준다. PPM1H 의 기전을 명확히 밝히기 위해서는 추가적인 연구가 필요하다.

주요단어: 유방암, 환자유래 이종이식 (PDX); PPM1H; p27
학번: 2016-22024