

Gene Expression Profiling from a Prostate Cancer PC-3 Cell Line Treated with Salinomycin Predicts Cell Cycle Arrest and Endoplasmic Reticulum Stress

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Abstract

Previously, we reported that salinomycin induces apoptosis of human prostate cancer cells through accumulated reactive oxygen species and mitochondrial membrane depolarization. To extend our understanding for the genome-wide expression pattern, we performed cDNA microarray analysis for gene expression profiles in prostate PC-3 cells treated with salinomycin. We found a couple of differences from gene expression profiles. First of them, cyclins and cyclin-dependent kinases were down-regulated, whereas cyclin dependent kinase inhibitors were up-regulated, implicating inhibition of cell cycle progression. Second, HSPA5/Bip, DDIT3/CHOP, TRIB3, ATF4 and ATF6 regarding endoplasmic reticulum (ER) stress and unfolded protein response (UPR) were increased at mRNA and protein levels, indicating salinomycin-induced growth inhibition of PC-3 cells seem to be mediated through induction of ER stress and activation of the UPR pathway. Our finding should be useful for understanding genome-wide expression patterns of salinomycin-mediated cell cycle arrest and ER stress response toward induction of apoptosis and be helpful for finding future cancer therapeutic targets in prostate cancer cells.

Keywords: Salinomycin; Prostate cancer; Apoptosis; Cell cycle; Proliferation; Endoplasmic reticulum stress; Gene expression analysis

Introduction

As of 2011, prostate cancer is the most frequently diagnosed cancer and the sixth leading cause of cancer death in males worldwide. Rates of detection of prostate cancers vary widely across the world, with South and East Asia detecting less frequently than in Europe and especially the United States [1]. Pharmacologic inhibition of the androgen receptor (AR) pathway by depletion of circulating androgen and by anti-AR drugs is a regular treatment as standard care, which induces apoptosis in androgen-dependent prostate cancer cells and brings the disease to remission. However, most hormone dependent cancers become refractory after one to three years and therapy resistance to conventional chemotherapy for a limited period. Thus finding new treatment options for therapy-resistant prostate cancer has been a critical challenge [2].

The complex network of cell cycle and apoptosis are closely linked [3,4]. Treatment of cancer cells with anticancer agents usually results in the arrest of the cell cycle, the cells subsequently entering into apoptosis. Checkpoint signaling may also result in activation of pathways leading to apoptosis if cellular damage cannot be properly repaired [5]. G₁ phase cyclin/cyclin-dependent kinase (CDK) complexes play a key role in the G₁/S checkpoint function. Three D type cyclins (cyclin D1, D2 and D3) bind to CDK4 and CDK6, which are essential for entry into G₁ phase [6]. Also cyclin E associated with CDK2 regulates progression from G₁ into S phase [7]. CDKs are negatively regulated by a group of functionally-related proteins, CDK inhibitors [8,9]. In recent studies, one of CDK inhibitors, p21^{Cip1} cleavage is a critical step in converting to growth arrest and apoptosis of cancer cells [10,11].

The main functions of endoplasmic reticulum (ER) are protein synthesis, folding, lipid synthesis and maintenance of Ca²⁺ homeostasis [12]. Conditions interfering with the function of ER, such as accumulation of unfolded proteins (unfolded protein response, UPR) and excessive protein traffic (ER overload response, EOR), are collectively called ER stress. The purpose of UPR is to restore normal function and activate the signaling pathway, but apoptotic cascade will be activated during prolonged or irresistible ER stress [13]. During ER stress, one of the three ER stress sensors; eukaryotic translation initiation factor 2 alpha (eIF2- α) kinase 3 (EIF2AK3/PERK) is a serine/threonine protein kinase and is activated by ER stress via dimerization and autophosphorylation upon the dissociation with Bip. Activated PERK phosphorylates eIF2 α , thereby attenuating global protein synthesis to reduce the protein load of ER [14]. However, translation of certain mRNAs is allowed, like mRNAs for activating transcription factor 4 (ATF4) and its downstream target C/EBP-homologous protein (CHOP) [15,16]. CHOP, which is also regulated by X-box binding protein 1 (XBP-1) [17], promotes apoptosis by down-regulating anti-

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Received November 01, 2012; **Accepted** December 05, 2012; **Published** December 07, 2012

Citation: Kim KY, Seo YK, Yu SN, Kim SH, Suh PG, et al. (2013) Gene Expression Profiling from a Prostate Cancer PC-3 Cell Line Treated with Salinomycin Predicts Cell Cycle Arrest and Endoplasmic Reticulum Stress. J Cancer Sci Ther 5: 023-030. doi:10.4172/1948-5956.1000180

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apoptotic factors like Bcl-2 [18] and up-regulating pro-apoptotic factors like the Bcl-2-interacting protein Bim [19].

Salinomycin is a monocarboxylic polyether antibiotic widely used as an anticoccidiosis agent in chickens [20,21]. A recent study showed that in high-throughput screening of ~16,000 small molecule chemicals, breast cancer stem cells (CSCs) were found to be inhibited selectively by salinomycin, showing at least 100 times more effective than paclitaxel in mice [22]. CSCs are a subpopulation of cells within the tumor mass that are thought to account for cancer recurrence by virtue of their refractivity to cytotoxic cancer treatment agents such as radiation and a wide variety of chemotherapeutic agents. Susceptibility of CSCs to salinomycin bolsters the possibility that this drug may target chemotherapy-resistant advanced human cancers. The mechanism of action by which salinomycin kills cancer stem cells specifically remains unknown. However, several studies show that the anticancer property of salinomycin has been recognized based on its ability to induce apoptosis and cause growth inhibition in diverse types of apoptosis and chemotherapeutic-resistant cancer cells [23]. In fact, salinomycin caused massive tumor cell apoptosis and associated regression of breast tumor growth and metastasis *in vivo* in a mouse xenograft tumor model [23]. This drug also triggers apoptosis by overcoming ABC transporter-mediated multidrug resistance, as was observed in the case of KG-1a human leukemia cells [24,25]. Description of the mechanisms that underlies cancer cell apoptosis by salinomycin is needed in order to rigorous assessment of the therapeutic potential of this drug as a novel cancer therapeutic.

Previously, we reported that proliferation of the androgen-dependent LNCaP and androgen-independent PC-3 prostate cancer cells markedly reduced in response to salinomycin treatment [26]. Its anti-proliferation resulted from induction of apoptosis through accumulated reactive oxygen species and mitochondrial membrane depolarization. In the present study, we have examined the mechanism that underlies prostate cancer cell death in the presence of salinomycin by gene expression using cDNA microarray. We present gene profiling for the first time that salinomycin mediates cell cycle arrest and ER stress response for apoptosis of prostate cancer cells.

Materials and Methods

Reagents and antibodies

Salinomycin and Propidium iodide (PI) were purchased from Sigma Aldrich (St. Louis, MO, USA). The ECL Western Kit was purchased from Amersham (Arlington Heights, IL, USA). Antibodies for cyclin D1, cyclin E, CDK2, CDK6 and ATF4 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for cyclin A, CDC2, p21^{cip1}, IRE1 α , Bip, and phospho-eIF2 α were purchased from Cell Signaling (Beverly, MA, USA). CDK4 and GADD153/CHOP were purchased from Stressgen (Victoria, BC, Canada) and USBiological (Swampscott, MA, USA), respectively.

Cell lines and cell culture

Androgen-independent PC-3 prostate cancer cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). These cells were maintained and cultured in Dulbecco's modified Eagle's medium (DMEM; WelGENE Inc., Daegu, Korea) supplemented with 10% fetal bovine serum (FBS) (WelGENE Inc.), 100 units/ml of penicillin and 100 μ g/ml of streptomycin (WelGENE Inc.). Cells were cultured in a humidified atmosphere with 5% CO₂ at 37°C.

Microarray analysis

For control and salinomycin-treated RNAs, the synthesis of target cDNA probes and hybridization were performed using Agilent's Low RNA Input Linear Amplification kit (Agilent Technology, Santa Clara, CA, USA) according to the manufacturer's instructions. Briefly, each 0.2 μ g total RNA was mixed with the diluted Spike mix (A and B, Agilent Technology) and T7 promoter primer mix and incubated at 65°C for 10 min. cDNA master mix (5X First strand buffer, 0.1 M DTT, 10 mM dNTP mix, RNase-Out, and MMLV-RT) was prepared and added to the reaction mixer. The samples were incubated at 40°C for 2 h and then the room temperature (RT) and dsDNA synthesis was terminated by incubating at 70°C for 10 min. The transcription master mix was prepared as the manufacturer's protocol (4X Transcription buffer, 0.1M DTT, NTP mix, 50% PEG, RNase-Out, Inorganic pyrophosphatase, T7-RNA polymerase, and Cyanine 3/5-CTP). Transcription of dsDNA was performed by adding the transcription master mix to the dsDNA reaction samples and incubating at 40°C for 2 h. Amplified and labeled cRNA was purified on RNase mini column (Qiagen, Hamburg, Germany) according to the manufacturer's protocol. Labeled cRNA target was quantified using ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). After checking labeling efficiency, each 750 ng of cyanine 3-labeled and cyanine 5-labeled cRNA target were mixed and the fragmentation of cRNA was performed by adding 10X blocking agent and 25X fragmentation buffer and incubating at 60°C for 30 min. The fragmented cRNA was resuspended with 2X hybridization buffer and directly pipetted onto assembled Agilent Human Oligo microarray. The arrays hybridized at 65°C for 17 h using Agilent Hybridization oven (Agilent Technology). The hybridized microarrays were washed as the manufacturer's washing protocol (Agilent Technology).

Data acquisition and analysis

The hybridization images were analyzed by Agilent DNA microarray Scanner (Agilent Technology) and the data quantification was performed using Agilent Feature Extraction software 9.3.2.1 (Agilent Technology). The average fluorescence intensity for each spot was calculated and local background was subtracted. All data normalization and selection of fold-changed genes were performed using GeneSpringGX 7.3.1 (Agilent Technology). Genes were filtered with removing flag-out genes in each experiment. Intensity-dependent normalization (LOWESS) was performed, where the ratio was reduced to the residual of the Lowess fit of the intensity vs. ratio curve. The averages of normalized ratios were calculated by dividing the average of normalized signal channel intensity by the average of normalized control channel intensity. >2- fold changed genes were selected and considered as significant genes. Functional annotation of genes was performed according to DAVID Bioinformatics Resources 6.7 (<http://david.abcc.ncifcrf.gov/>). All experiments have been submitted to Array-Express (EBML-EBI, Cambridge, UK).

Cell cycle analysis

PC-3 cells were plated in 6-well plates at a density of 1 \times 10⁵/well for 24 h after serum starvation overnight. The culture medium was refreshed with new medium and then the cells were treated with various concentrations of salinomycin for 48 h. And the cells were then fixed in 70% ethanol, washed with PBS twice and finally incubated in RNase A (200 μ g/ml) at 37°C for 30 min. DNA content per cell was evaluated in flow cytometer (Becton Dickinson Co., Franklin Lakes, NJ, USA) after staining cells with 1 mg/ml propidium iodide solution. Data collection

and analysis of the cell cycle distribution were performed using Cell Quest and Modifit software (Becton Dickinson Co.).

Semiquantitative Revers Transcriptase (RT)-PCR

PC-3 cells were seeded in 60 cm² cell culture dish (1×10⁶ cells). After 24 h incubation, cells were treated with salinomycin (1.33 μM) for various times. Cell harvesting and total RNA isolation was performed using the RiboEX_column Total RNA purification Kit (GeneAll, Seoul, Korea). Semiquantitative RT-PCR amplification was performed using primers for CDKN2B, CDKN1C, CDKN2A, CCNB1, CCNB2, CCND1, CCND3, CCNE1, CDK2, HSPA5, DDIT3, TRIB3, ATF4, ATF6, XBP1 and GAPDH. RT-PCR primers were designed by primer 3 (<http://frodo.wi.mit.edu>) (Supplementary Table 1). For semiquantitative analysis, RT-PCR was performed at low cycle numbers to avoid saturation, in triplicate samples.

Western blotting

Cell extracts were prepared by incubating the cells in lysis buffer [150 mM NaCl, 10 mM Tris (pH 7.4), 5 mM EDTA (pH 8.0), 1% Triton X-100, 1 mM PMSF, 20 mg/ml aprotinin, 50 μg/ml leupeptin, 1 mM benzidine, and 1 mg/ml pepstatin]. Forty micrograms of proteins were electrophoretically separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12-15% gel and transferred to a polyvinylidene fluoride (PVDF) membrane (Amersham). After blocking with TBS-T buffer [20 mM Tris (pH 7.4), 150 mM NaCl, 0.1% Tween 20] containing 5% skim milk, the membranes were incubated with primary and secondary antibodies. The membranes were then washed with TBS-T buffer and visualized with ECL Western blotting detection reagents (Amersham). The density of each band was determined with a fluorescence scanner (LAS 3000, Fuji Film, Tokyo, Japan) and analyzed with Multi Gauge V3.0 software.

Statistical analysis

Experiments were repeated at least 3 times with consistent results. Unless otherwise stated, data are expressed as the mean ± SD. ANOVA was used to compare the experimental groups with the control; whereas comparisons among multiple groups were performed using a Tukey's multiple comparison tests. Results were statistically significant at p<0.05.

Results

Salinomycin changes the genome-wide expression in PC-3 prostate cancer cells

An advanced prostate cancer cell line, PC-3 cells were cultivated in the absence or presence of 1.33 μM salinomycin for 48 h, followed by gene expression profiling. The gene expression data were compared in order to identify the differentially expressed genes between absence and presence of salinomycin treated group. The Agilent Feature Extraction software 9.3.2.1 contains 44,000 probe sets according to the manufacturer. Among these transcripts, 4,852 probe sets were detected as over 2-fold changed in both absence and presence salinomycin. Of all the present transcripts, 2,541 genes were up-regulated and 2,311 genes were down-regulated (Supplementary Figure 1). We analyzed the changed genes using the DAVID Gene Ontology (GO) resource (<http://david.abcc.ncifcrf.gov/>) and grouped them into enriched broad categories using a term of Biological Process [27].

Salinomycin induces G₁-phase cell cycle arrest in PC-3 cells

To confirm the cell cycle arrest related gene profiling, we subsequently analyzed cell cycle progression in salinomycin-exposed PC-3 cells using flow cytometry after propidium iodide (PI) staining. Treatment with salinomycin for 48 h in dose-dependent manner resulted in the accumulation of cells in the G₀/G₁ phase (Figure 1A). In the presence of 1.33 and 4.00 μM of salinomycin, the proportion of cells in G₀/G₁ phase increased gradually over 50% (Figure 1B). The proportion of cells in G₂/M phase after 4.00 μM salinomycin treatment was reduced under approximately 10% as compared to almost 20% of the untreated cells. These results were consistent with previous microarray analysis to show regulatory gene expression involved in G₁/S transition. This observation is important because the molecular analyses of human cancers have revealed the cell cycle regulators frequently mutated in most common malignancies and inhibition of the cell cycle has been appreciated as a target for the management of cancer.

Salinomycin induces extensive changes at mRNA and protein level implicating cell cycle arrest

When the over 2-fold changed genes were analyzed by GO software, clusters for cell cycle arrest and apoptosis were enriched unexpectedly. There was a strong enrichment for genes in cell cycle and apoptosis and proliferation (Supplementary Table 2), and the most significantly enriched genes were shown in Tables 1 and 2. In order to validate the accuracy of GO analysis, we randomly picked genes of cell cycle regulators and determined cDNA expression patterns of

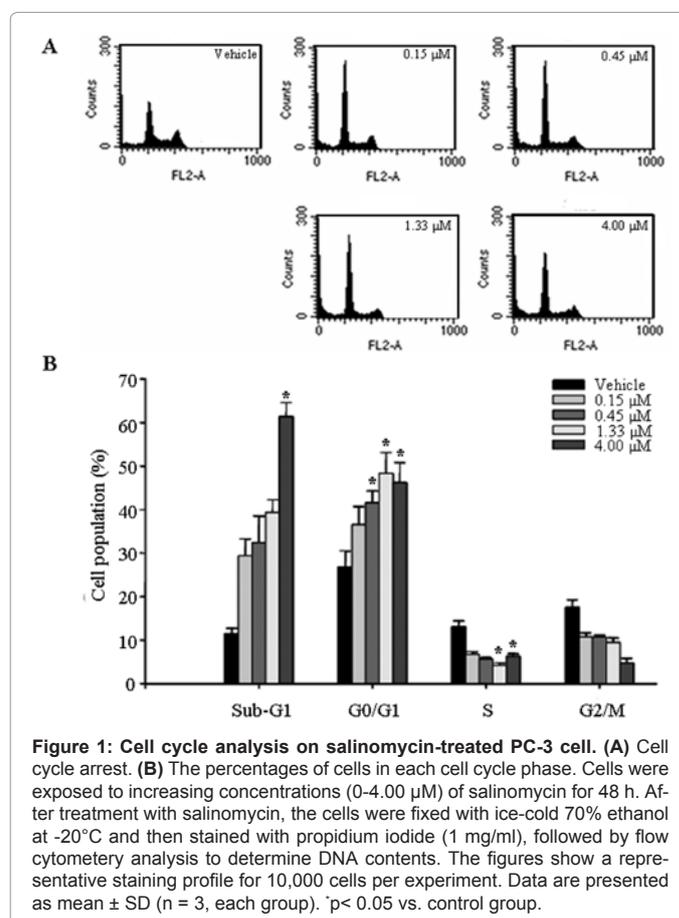


Figure 1: Cell cycle analysis on salinomycin-treated PC-3 cell. (A) Cell cycle arrest. **(B)** The percentages of cells in each cell cycle phase. Cells were exposed to increasing concentrations (0-4.00 μM) of salinomycin for 48 h. After treatment with salinomycin, the cells were fixed with ice-cold 70% ethanol at -20°C and then stained with propidium iodide (1 mg/ml), followed by flow cytometry analysis to determine DNA contents. The figures show a representative staining profile for 10,000 cells per experiment. Data are presented as mean ± SD (n = 3, each group). *p < 0.05 vs. control group.

Gene Symbol	Fold Change	Description	Genbank
CDKN2B	8.61	cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	NM_078487
PIM1	8.07	pim-1 oncogene	NM_002648
JUN	7.30	jun oncogene	NM_002228
CDKN1C	6.02	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	NM_000076
TNFSF15	4.58	tumor necrosis factor (ligand) superfamily, member 15	NM_005118
BCL2L1	4.32	BCL2-like 1	NM_001191
VEGFA	4.28	vascular endothelial growth factor A	NM_001025366
ARHGEF2	4.23	rho/rac guanine nucleotide exchange factor (GEF) 2	NM_004723
DUSP1	3.59	dual specificity phosphatase 1	NM_004417
FOXO4	3.32	forkhead box O4	NM_005938
TNFRSF9	3.14	tumor necrosis factor receptor superfamily, member 9	NM_001561
TXNIP	3.14	thioredoxin interacting protein	NM_006472
HMOX1	2.98	heme oxygenase (decycling) 1	NM_002133
CDKN1A	2.89	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	NM_000389
PRUNE2	2.81	prune homolog 2 (Drosophila)	NM_015225
MAGED1	2.57	melanoma antigen family D, 1	NM_001005333
CDKN2A	2.53	cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	NM_058197
TNF	2.44	tumor necrosis factor (TNF superfamily, member 2)	NM_000594
SOD2	2.38	superoxide dismutase 2, mitochondrial	NM_001024465
PTPRF	2.04	protein tyrosine phosphatase, receptor type, F	NM_002840

Table 1: List of 2-fold up genes involved in apoptosis, cell cycle and proliferation.

Gene Symbol	Fold Change	Description	Genbank
BARD1	0.27	BRCA1 associated RING domain 1	NM_000465
CCNA2	0.15	cyclin A2	NM_001237
CCNB1	0.12	cyclin B1	NM_031966
CCNB2	0.17	cyclin B2	NM_004701
CCND1	0.40	cyclin D1	NM_053056
CCND3	0.46	cyclin D3	NM_001760
CCNE1	0.22	cyclin E1	NM_001238
CDC2	0.12	cell division cycle 2, G1 to S and G2 to M	NM_001786
CDC6	0.32	cell division cycle 6 homolog (S. cerevisiae)	NM_001254
CDC7	0.28	cell division cycle 7 homolog (S. cerevisiae)	NM_003503
CDK10	0.45	cyclin-dependent kinase (CDC2-like) 10	NM_052987
CDK2	0.24	cyclin-dependent kinase 2	NM_001798
CDK4	1.05	cyclin-dependent kinase 4	NM_000075
CDK6	0.84	cyclin-dependent kinase 6	NM_001259
CDKN2C	0.32	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	NM_078626
CDKN3	0.14	cyclin-dependent kinase inhibitor 3 (CDK2-associated dual specificity phosphatase)	NM_005192
CHEK1	0.19	CHK1 checkpoint homolog (S. pombe)	NM_001274
ERBB2	0.14	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	NM_001005862
RPA1	0.37	replication protein A1, 70kDa	NM_002945
SKP2	0.41	S-phase kinase-associated protein 2 (p45)	NM_032637

Table 2: List of 2-fold down genes involved in apoptosis, cell cycle and proliferation.

several cell cycle arrest genes with specific primers (Supplementary Table 1 and Figure 2). Cyclins (CCNs) and cyclin dependent kinases (CDKs) are known to be positively regulated. In gene expression profiling, transcripts for CCNA2, CCNB1, CCNB2, CCND1, CCND3 and CCNE1 were down-regulated in gene expression profiling (Table 2). The mRNA levels of cyclin B1 (CCNB1) were slightly down-regulated, while cyclin E1 (CCNE1) was obviously down-regulated upon salinomycin treatment, as shown in Figure 2A. Those of cyclin B2 (CCNB2) and cyclin D3 (CCND3), however, were not significantly changed (Figure 2A). The treatment 1.33 μ M of salinomycin for 6 to 48 h induced a time-dependent decrease in cyclin A, cyclin D1 and cyclin E protein levels compared to the controls (Figure 2D).

Also, transcripts for CDK1/cell division cycle 2 (CDC2), CDC6, CDC7, CDK10 and CDK2 were down-regulated (Table 2). CDC2/CDK1, CDK2, CDK4 and CDK6 protein expression were decreased in time dependent manner (Figures 2B and 2E).

The CDK inhibitor 1A (CDKN1A), CDKN1C, CDKN2A, and CDKN2B, which are known to be negative regulators of cell cycle, were increased in gene expression profiling (Table 1). On the RT-PCR, the p15, as CDK4 inhibitor, was increased significantly in time dependent manner, but p16 and p57 were not significantly changes (Figure 2C). Furthermore, p21^{Cip1} was increased significantly in cells time dependent manner (Figure 2F), consistent with cell arrest at G₁ phase by inhibiting CDK4 activation following E2F1 inactivation since the CDK inhibitor, p21^{Cip1}, is a key component in the induction of cell cycle arrest and apoptosis. At 48 h, there was a huge accumulation of the expression of p21^{Cip1} on salinomycin-treated cells compared to the

control. Since the cell cycle proteins cyclin D1 and CDK4 control G₁ cell cycle progression, whereas cyclin E and its partner CDK2 dominate cell cycle progression from G₁ to S phase. These findings, therefore, implicate that salinomycin suppressed cell proliferation in the PC-3 cells through the regulation of the cyclin-protein expression required in G₁ cell cycle progression, leading to induction of apoptosis.

Salinomycin changes expression of mRNA and proteins involved in ER stress and apoptosis

ER stress is associated with unfolded protein response activation which is associated with the production of protective chaperones. However, excessive ER stress can cause cellular damage via the activation of the ER-dependent apoptotic pathway. When the genes over 2-fold changed were analyzed by GO software, clusters for endoplasmic reticulum unfolded protein response, response to unfolded protein and response to endoplasmic reticulum (ER) stress were enriched. There was a strong enrichment for genes in ER stress and UPR (Supplementary Table 3), and the most significantly enriched genes shown in Tables 3 and 4. After incubation with salinomycin, the transcription level of several genes involved in ER stress and UPR were affected. The ER stress sensors inositol-requiring enzyme 1 (IRE1) and downstream targets of these, like XBP-1, were up-regulated, as shown in Table 3. However, as known HSPA4 and HSPA4L, heat shock protein (HSP) 110 family, were down-regulated (Table 4).

As shown in Figure 3, the effects of salinomycin on some genes were involved in ER stress and apoptosis. After salinomycin treatment, phosphorylated eIF2 α (eIF2 α -P), which is a hallmark of ER stress, was found significantly increased time dependent manner (Figure 3A). Phosphorylation of eIF2 α induces transcription of ATF4 and its downstream targets, like ATF3, CHOP, growth arrest and TRIB3 [12,28] and heme oxygenase 1 (HMOX1) [29]. Moreover, the mRNA and protein levels of CHOP (Figures 3A and 3B), TRIB3 (Figure 3A) and ATF4 (Figures 3B and 3D), were also increased. ATF4 is known to induce genes involved in amino acid synthesis and several transcripts involved in amino acid synthesis and transport were also up-regulated (Table 3). Therefore, our results suggest salinomycin induces ER stress and UPR by regulated a variety of chaperone proteins and transcription factors and leads to induction of apoptosis in PC-3 prostate cancer cells.

Discussion

Several studies have shown that salinomycin inhibits the growth of various cancer cells in vitro and in vivo [22,24,25]. We have previously shown that salinomycin induced apoptosis of human prostate cancer cells due to accumulated reactive oxygen species and mitochondrial membrane depolarization [26]. Also, some studies have indicated that salinomycin affects multiple biochemical pathways that might play a role in its apoptosis and growth-limiting effect on diverse type of apoptosis- and chemoresistant- cancer cells. Furthermore, recent preclinical study demonstrated that salinomycin-based various anticancer agents combination treatment could be beneficial for development of p-glycoprotein inhibiting drug or radiation therapy [30,31]. However, the exact mechanisms underlying salinomycin induced cell death are not yet clear.

Our results showed that salinomycin affects several transcripts involved in cell cycle progression. In microarray data, positive cell cycle regulators, such as cyclin-dependent kinases (CDKs), cyclins, and cell division cycle (CDC), were down-regulated and negative regulators such as CDKs inhibitors were up-regulated. Also, salinomycin affected

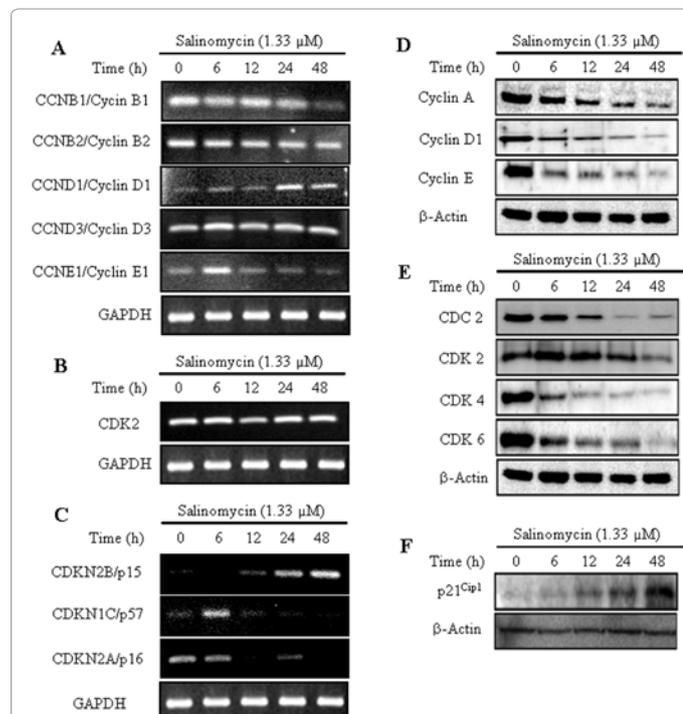


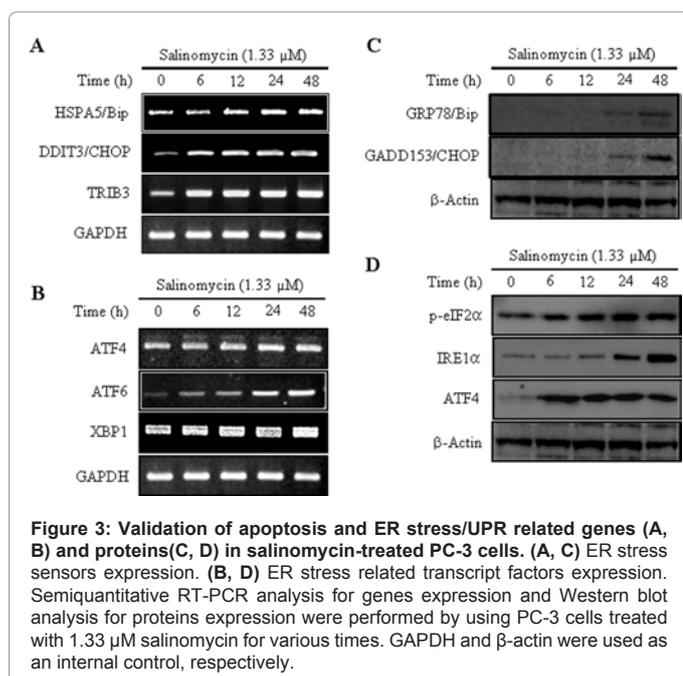
Figure 2: Validation of apoptosis and cell cycle arrest related genes (A, B, C) and proteins (D, E, F) in salinomycin-treated PC-3 cells. (A, D) Cyclin expression, (B, E) Cyclin-dependent kinase expression, (C, F) Cyclin-dependent kinase inhibitors expression. Semiquantitative RT-PCR analysis for genes expression and Western blot analysis for proteins expression were performed by using PC-3 cells treated with 1.33 μ M salinomycin for various times. GAPDH and β -actin were used as an internal control, respectively.

Gene Symbol	Fold Change	Description	Genbank
ATF4	2.18	activating transcription factor 4 (tax-responsive enhancer element B67)	NM_001675
ATF6	1.78	activating transcription factor 6	NM_007348
DDIT3	6.33	DNA-damage-inducible transcript 3	NM_004083
EDEM1	3.60	ER degradation enhancer, mannosidase alpha-like 1	NM_014674
EIF2A	1.16	eukaryotic translation initiation factor 2A, 65kDa	NM_032025
EIF2AK3	4.02	eukaryotic translation initiation factor 2-alpha kinase 3	NM_004836
FAM129A	2.26	family with sequence similarity 129, member A	NM_052966
HERPUD1	8.78	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	NM_014685
HERPUD2	2.13	HERPUD family member 2	NM_022373
HMOX1/HSP32	2.98	heme oxygenase (decycling) 1	NM_002133
HSPA1A	2.31	heat shock 70kDa protein 1A	NM_005345
HSPA1L	2.29	heat shock 70kDa protein 1-like	NM_005527
HSPA5	3.54	heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	NM_005347
IRE1/ERN1	4.06	endoplasmic reticulum to nucleus signalling 1	NM_001433
SEC63	2.15	SEC63 homolog (S. cerevisiae)	NM_007214
SQSTM1	4.44	sequestosome 1	NM_003900
TRIB3	2.07	tribbles homolog 3 (Drosophila)	NM_021158
TXNDC4	2.03	thioredoxin domain containing 4 (endoplasmic reticulum)	NM_015051
XBP1	2.66	X-box binding protein 1	NM_005080

Table 3: List of 2-fold up genes involved in ER stress and UPR.

Gene Symbol	Fold Change	Description	Genbank
ATG10	0.45	ATG10 autophagy related 10 homolog (S. cerevisiae)	NM_031482
HSPA4	0.38	heat shock 70kDa protein 4	NM_002154
HSPA4L	0.27	heat shock 70kDa protein 4-like	NM_014278
TOR1A	0.41	torsin family 1, member A (torsin A)	NM_000113

Table 4: List of 2-fold down genes involved in ER stress and UPR.



several cell cycle transcripts and proteins like CDKs, cyclins and CDKs inhibitors and arrests significantly the cells in G₁ phase of the cell cycle. However, CDK4 and CDK6 did not change mRNA levels in microarray analysis but protein expression was confirmed to be reduced in a time-dependent manner. Salinomycin-induced cell cycle arrest might be associated with reduction of cyclins E and B, CDK2 and CDK4. Furthermore, several studies reported that expression of p21^{cip1} was increased by p53 activation [9]. But salinomycin induced p21^{cip1} activation in even p53-independent PC-3 cells. These results indicated that one of the mechanisms which salinomycin may regulate is the proliferation of cancer cells by inhibiting cell cycle progression.

This is the first study demonstrating that salinomycin induces endoplasmic reticulum (ER) stress and substantial activation of ER stress-dependent gene expression in a prostate cancer cell. Transcripts involved in ER stress and downstream of all three ER stress sensors, such as inositol-requiring enzyme 1 (IRE1), X-box binding protein 1 (XBP1), activating transcription factor 4 (ATF4) and ATF6 were found to be increased after treating PC-3 cells with salinomycin. Also, the increased expression of heat shock proteins (HSPs) indicated that the cells try to increase their protein folding capacity. The up-regulation of ER degradation enhancer, mannosidase alpha-like 1 (EDEM1) and sequestosome 1 (SQSTM1), proteasomal and ubiquitin-related

transcripts indicated activation of ER-associated degradation (ERAD). The eukaryotic translation initiation factor 2 alpha (eIF2 α) kinase 3 (EIF2AK3/PERK) branch of unfolded protein response (UPR) was found to be activated, supported by the increased protein level of p-eIF2 α and ATF4, as well as up-regulation of several downstream targets of ATF4, such as ATF3, ASNS, DNA-damage-inducible transcript 3 (DDIT3/CHOP) and tribbles homolog 3 (TRIB3) at mRNA level, which are known to link ER stress to ER stress induced cell death. During ER stress, activation of the PERK-dependent pathway leads to phosphorylation of eIF2 α [32,33]. Phosphorylation of this protein increases translation of a number of mRNAs, one of which is ATF4, and results in the reduction of ER protein load [33,34]. Our observations of salinomycin-induced increases in the expression of ATF4 protein and transcript were consistent with prior reports that this transcription factor is induced under conditions of ER stresses. The expression of CHOP in response to stress is regulated at the transcriptional level through the transcription factor ATF2, 4 and 6 [17]. Numerous pro-apoptotic and anti-apoptotic genes, including Bcl-2, GADD34, and TRIB3, were regulated by CHOP, which is a transcription factor with a well-established role in ER stress, particularly in the induction of apoptosis [35]. CHOP directly binds the promoter of TRIB3 gene and up-regulates its expression, which results in apoptosis via inhibition of AKT activation [35]. Intriguingly, TRIB3 also regulates CHOP expression through negative feedback [36]. Also, CHOP has been associated with mitochondrial stress induced by accumulation of unfolded proteins [37]. Originally we reported that salinomycin induced the apoptosis of prostate cancer cell lines through accumulated reactive oxygen species (ROS) and mitochondrial membrane depolarization [26]. At this time, analysis of complete gene profiling in our cDNA microarray was consistent with our previous study. That is to say, salinomycin induced several oxidation stress- and mitochondrial dysfunction-related genes expression (data not shown) but, in this study, we didn't research the genes expression related with ROS and mitochondrial membrane potentials. Because our data implicate that salinomycin seems to cause ER stress through mitochondrial stress and oxidative stress, along with our previous data [26], we will handle those kinds of genes expression to confirm the cDNA microarray data in next study.

In summary, the present study demonstrated that a dose of salinomycin causes changes in the transcription of genes or induction of proteins that are involved in the regulation of an array of cellular functions. These include activation of genes that participate in cell cycle arrest and cellular responses to ER stresses, observing a gene expression analysis using microarray technology, which lead to induction of apoptosis. Additional studies are required to evaluate these cellular phenomena further.

Acknowledgments

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2012R1A1A2022587).

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