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 genomewide 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 genomewide 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, p21WAF1 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 eIF2α, 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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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 refractoriness 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 ATR were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for cyclin A, CDC2, p21(WAF1), IRE1α, Bip, and phospho-eIF2α were purchased from Cell Signaling (Beverly, MA, USA). CDK4 and GADD153/CHOP were purchased from Santa Cruz Biotechnology (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 μg/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 cDNAs 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 μg 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 mixture. 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 1×10⁵/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 μg/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 Modfit software (Becton Dickinson Co.).

**Semi-quantitative 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). Semi-quantitative RT-PCR amplification was performed using primers for CDKN2B, CDKN1C, CDKN2A, CCNB1, CCNB2, CCND1, CCND3, CCNE1, CDK2, HSPA5, DDIT3, TRIB3, ATF4, ATF6, XBPI, and GAPDH. RT-PCR primers were designed by primer 3 (http://frodo.wi.mit.edu) (Supplementary Table 1). For semi-quantitative 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 over 2-fold changed in both absence and presence salinomycin. Of 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 G1/G0 phase (Figure 1A). In the presence of 1.33 and 4.00 μM of salinomycin, the proportion of cells in G1/G0 phase increased gradually up to 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...
### Table 1: List of 2-fold up genes involved in apoptosis, cell cycle and proliferation.

| 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 |
| HOX1        | 2.98        | heme oxygenase (decycling) 1 | NM_002133 |
| CDKN1A      | 2.89        | cyclin-dependent kinase inhibitor 1A (p21, Cip1) | NM_00389 |
| 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 2: List of 2-fold down 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_005356 |
| 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        | 0.26        | 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_055192 |
| 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 |
salinomycin-induced cell death are not yet clear. However, the exact mechanisms underlying salinomycin development of p-glycoprotein inhibiting drug or radiation therapy [26]. Also, some studies have indicated shown that salinomycin induced apoptosis of human prostate cancer various cancer cells in vitro and in vivo [22,24,25]. We have previously shown that salinomycin inhibited apoptosis of human prostate cancer cells due to accumulated reactive oxygen species and mitochondrial membrane depolarization [26]. Also, some studies have indicated that salinomycin inhibits 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 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 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 μM salinomycin for various times. GAPDH and β-actin were used as an internal control, respectively.

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

| 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 (decrystallizing) 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 4: List of 2-fold down 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 |

Figure 3: Validation of apoptosis and ER stress/UPR related genes (A, B) and proteins (C, D) in salinomycin-treated PC-3 cells. (A, C) ER stress sensors expression. (B, D) ER stress related transcript factors expression. Semi-quantitative RT-PCR analysis for genes expression and Western blot analysis for proteins expression were performed by using PC-3 cells treated with 1.33 μM salinomycin for various times. GAPDH and β-actin were used as an internal control, respectively.

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<sup>WAF1</sup> was increased by p53 activation [9]. But salinomycin induced p21<sup>WAF1</sup> 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 (eIF2α) kinase 3 (EIF2AK3/PERK) branch of unfolded protein response (UPR) was found to be activated, supported by the increased protein level of p-eIF2a and ATF4, as well as up-regulation of several downstream targets of ATF4, such as ATF3, ANS3, 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 eIF2a [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 stress. 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.

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