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A Comparison of Proteomic Profiles Changes during 17β-Estradiol Treatment in Human Prostate Cancer PC-3 Cell Line

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Abstract. *Human telomerase reverse transcriptase (hTERT) is overexpressed in prostate cancer. Estrogen plays a central role in the development of prostate cancer. hTERT activity has been shown to be increased after estrogen treatment. Although significant efforts have been made to understand the role of estrogen, the telomerase connection with estrogen is poorly understood. In this report, we describe a proteomics approach for investigating the global changes in protein expression in estrogen-treated human prostate cancer PC-3 cells. PC-3 cells were seeded in medium and then treated with estrogen; the protein extract from these cells was used for two-dimensional (2D) gel electrophoresis. The protein spots were subjected to comparative analysis by liquid chromatography/mass spectrometry (LC/MS). We observed that the expression of 17 proteins, including stress-induced phosphoprotein 1 and lamin-A/C was down-regulated, and that the expression of proteins such as subunit α of T-complex protein 1, tubulin alpha-1B, and other 13 proteins was up-regulated. These proteins may have been closely associated with estrogen-induced hTERT activity. The expression level of these proteins could be a useful parameter for evaluating the estrogen-induced hTERT activity in clinical specimens of human prostate cancer.*

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Prostate cancer has become one of the major causes of male deaths, and is the second leading cause of cancer death in males in the United States (1). Although the use of prostate-specific antigens for early detection of prostate cancer has proven to be advantageous, this screening method may not always be effective due to its low specificity and sensitivity. Therefore, much effort has been directed toward developing better diagnostic and/or prognostic biomarkers (2). Proteomic analysis has great potential for developing such markers; it has been applied for the detection of characteristic proteins (other than the prostate-specific antigen) present in the tissue or serum of prostate cancer patients (3-7).

Telomerase is a ribonucleoprotein complex responsible for the complete replication of chromosome ends, and its activation is considered to play a crucial role in not only cell proliferation but also carcinogenesis (8-11). Previous studies have demonstrated an enhanced telomerase activity in more than 85% of human cancers, but only in a few normal somatic cells (9, 10). Human telomerase reverse transcriptase (hTERT) positively regulates telomerase activity at the transcriptional level and is selectively overexpressed in proliferating neoplastic tissues and cells (8-11).

An important impediment in the treatment of prostate cancer is the refractory response to androgens that develops after chemotherapeutic treatment. Although in the initial stages, prostate cancer responds well to androgen deprivation, it often progresses to an androgen-independent phenotype after treatment (12). In contrast, 17β-estradiol (E2) treatment promptly induces the expression of the mRNA encoding the catalytic subunit of hTERT and telomerase activity in the human prostate cancer cell line PC-3 (13). Binding of the endothelial nitric oxide synthase (eNOS)/estrogen receptor complex to the estrogen response element of hTERT activates eNOS, and this ligand-activated estrogen receptor is involved

in the regulation of hTERT expression in the endothelium (14). The present study investigated a comparison of proteomic profiles before and after 7β-estradiol treatment in human prostate cancer PC-3 cell line.

Materials and Methods

Cell culture and hormones. The human prostate cancer PC-3 cells were provided by American Type Culture Collection (Rockville, MD). The PC-3 cells were maintained in Ham's F-12K medium, supplemented with 10% fetal bovine serum (FBS), and cultured in 100-ml flasks. The cells were treated with E2 (CALBIOCHEM).

In vitro treatment of cells. The PC-3 cells were treated with E2 (10^{-7} mol/L) according to the manufacturer's instructions.

Real-time reverse transcription polymerase chain reaction. An RNeasy kit (QIAGEN GmbH, Hidden, Germany) was used for isolation of total RNA from PC-3 cells after treatment with E2 for 12 hours and 24 hours. Non-treated PC-3 cells were used as the control. Quantification of hTERT and hTR was carried out by PCR amplification with a commercial kit (Roche Diagnostics, Indianapolis, IN), as previously described. Approximately 100 μg of total RNA were used for this experiment.

Sample preparation and 2D polyacrylamide gel electrophoresis. Human PC-3 cells were seeded in the culture medium at approximately 20% confluence, and after 4 days the cells attained 70-80% confluence. Samples of the non-treated PC-3 cells and PC-3 cells treated with E2 at 12 hours and 24 hours were prepared as follows. cells were suspended in ice-cold sample buffer containing 40 mM Tris, 7 M urea, 2 M thiourea, 4% CHAPS, 100 mM 1,4-dithioerythritol, and protease inhibitor cocktail (Roche, Mannheim, Germany). The suspensions were incubated for 60 min on ice and centrifuged at 13,000 × g for 10 min. The supernatant of each clone was used for each electrophoresis. Total protein (20 μg) in sample buffer was applied to immobilized pH gradient (pH 3-10) nonlinear strips. Isoelectrofocusing was carried out by increasing the voltage in a stepwise manner up to 12,000 V, and then continuing for 2 h at 12,000 V. The second dimension was carried out in a 9-16% linear gradient polyacrylamide gel at 40 mA at a constant current for ~5 h, until the dye front reached the bottom of the gel.

Protein visualization and image analysis. After protein fixation in 40% methanol and 5% phosphoric acid for 12 h, the gels were stained using a Dodeca Silver Stain Kit (Bio-Rad) according to the manufacturer's instructions. The gels were destained using the Silver Stain MS Kit (Wako, Osaka, Japan), and then were scanned in a Gel Doc XR 170-8170 (Bio-Rad). All data were converted into electronic files, which were then analyzed using the Finger printing II Software program (Bio-Rad).

In-gel digestion of proteins and their identification by liquid chromatography-mass spectrometry. In-gel digestion of protein spots on the silver-stained gels was performed essentially as described by Shevchenko *et al*. (1996), with minor modifications (15). The resulting tryptic fragments were eluted from the excised gels by allowing their diffusion into 5% trifluoroacetic acid (TFA) and 50% acetonitrile, and then sonicated twice in an ultrasonic water bath. Ultrasonication facilitated diffusion of the peptides. The peptides obtained by sonication were treated with 0.1% formic acid and 50% acetonitrile and introduced into microtitre plates.

For liquid chromatography-mass spectrometry (LC-MS) analysis, 0.5 μl of the tryptic peptide mixture along with 0.5 μl matrix solution containing 10% (mg/ml) α-cyano-4-hydroxy-cinnamic acid (CHCA), 0.1% TFA, and 50% acetonitrile, were loaded onto the LC/MS column. Spectra were obtained by liquid chromatography-tandem mass spectrometry using the LC/MSD Trap XCT Ultra (Agilent Technologies, Santa Clara, CA, USA). Analysis was performed under conditions in which the autolytic trypsin fragments were abundant. For each sample, averages of 18–20 spectra were acquired in the delayed-extraction and reflector modes. The protein database search was performed using the Spectrum Mill Ms Proteomics Workbench software program (http://www.proteomesoftware.com).

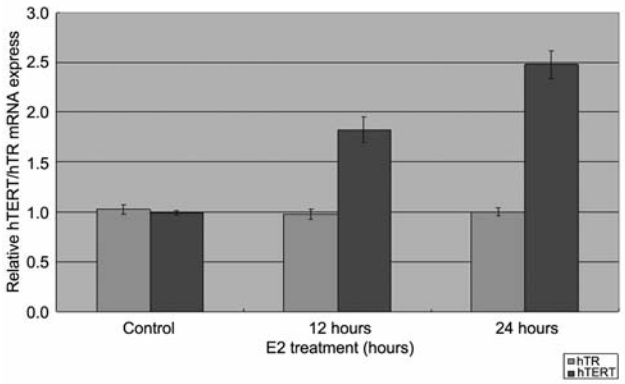

Figure 1. *Induction of hTERT mRNA by E2 treatment (10^{-7} M) in human prostate cancer PC-3 cells. Real-time RT-PCR analysis was performed to determine the hTERT and hTR mRNA levels in PC-3 cells treated with E2 at 12 hours and 24 hours. Average values from 5 independent experiments and expressed as the relative hTERT mRNA calculated as the ratio to the internal control.*

Results

hTERT expression and telomerase activity in non-treated PC-3 cells and in cells transformed by E2 treatment. The hTERT mRNA levels in E2-treated and non-treated cells were evaluated by real-time reverse transcriptase-polymerase chain reaction (RT-PCR); the extent to which the mRNA expression was induced was measured by densitometry. It was observed that hTERT mRNA expression was rapidly induced in all the samples after E2 treatment. After E2 treatment 24 hours, a reproducible increase was observed in the hTERT mRNA levels in PC-3 cells (average stimulation ranging from 2.2 to 2.8-fold). No significant change in hTR mRNA levels was elicited in all samples by E2 treatment (Figure 1).

Protein analysis of non-treated and E2-treated human PC-3 cells. In this study, we used silver staining for the detection of proteins on the gel. Silver staining revealed more than 1,000 spots on the 2D gel (Figure 2); the samples obtained at each

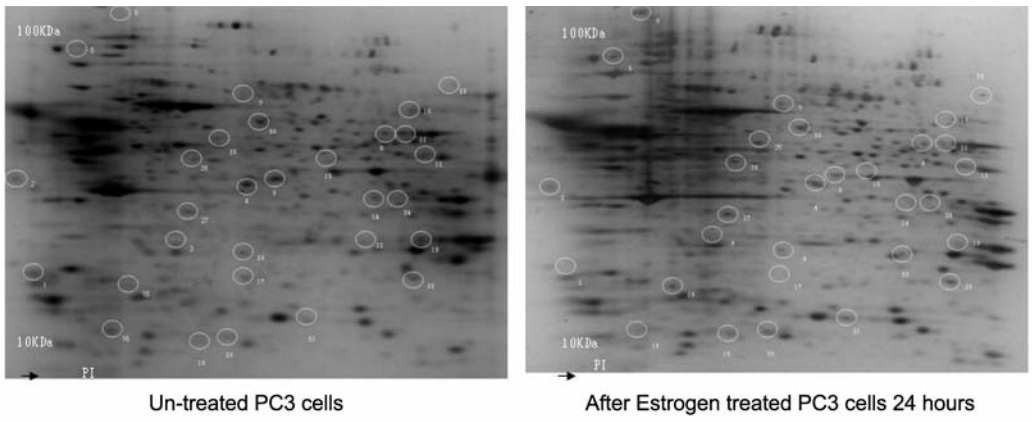

Figure 2. *2D Gel electrophoresis profiles showing the identified proteins in estrogen-treated and non-treated human PC-3 cells.*

time point (24 hours after E2 treatment) were analyzed on 3 or more gels by using the Melanie III software, and only those gels for which the normalized volumes of the protein spots were similar were selected for further investigation. Taken together, these results indicate that the complex protein mixtures in the E2-treated PC-3 cells were well resolved with the 2D gel electrophoresis system used in our study, and that highly reproducible patterns were obtained. However, more than 30 of the excised spots could not be identified; this was usually because the amount of protein available, even after pooling the proteins from several gels, was insufficient. The identity of the extracted proteins was also confirmed by western blotting.

Proteomic profiles of non-treated and E2-treated PC-3 cells by LC-MS analysis. Analysis of the profiles of 2D gels also demonstrated the down-regulation of the expression of 17 proteins (Table I) such as Sirt-1, Lamin-A/C subunit-σ and subunit-α of T-complex protein 1, regulatory subunit 8 of the 26S protease, Rab-GDP dissociation inhibitor-β, adenosyl-homocysteinase, transketolase, pyruvate kinase isozymes M1/M2, mitochondrial precursor of fumarate hydratase, multifunctional protein ADE2, annexin A7, and actin-related protein-3. Among these, the expression of 13 proteins (Table II), including subunit-α of the T-complex protein 1, tubulin α-1B, the precursor of Calreticulin, and Desmoplakin was up-regulated in response to estrogen treatment.

Discussion and Conclusion

In 2002, Simona Nanni *et al*. reported that E2 treatment promptly induced the expression of mRNA encoding the catalytic subunit of hTERT and telomerase activity in the PC-3 cell lines (13). The expression of many proteins was altered by treatment of the PC-3 cells with estrogen; this might have lead to the increase in hTERT activity. For example, a recent study has implicated stress-induced phosphoprotein 1 in the induction of hTERT activity (16).

The level of this protein was found to be reduced in PC-3 cells after estrogen treatment. For example: Stress-induced phosphoprotein 1 is the mammalian ortholog of silent information regulator (SIR2); the SIR2 family of genes is a highly conserved group of genes that encode nicotinamide adenine dinucleotide (NAD)-dependent protein deacetylases, also known as class III histone deacetylases (17, 18). The *Saccharomyces cerevisiae* SIR2 gene was the first gene of this type to be discovered, and is one of the best characterized genes; it is involved in the silencing of mating type loci, telomere maintenance, response to DNA damage, and cell aging (19). It is a nuclear protein that is involved in regulation of many cellular processes, including apoptosis, cellular senescence, endocrine signaling, glucose homeostasis, and aging and longevity (20, 21). The acetylated form of p53 is a target of stress-induced phosphoprotein 1. Deacetylation of p53 and forkhead box (Fox0) transcription factors represses apoptosis and increases cell survival (17-21). However, the function of the phosphorylation sites on these proteins has not yet been determined. In 1992, Celis *et al*. showed in a classical microsequencing/genomic study that the expression of this transformation-sensitive protein was up-regulated in transformed embryonic lung fibroblasts. Interestingly, they noted that Stip-1 was mainly localized in the Golgi in normal fibroblasts, but in the case of transformed cells, it was localized in the nucleus (22, 23).

From the results of this proteomic study, it can be speculated that the expression of stress-induced phosphoprotein 1, lamin A/C, junction plakoglobin, α subunit of hemoglobin, annexin A7, and Desmoplakin was different between the estrogen-treated and non-treated PC-3 cells. However, further studies are required to prove this and to understand the molecular mechanism underlying increased hTERT activity in E2-treated PC-3 cells. To this end, we are studying the influence of estrogen on hTERT activity by transfecting these cells with small interference RNA.

Table I. *Proteins that were down-regulated in human PC-3 cells after estrogen treatment for 24 hours.*

Spot	Protein name	Database accession	Protein pI	Protein MW (Da)	Mean peptide spectral intensity
1a	T-complex protein 1 subunit delta	P50991	7.96	57924.6	9.46e+006
1b	Pyruvate kinase isozymes M1/M2	P14618	7.95	57937.2	4.56e+006
1c	Pyruvate kinase isozymes R/L	P30613	7.65	61830.5	4.94e+006
2a	26S protease regulatory subunit 8	P62195	7.11	45626.3	5.67e+006
2b	Heterogeneous nuclear ribonucleoprotein D0	Q14103	7.61	38434.4	5.64e+006
3a	Rab GDP dissociation inhibitor beta	P50395	6.11	50663.5	1.55e+008
3b	Rab GDP dissociation inhibitor alpha	P31150	5.00	50583.0	2.04e+008
4a	Adenosylhomocysteinase	P23526	5.92	47716.4	9.48e+006
4b	Putative adenosylhomocysteinase 3	Q96HN2	7.13	66721.5	1.47e+007
4c	Eukaryotic translation initiation factor 3 subunit G	O75821	5.87	35611.2	8.46e+006
8a	T-complex protein 1 subunit alpha	P17987	5.80	60343.9	8.98e+006
8b	UDP-N-acetylhexosamine pyrophosphorylase	Q16222	5.92	58769.3	4.17e+006
9	Transketolase	P29401	7.58	67878.0	1.15e+007
10	Pyruvate kinase isozymes M1/M2	P14618	7.95	57937.2	5.91e+006
11	Fumarate hydratase, mitochondrial precursor	P07954	8.85	54637.3	7.32e+006
12a	Stress-induced-phosphoprotein 1	P31948	6.40	62639.6	3.18e+006
12b	Bifunctional purine biosynthesis protein PURH	P31939	6.27	64616.2	8.18e+006
13	Lamin-A/C	P02545	6.57	74139.8	6.42e+006
14	Multifunctional protein ADE2	P22234	6.94	47079.5	4.31e+006
15	Annexin A7	P20073	5.52	52739.5	7.88e+006
16	Actin-related protein 3	P61158	5.61	47371.4	7.15e+006
17	Hemoglobin subunit alpha	P69905	8.72	15257.6	4.81e+006
28	Fumarate hydratase, mitochondrial precursor	P07954	8.85	54637.3	7.25e+006
29	Elongation factor 1-gamma	P26641	6.25	50119.1	1.21e+007
30	Synaptic vesicle membrane protein VAT-1 homolog	Q99536	5.88	41920.5	2.64e+006

Table II. *Proteins that were up-regulated in human PC-3 cells after estrogen treatment for 24 hours.*

Spot	Protein name	Database accession	Protein pI	Protein MW (Da)	Mean peptide spectral intensity
5a	T-Complex protein 1 subunit alpha	P17987	5.80	60343.9	8.98e+006
5b	UDP-*N*-acetylhexosamine pyrophosphorylase	Q16222	5.92	58769.3	4.17e+006
6a	Tubulin alpha-1B	P68363	4.94	50151.9	3.11e+008
6b	Tubulin alpha-1A chain	Q71U36	4.94	50135.9	3.11e+008
7	Calreticulin precursor	P27797	4.29	48141.8	1.90e+007
18	Macrophage-capping protein	P40121	5.89	38517.8	4.11e+006
19	Tubulin alpha-1C chain	Q9BQE3	4.96	49895.6	7.88e+006
20	Desmoplakin	P15924	6.43	331775.6	7.91e+006
21	Vimentin	Q5R1W8	5.03	53652.9	4.07e+007
22	40S ribosomal protein SA	Q4GWZ2	4.80	32928.3	9.25e+006
23	Cytochrome *b-c*1 complex subunit 1, mitochondrial	P31930	5.94	52646.1	4.40e+006
24a	Junction plakoglobin	P14923	5.95	81630.2	1.64e+007
24b	Galectin-7	P47929	7.02	15075.1	1.06e+007
25a	Aldehyde dehydrogenase 1A3	P47895	6.99	56108.8	2.55e+006
25b	UDP-glucose 6-dehydrogenase	O60701	6.73	55024.4	6.31e+006
26	DNA polymerase delta catalytic subunit	P28340	6.64	123631.8	5.44e+006
27a	Aldehyde dehydrogenase 1A3	P47895	6.99	56108.8	1.66e+006
27b	Nucleoporin p54	Q7Z3B4	6.53	55435.7	8.03e+005

In conclusion, we identified 158 proteins in estrogen-treated and non-treated PC-3 cells by proteomic analysis with LC-MS, and categorized them according to their functions. Several of these proteins might be involved in increasing the expression and activity of hTERT in human prostate cancer cells. Moreover, some of these proteins may play an important role in increasing the hTERT activity of estrogen-induced cells; further studies are required for

clarifying the mechanism underlying this effect. The expression level of these proteins could be used to evaluate the estrogen-induced hTERT activity in clinical specimens of human prostate cancer.

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