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# Tamoxifen Suppresses Clusterin Level through Akt Inactivation and Proteasome Degradation in Human Prostate Cancer Cells

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**Abstract** − Clusterin is a heterodimeric sulfated glycoprotein and plays a role in many different types of cancer as a cell survival factor and helps cancerous cells to evade stress-induced apoptosis. To investigate whether the regulation of clusterin expression is involved in the mechanism of anticancer agent, we studied the effect of tamoxifen on clusterin expression in human prostate cancer PC-3 cells. Treatment of PC-3 cells with tamoxifen reduced cellular proliferation. Western blot analyses showed that treatment with tamoxifen suppressed clusterin expression in a concentration-dependent manner. Transfection with clusterin siRNA plasmid showed that clusterin is required for PC-3 cell survival. We found that tamoxifen resulted in a rapid decrease in the phosphorylation of Akt on Ser473 leading to prevent kinase activity. Expression of myristoylated Akt prevented tamoxifen-mediated clusterin downregulation. Interestingly, MG132, a well-known proteasome inhibitor also recovered clusterin expression suppressed by tamoxifen. These data indicate that clusterin expression may be regulated by activation of Akt and ubiquitin-proteasome pathway plays an important role in tamoxifen-mediated clusterin suppression.

**Keywords:** Tamoxifen, Clusterin, Akt, Proteasome

## INTRODUCTION

Clusterin/Apolipoprotein J (CLU) is a heterodimeric ubiquitously expressed glycoprotein of 75-80 kDa that is implicated in a variety of physiological processes such as tissue remodeling, reproduction, lipid transport, complement regulation, and apoptosis (Blaschuk *et al.,* 1983; Wilson and Easterbrook-Smith, 2000; Jones and Jomary, 2002). It was first identified as a secreted glycoprotein in ram rete testis fluid enhancing cell aggregation in vitro (Blaschuk *et al.,* 1983; Collard and Griswold, 1987). The primary product of the clusterin gene is a polypeptide of 449 amino acids and after proteolytic processing to remove the first 22 amino acids hydrophobic secretory signal sequence, clusterin is cleaved to produce its $\alpha$ and $\beta$ subunits prior to secretion from the cells (Leskov *et al.,* 2003). Clusterin gene is differentially expressed in various severe physiological disturbance states, including kidney degenerative disease, neurodegenerative conditions, and carcinogenesis.

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Increased CLU mRNA and protein levels have been detected in various tissues undergoing stress, including heart, brain, liver, kidney, and retinal tissues both in vivo and in vitro. There is extensive evidence of a correlation between clusterin expression and human prostate cancer (Miyake *et al.,* 2000, 2004; Bettuzzi *et al.,* 2002; Zellweger *et al.,* 2002). Up-regulation of clusterin expression was shown in prostate carcinomas when compared with normal tissues.

A close relationship between clusterin expression and apoptosis has been studied. Clusterin may be associated with cell survival and disease progression in prostate cancer because it increases in regressing normal prostatic epithelial cells and prostate cancer xenografts after treatment with various apoptotic stimuli (Miyake *et al.,* 2000). Other studies with antisense oligonucleotide inhibiting clusterin expression also provide evidences regarding its function during apoptosis (Trougakos *et al.,* 2004; So *et al.,* 2005; Sowery *et al.,* 2008). However, arguments have been advanced proposing clusterin as a marker for apoptosis because other studies have providing conflicting information that clusterin is not directly related to apoptosis (Petropoulou *et al.,* 2001; Dumont *et al.,* 2002).

Tamoxifen is well known as a selective estrogen receptor modulator and currently used for the treatment of early and advanced estrogen receptor positive breast cancer (Jordan, 2006, 2007). Tamoxifen also leads to the inhibition of the growth and invasion of prostate cancer cells by inducing apoptosis (El Etreby *et al.,* 2000). Because clusterin can prevent apoptosis, we were interested in studying regulation of clusterin expression by tamoxifen in PC-3 human prostate cancer cells. The data presented in this report show that clusterin expression may be regulated by Akt-mediated signal pathway and tamoxifen downregulates clusterin expression through ubiquitin-proteasomal degradation.

## MATERIALS AND METHODS

### Cell culture

Human prostate cancer PC-3 cells were grown in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37°C in a humidified atmosphere of 5% $CO_2$. After incubation, the cells were harvested by scrapping and solubilized in 50 mM Tris-HCl (pH 8.0) containing 150 mM NaCl, 1% nonidet P-40, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Cells were centrifuged at 1,000 × g for 5 min at 4°C and the pellets were resuspended and stored in −70°C.

### Cell viability assay

Cells ($1\times10^4$/well) were plated onto 96-well plates and incubated at 37°C in a 5% $CO_2$ atmosphere. After incubation for the designated time, 10 μg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added per well. After incubating at 37°C for 4 h, cells was centrifuged at 1,000 × g for 5 min, the medium was removed by aspiration, and then MTT formazan crystal formed was dissolved by adding 0.15 ml of DMSO and shaking for 15 min. The absorbance at 590 nm was measured using a GENios microplate reader (Tecan, Männedorf, Switzerland). The percentages of cells surviving from each group relative to control, defined as 100% survival, were calculated.

### Construction of siRNA

For design of siRNA oligonucleotides, the specific sequence for the human clusterin gene (5'-AATCGACAGAAGGACATCTAC-3') was selected using siRNA target finder from Ambion (Austin, TX) web sources. Oligonucleotides were synthesized and annealed at 15°C for 12 h. Annealed oligonucleotides were ligated with pSilencer 2.0-U6 plasmid (Ambion), digested with *BamH*I and *Hind*III. A nonspecific scrambled siRNA was used as a negative control. The nucleotide sequences of siRNA were determined by direct DNA sequencing.

### Transient transfection

For transient transfection, cells were transfected with WelFect-Q™ transfection reagent (Welgene, Daegu, Korea) using clusterin siRNA plasmid or mys-Akt plasmid as recommended by the manufacturer. Briefly, cells were washed in PBS and then resuspended in Opti-MEM serum free medium. Transfection was carried out with 3 μg of plasmid DNA and 12.5 μl of transfection reagent for 5 h at 37°C. After transfection, cells were maintained in RPMI medium containing 20% FBS for 24 h or 48 h.

### RT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen). RT-PCR was performed using an Access RT-PCR system purchased from Promega (Madison, WI) according to the manufacturer's instructions. Human clusterin cDNA was amplified by PCR using a sense primer (5'-GTCAACGGGGTGAAACAGAT-3') and an antisense primer (5'-TCAGGCAGGGCTTACACTCT-3') with 30 cycles by denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. Human GAPDH cDNA was amplified using a sense primer (5'-TGCACCACCAACTGCTTAG-3') and an antisense primer (5'-GATGCAGGCATGATGTTC-3') with 25 cycles by denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 30 s. The number of amplification cycles was optimized in preliminary experiments to ensure that the PCR has not reached its plateau. The amplified PCR products were analyzed by 2% agarose gel electrophoresis and ethidium bromide staining.

### Western blot analysis

Cell lysates (30 μg of protein) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to PVDF membranes. Membranes were blocked in 5% (w/v) nonfat dried milk in Tris-buffered saline with 0.05% tween 20 (TBST) overnight at 4°C. Membranes were then incubated for 1 h with primary antibodies against clusterin, Akt, phospho-Akt, PP2A, and GAPDH at a 1:1,000 dilution in TBST. After subsequent incubation with a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody, proteins were visualized by an ECL method using WEST-one Western Blot detection system (iNtRON, Korea). Protein concentrations were esti-

mated using the bicinchoninic acid method according to the supplier's recommendations (Pierce Chemical Co., Rockford, IL) using BSA as a standard. Antibodies against Akt, phospho-Akt, PP2A, and GAPDH were obtained Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against human clusterin $\alpha$ chain was purchased from Upstate Biotechnology (Lake Placid, NY).

### Data analysis

Statistical analysis was performed by using one-way analysis of variance, followed by Dunnett's pairwise multiple comparison t-test with GraphPad Prism software (GraphPad Software Inc., San Diego, CA) when appropriate. The difference was considered statistically significant at $p < 0.05$.

## RESULTS

### Suppression of clusterin expression by tamoxifen

To investigate the effects of tamoxifen on cell viability, PC-3 cells were treated with tamoxifen for 4 h and then MTT assay was performed. As shown in Fig. 1, tamoxifen exerted a concentration-dependent inhibition of PC-3 cell proliferation. To elucidate the mechanism of tamoxifen cytotoxicity, we determined the expression level of clusterin by tamoxifen by Western blot. Fig. 2A showed that treatment with tamoxifen for 4 h caused a significant decrease in clusterin expression. To determine whether clusterin expression is necessary for PC-3 cell growth, siRNA for clusterin was treated for 48 h. Clusterin siRNA showed a significant decrease in clusterin expression and also reduced PC-3 cell viability to 65% (Fig. 2B).

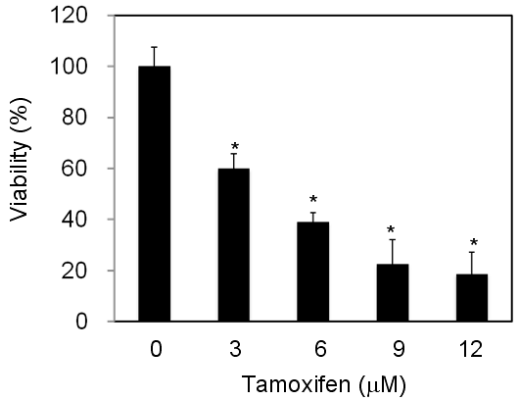


**Fig. 1.** Cytotoxic effect of tamoxifen in PC-3 human prostate cancer cells. Cells were treated with various concentrations of tamoxifen for 4 h. The percentage of cells surviving from each groups relative to controls were determined by MTT assay. Results are the mean ± S.D. of three separate experiments. *Significantly different from the untreated control group ($p < 0.05$).

### Tamoxifen inactivates Akt activity

To explore the molecular basis for the tamoxifen-mediated suppression of clusterin expression, we tested the status of Akt, as activation of Akt plays an important role in cellular functions by phosphorylating a variety of substrates involved in the promotion of cell proliferation and prevention of apoptosis. The results showed that tamoxifen significantly prevented phosphorylation of Akt on Ser 473 as well as Akt level itself (Fig. 3A). These data suggest that the level of Akt expression and phosphorylation are correlated with clusterin expression.

### Transfection of myristoylated Akt in PC-3 cells leads to recover clusterin suppression by tamoxifen

To determine whether clusterin expression is correlated with high levels of Akt phosphorylation in PC-3 cells, myristoylated Akt1 was transiently transfected into PC-3 cells. The status of clusterin expression and Akt phosphorylation

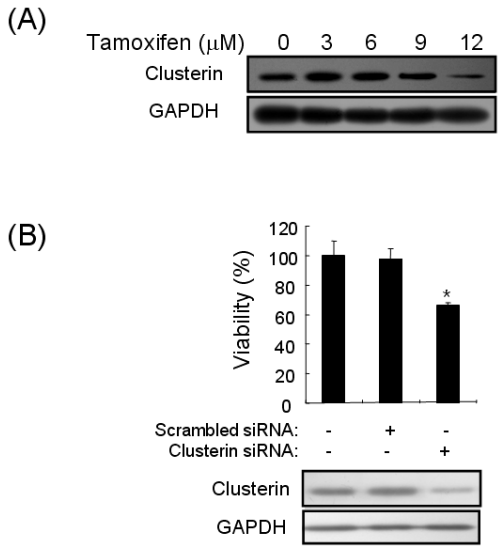


**Fig. 2.** (A) Effect of tamoxifen on clusterin protein level of PC-3 cells. Cells were treated with various concentrations of tamoxifen for 4 h. Total cellular sonicates were prepared and clusterin expression was examined by Western blot analysis. GAPDH level was measured as a loading control. (B) Inhibition of clusterin expression and PC-3 viability by clusterin siRNA. Cells were transfected with clusterin siRNA plasmid (3 μg) or scrambled siRNA plasmid (3 μg) for 48 h and cell viability was determined and clusterin expression was examined by Western blot analysis. Results are the mean ± S.D. of three separate experiments. *Significantly different from the untreated control group ($p < 0.05$). GAPDH level was measured as a loading control.

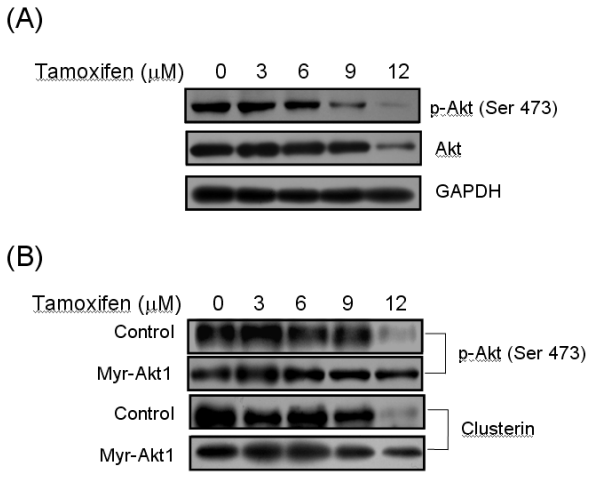


**Fig. 3.** Effect of tamoxifen on Akt phosphorylation and clusterin expression in PC-3 cells. (A) Effect of tamoxifen on Akt phosphorylation of PC-3 cells. Cells were treated with various concentrations of tamoxifen for 4 h. Total cellular sonicates were prepared and phospho-Akt and total Akt level was examined by Western blot analysis. GAPDH level was measured as a loading control. (B) Effect of overexpression of Akt1 on tamoxifen effects. Cells were transfected with myristoylated Akt1 plasmid (3 μg) for 48 h and Akt phosphorylation and clusterin expression was examined by Western blot analysis.

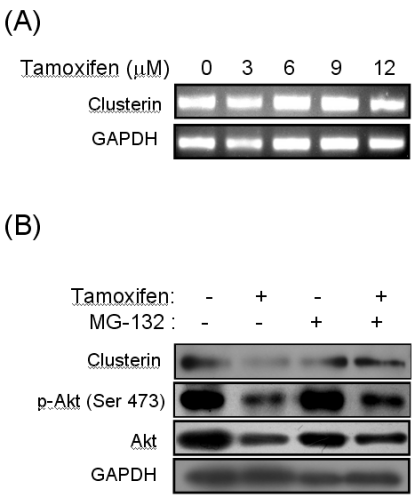


**Fig. 4.** Expression of clusterin mRNA and protein by tamoxifen in PC-3 cells. (A) Expression of clusterin mRNA was detected by RT-PCR. Cells were treated with various concentrations of tamoxifen for 4 h. Total RNA was isolated and clusterin mRNA level was examined by RT-PCR analysis. GAPDH mRNA level was measured as a loading control. (B) Effect of a proteasome inhibitor MG-132. Cells were pretreated with MG-132 (10 μM) for 30 min and then treated with 12 μM tamoxifen for 4 h. Total cellular sonicates were prepared and the expression levels of clusterin, phospho-Akt and total Akt were examined by Western blot analysis. GAPDH level was measured as a loading control.

were examined after 48 h (Fig. 3B). We found that suppressed Akt phosphorylation on Ser473 by tamoxifen was enhanced by myristoylated Akt1 transfection. Moreover, the clusterin expression suppressed by tamoxifen (12 μM) was also significantly recovered by myristoylated Akt1 expression (Fig. 3B).

### Ubiquitin pathway may be involved in tamoxifen-mediated clusterin degradation

To address the question of how tamoxifen can decrease clusterin expression, we first examined whether tamoxifen suppresses the clusterin mRNA expression in cells. Total RNAs were extracted and RT-PCR analyses were performed to examine clusterin gene expression. As shown in Fig. 4A, tamoxifen did not cause significant change in clusterin mRNA level up to 12 μM although tamoxifen strongly suppressed clusterin protein expression at 12 μM. Then, we examined the possible involvement of ubiquitin-mediated proteolytic pathways. To this aim, PC-3 cells were pretreated with the proteasome inhibitor MG132 (10 μM) for 30 min and then treated with tamoxifen (12 μM) for 4 h. Treatment with MG132 effectively rescued degradation of clusterin as well as Akt (Fig. 4B).

## DISCUSSION

In the present study, we have studied how tamoxifen affects clusterin expression in human prostate cancer cells. We found that clusterin protein expression was significantly suppressed by tamoxifen although mRNA level was almost unchanged. Additionally, our data suggested the involvement of Akt activation in clusterin expression.

The role of clusterin in cell growth and death of prostate cancer cells is controversial. Cleavage of clusterin precursor protein causes the generation of the alternative isoforms of clusterin such as nuclear clusterin (nCLU) and secreted clusterin (sCLU). sCLU is the full length glycosylated secreted heterodimer with antiapoptotic function, while nCLU is a 55 kDa nonglycosylated form to translocate from the cytoplasm to the nucleus following certain cytotoxic events and would be a proapoptotic factor (Bettuzzi *et al.,* 2002; Caccamo *et al.,* 2003; Trougakos *et al.,* 2005).

Various recent studies provide strong evidence that clusterin is an inhibitor of apoptosis with a cytoprotective function (Zhang *et al.,* 2005). sCLU was reported to interact and inhibit activated Bax, thereby inhibiting cytochrome c release and apoptosis. The overexpression of clusterin

has been shown to result in resistance for anticancer drugs such as paclitaxel, doxorubicin, and radiation therapy (Cervellera *et al.,* 2000; Miyake *et al.,* 2000; Zellweger *et al.,* 2002). Moreover, downregulation of clusterin expression by antisense or siRNA expression increases the sensitivities of anticancer agents (Gleave *et al.,* 2001; Trougakos *et al.,* 2004; So *et al.,* 2005; Sowery *et al.,* 2008). Interestingly, it was shown that a strong positive association between elevation of clusterin expression and loss of tumor suppressor Apc function in human colon tumor cells (Chen *et al.,* 2003). Our results also indicated that silencing of clusterin gene expression by siRNA resulted in significant retardation of PC-3 cell growth. These findings confirm the cytoprotective function of clusterin in prostate cancer cells. Thus we proposed that cytotoxic effect of tamoxifen in PC-3 cells may be mediated in part through clusterin suppression.

Because clusterin mRNA level was not detectably affected by tamoxifen but protein expression was significantly suppressed after treatment with tamoxifen in our data, we therefore focused on the possibilities that ubiquitin-proteasome pathway may play an important role in tamoxifen-mediated clusterin suppression. Treatment with MG132, a well-known cell permeable proteasome inhibitor restored downregulation of clusterin by tamoxifen. MG132 is a peptide aldehyde that acts as a potent competitive inhibitor of the chymotrypsin-like activity of the 26S proteasome complex (Tsubuki *et al.,* 1993; Hilt and Wolf, 1996). These results suggest that tamoxifen is able to induce clusterin degradation through ubiquitin-26S proteasome proteolysis. Previous report demonstrated that tamoxifen accelerates the degradation of the $O^6$-methylguanine DNA methyltransferase through the ubiquitin-dependent proteasomal pathway (Kuo *et al.,* 2007). Our data showed that Akt expression and phosphorylation decreased by tamoxifen are also recovered by MG132. In MDA-MB231 cells, clusterin levels increase significantly following treatment with proteasome inhibitors such as MG132 or epoxomycin (Ranney *et al.,* 2007).

Akt is a major regulator of the survival signaling pathways mediated by growth factors and cytokines, which exerts its antiapoptotic effects (Kennedy *et al.,* 1997; Zhou *et al.,* 2000). Akt inactivates proapoptotic proteins such as Bad, caspase-9, and Apaf-1 through phosphorylation. Akt also phosphorylates and inactivates the Forkhead transcription factor FKHRL1, resulting in decreased Fas ligand transcription and promoting cell survival (Brunet *et al.,* 1999). Our results indicate that clusterin expression may be controlled by Akt signal pathway because overexpression of myristoylated Akt1 in cells induces clusterin expression. Because tamoxifen induces down-regulation of phospho-Akt at Ser473 and promotes proteasomal degradation of clusterin, there is a possibility that Akt phosphorylation may control ubiquitin-proteasome pathway. It has been previously shown that Akt regulates ubiquitination and degradation of proteins such as Mdm2 and XIAP (X-linked inhibitor of apoptosis protein), which leads to the increased cell survival (Dan *et al.,* 2004; Feng *et al.,* 2004).

Recent work has shown that the survival effect of clusterin on rat prostate cells is mediated in part by activation of the PI3K/Akt pathway (Ammar and Closset, 2008). In neuroblastoma cells, the mRNA levels of clusterin were enhanced by B-Myb overexpression, a nuclear transcription factor that recognize the Myb consensus sequence (C/T)AACNG in viral and cellular promoters (Cervellera *et al.,* 2000). B-Myb function appears to be associated with phosphatidylinositol 3-kinase/Akt pathway (Tanno *et al.,* 2002). Thus, it is possible that activation of Akt pathway may affect clusterin expression though B-Myb-dependent pathway. We also found previously that exposure of PC-3 cells to clusterin siRNA resulted in loss of Akt phosphorylation (data not shown). These suggest that there will be cross-talk between clusterin expression and Akt phosphorylation.

Previous studies reported that activation of Akt is increased in the tamoxifen-resistant MCF-7 human breast cancer cell line (Jordan *et al.,* 2004; Yoo *et al.,* 2008). Our study showed that tamoxifen at higher than 9 μM clearly inhibits Akt phosphorylation on Ser473. This suggests that the anticancer effect of tamoxifen may be partially mediated by inhibiting Akt activation. Clark *et al.* (2002) showed that tamoxifen (160 nM) significantly reduced Akt phosphorylation on Ser473 although lower concentration of tamoxifen (80 nM) increased Akt phosphorylation in ZR75-1 breast cancer cells. Therefore, in our studies, tamoxifen is able to block Akt activation by inhibiting Ser473 phosphorylation and overexpression of Akt can prevent tamoxifen-mediated cellular response such as clusterin downregulation.

In conclusion our data show for the first time that downregulation of clusterin expression by tamoxifen is mediated through ubiquitin-proteasome pathway and this mechanism may be controlled by Akt-dependent pathway. The detailed mechanism for cross-talk between clusterin expression and Akt activation will need to be determined to understand the role of clusterin in signal pathways in PC-3 cells.

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