**Overexpression of Glycogen Synthase Kinase-3 in Ovarian Carcinoma Cells With Acquired Paclitaxel Resistance**

**Yunfeng Fu, PhD,* Dongxiao Hu, MD,* Jian Qiu, MD,* Xing Xie, MD,*† Feng Ye, PhD,† and Wei-Guo Lu, PhD*†**

**Introduction:** Acquired resistance to paclitaxel, including regimens, is one of the most significant reasons for treatment failure and death in patients with ovarian cancer, but the causes of this resistance remain unclear. However, cell cycle regulation is a key mechanism by which most chemotherapeutic agents exert their cytotoxic effects.

**Methods:** We created a paclitaxel-resistant ovarian carcinoma cell line from SKOV3 cell line, and the difference of cell cycle distribution was analyzed using flow cytometry. Analysis of human cell cycle pathway complementary DNA array was performed to identify candidate genes associated with paclitaxel resistance. Gene expression changes were validated at the messenger RNA and protein levels by real-time reverse transcriptase polymerase chain reaction and Western analysis, respectively.

**Results:** The ratio of Gap0/Gap1 phase in SKOV3-TR30 was significantly lower than that in SKOV3 (54.8% ± 6.3% vs 72.7% ± 7.6%, *P* = 0.035), and the ratio of G2/M phase in SKOV3-TR30 was significantly higher than that in SKOV3 (24.9% ± 6.0% vs 10.2% ± 3.5%, *P* = 0.021). Complementary DNA microarray analysis demonstrated enhanced glycogen synthase kinase-3α (GSK-3α) expression in paclitaxel-resistant ovarian carcinoma cells. Real-time reverse transcriptase polymerase chain reaction analysis revealed that the paclitaxel-resistant subline exhibited a 7.0 ± 1.8-fold increase in GSK-3α messenger RNA expression. There was a 3.34 ± 0.47-fold increase of total GSK-3 protein (GSK-3α/β) in SKOV3-TR30 cells validated by Western analysis.

**Conclusions:** This study demonstrates that enhanced expression of GSK-3 is associated with acquired resistance to paclitaxel in ovarian carcinoma cells. Glycogen synthase kinase-3 overexpression may probably be a significant contributor to chemoresistance.

**Key Words:** Ovarian carcinoma, Paclitaxel, Drug resistance, Glycogen synthase kinase-3, Gene expression

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It is DNA at 703 SKOV3-TR30 cells. Two isoforms of GSK-3β have been implicated in tumorigenesis and cancer progression. It also interacts with the cell cycle machinery that controls the cell cycle and molecular pathways that mediate the sequent cell death. It is obvious that alterations of cell cycle control can create drug resistance, thereby reducing chemotherapeutic efficacy. Thus, global analysis of genes of cell cycle control by microarray-based technologies in clinical phenotype is critical to clarify molecular mechanisms for chemotherapy resistance and may help to identify potential targets for improved cancer therapies.

Glycogen synthase kinase-3 (GSK-3) is a multifunctional protein kinase known to play a pivotal role in the regulation of metabolism, embryonic development, cell differentiation, and apoptosis. It also interacts with the cell cycle through the regulation of a number of cell cycle regulatory genes such as cyclin D1 and P21. Two isoforms of GSK-3, GSK-3α and GSK-3β, have been identified with high homology and similar but not identical biochemical properties. The dysregulation of GSK-3β has been implicated in tumorigenesis and cancer progression. However, it remains controversial whether GSK-3β is a tumor promoter or a tumor suppressor. Whether modulation of GSK-3 expression plays a role in the development of paclitaxel resistance in ovarian carcinoma cells remains uncertain. This study focuses on the role played by GSK-3α in paclitaxel resistance, at RNA and protein levels, in an in vitro ovarian carcinoma model of acquired paclitaxel resistance. In addition, our use of complementary DNA (cDNA) microarrays allowed identification of other candidate genes, which may be important for subsequent evaluation of paclitaxel resistance.

**MATERIALS AND METHODS**

**Cell Lines and Cell Culture**

Human ovarian carcinoma cell line SKOV3 was obtained from the American Type Culture Collection (ATCC) and maintained in McCoy’s 5A medium (Gibco, Tulsa, OK) supplemented with 10% fetal bovine serum, 100-U/mL penicillin, and 100-µg/mL streptomycin at 37°C and 5% CO2. Paclitaxel-resistant cell subline, SKOV3-TR30, was developed by exposing parental SKOV3 cells to increased concentration of paclitaxel as described previously. SKOV3-TR30 cells demonstrated a 27.5-fold greater resistance to paclitaxel than SKOV3 cells.

**Cell Cycle Analysis**

Cell cycle distribution was analyzed using flow cytometry. Cells were harvested, washed twice with PBS, stained with the cell cycle analysis kit (Beckman Coulter, Brea, Calif) according to the manufacturer’s instruction, and analyzed using flow cytometry. The percentage of cells in different phases of the cell cycle was estimated using the Multicycle for Windows software (Beckman Coulter).

**Analysis of Human Cell Cycle Pathway Array**

Total RNA was isolated from the cell lines using Trizol reagent (Invitrogen, Carlsbad, Calif) in accordance with the manufacturer’s instructions. RNA quality was enhanced using the RNeasy Mini Kit (Qiagen, Germany), and the integrity of RNA samples were assessed qualitatively on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, Calif). For a high-quality total RNA sample, 2 well-defined peaks corresponding to the 18S and 28S ribosomal RNAs should be observed, similar to a denaturing agarose gel, with ratios approaching 2:1 for the 28S to 18S bands (Fig. 1). In addition, the purity of RNA was determined by measuring the absorbance at 260 and 280 nm using Du-640 spectrophotometry (Beckman). Expression of 283 genes associated with cell cycle was evaluated using human cell cycle pathway array (SBC-RH-102-10, National Engineering Center for Biochip, Shanghai, China) in accordance with the manufacturer’s instructions.

In brief, Cy3/Cy5-labeled cDNA probes were generated from 50 µg total RNA using oligo-(dT)15 primers for reverse transcription using Superscript II reverse transcriptase (Invitrogen). The Cy3/Cy5-labeled cDNA probes were purified using QIAquick Nucleotide Removal Kit (Qiagen). The cDNA probe was denatured by heating at 94°C for 5 minutes after incubated with human Cot-1 DNA at 70°C for 45 minutes; the cDNA probe supplied with hybridization buffer was hybridized with the cDNA membrane array at 42°C for 18 hours. The membrane was washed accordingly and then dried by centrifuge. After this, the membrane was scanned with an Agilent G2655AA scanner, and the scanned image was converted into single fluorescence image using the Split-tiff software and analyzed with the ImagenGene3.0 software.

**Real-Time Semi-Quantitative Reverse Transcriptase Polymerase Chain Reaction**

A real-time semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) was done using the ABI Prism 7000 system (ABI, Los Angeles, Calif) to assess the expression levels of GSK-3α messenger RNA (mRNA). Total RNA was isolated, and cDNA was prepared starting from 2 µg of total RNA using the Moloney Murine Leukemia Virus Rnase H- reverse transcriptase (Takara, Japan). Amplifications were carried out using the following primers (GSK-3α, forward 5'-GGCGAGAAGAAAGACGA-3', reverse 5'-CTTGCATAGGAGTGGAG-3'; β2-microglobulin (β2-M), forward 5'-ACCCCCACTGAAAA GATGA-3', reverse 5'-ATCTTCAAACCTCCATATG-3'); and the SYBR Premix Ex Taq kit (Takara) in a final volume of 25 µL according to the manufacture’s instructions, starting with a 10-second template denaturation step at 95°C followed by 40 cycles of 5 seconds at 95°C and 31 seconds at 60°C. Standard curves were generated using a serial dilution of the initial amount of cDNA to determine the range of template concentrations, which showed a good linearity and efficiency for the different reactions. Melt curves of the reaction products were also generated to assess the specificity of the measured fluorescence. Samples were run in triplicate, and the mean of threshold cycles (Ct) for each specimen was used to obtain the fold change of GSK-3α expression level applying the following equation:

\[
\text{Fold change} = 2^{-\Delta\Delta Ct}, \quad \text{where} \quad \Delta Ct = Ct_{\text{GSK-3a}} - Ct_{\beta2-M} \quad \text{and} \quad \Delta\Delta Ct = \Delta Ct_{\text{SKOV3-TR30}} - \Delta Ct_{\text{SKOV3}}.
\]
fold change equal to 1 represents the resistant cell with an expression level equal to the control. To gain accuracy, 3 independent real-time RT-PCR experiments were repeated.

**Western Blotting**

Cells were harvested and lysed in modified protein lysis buffer (50-mmol/L Tris-HCl, pH 8.0; 150-mmol/L sodium chloride; 0.1% sodium dodecyl sulfate; 1% nonyl phenoxypolyethoxylethanol-40; and 0.02% sodium azide) added with 1% proteinase inhibitor cocktail (Sigma USA). The protein concentration was measured by the Bradford method. Equal amounts of sample lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat dried milk in tris-buffered saline with 0.1% (vol:vol) tween 20 buffer (20-mmol/L Tris-HCl, pH 7.4; 150-mmol/L sodium chloride; and 0.1% Tween-20) and incubated overnight at 4°C with the antibodies against GSK-3α/β (1:150, Sigma) and β-actin (1:500, Santa Cruz Biotechnology, Santa Cruz, Calif), respectively. The membrane was washed with TBST buffer and incubated with appropriate secondary antibodies. The protein bands were visualized using the enhanced chemiluminescence kits (Pierce USA). Signal data were normalized for β-actin bands, and a mean value was calculated from 3 independent experiments.

**Statistical Analysis**

Statistical analysis was performed with the SPSS 10.0 software package. All experiments were repeated 2 (microarray) or 3 times. Differences in data among treatment groups were

FIGURE 1. Good-quality total RNA samples are shown on electropherogram from the Agilent 2100 Bioanalyzer for total RNA of SKOV3 and SKOV3-TR30 cells.

FIGURE 2. Flow cytometric analysis on cell cycle. Increased G2-M phase proportion was shown in SKOV3-TR30 than in SKOV3 cells.
TABLE 1. Comparison of cell cycle distribution between SKOV3-TR30 and SKOV3

<table>
<thead>
<tr>
<th>Distribution</th>
<th>SKOV3</th>
<th>SKOV3-TR30</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>G0/G1</td>
<td>72.7% ± 7.6%</td>
<td>54.8% ± 6.3%</td>
<td>0.035</td>
</tr>
<tr>
<td>S</td>
<td>17.1% ± 10.3%</td>
<td>20.3% ± 4.5%</td>
<td>0.652</td>
</tr>
<tr>
<td>G2/M</td>
<td>10.2% ± 3.5%</td>
<td>24.9% ± 6.0%</td>
<td>0.021</td>
</tr>
</tbody>
</table>

Results represent the average of 3 independent experiments; analysis of variance (ANOVA).

analyzed using 1-way analysis of variance. P < 0.05 was considered statistically significant.

RESULTS

Different Cell Cycle Distributions Between Paclitaxel-Resistant and Paclitaxel-Sensitive Cell Lines

The ratio of G0/G1 phase in SKOV3-TR30 was significantly lower than that in SKOV3 (54.8% ± 6.3% vs 72.7% ± 7.6%, P = 0.035) and the ratio of G2/M phase in SKOV3-TR30 was significantly higher than that in SKOV3 (24.9% ± 6.0% vs 10.2% ± 3.5%, P = 0.021). However, there was no significant difference between the ratio of S phase in SKOV3-TR30 and SKOV3 cells (Fig. 2, Table 1).

Cell Cycle cDNA Microarray Identifies GSK-3α as a Candidate Gene Associated With Paclitaxel Resistance

To identify genes associated with paclitaxel resistance, we applied a cDNA expression array to our in vitro paclitaxel-resistant ovarian carcinoma model. The human cell cycle pathway array contained 283 genes that are involved in cell cycle control, including cyclin, cyclin-dependent kinase, cyclin-dependent kinase inhibitor; genes involved in mitosis, meiosis, and cytokinesis; and other genes that interacted with cell cycle. There was a 5.2-fold up-regulation of GSK-3α in SKOV3-TR30 cells as compared with the parental cells (Table 2). Because GSK-3 signaling is critically important in cancer and the participation of GSK-3 in paclitaxel resistance is unclear, we selected this as a candidate for the present study. In addition, there were further changes in gene expression as illustrated in Table 2.

Expression of GSK-3α mRNA Is Increased in Paclitaxel-Resistant Ovarian Carcinoma Cell Line

To confirm the results obtained using cDNA microarrays, the expression of GSK-3α at the mRNA level was determined by real-time semi-quantitative RT-PCR analysis. In comparison with SKOV3 cells, the paclitaxel-resistant sublines exhibited a 7.0 ± 1.8-fold increase in GSK-3α mRNA expression (Fig. 3).

Up-Regulation of GSK-3 Protein in Paclitaxel-Resistant Ovarian Carcinoma Cells

The GSK-3 expression difference between SKOV3 and SKOV3-TR30 cells was also validated at the protein level by Western analysis. It was observed that both GSK-3α and GSK-3β protein expressions were significantly increased in paclitaxel-resistant cell line. However, no visible GSK-3α protein was detected in SKOV3 cells (Fig. 4). There was a 3.34 ± 0.47-fold increase of total GSK-3 protein (GSK-3α/β) in SKOV3-TR30 cells (Fig. 4).

DISCUSSION

This study is the first to demonstrate that increased GSK-3 expression is associated with acquired resistance to paclitaxel in ovarian carcinoma cells in vitro. There may be several mechanisms that are involved in this process, and that may be the result of either transcriptional or translational regulation, with modulation of gene expression and/or resultant production of the protein.

TABLE 2. Gene expression differences in paclitaxel-resistant ovarian carcinoma cells

<table>
<thead>
<tr>
<th>Genbank_ID</th>
<th>Gene Name</th>
<th>Gene Symbol</th>
<th>Gene Expression*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_003914</td>
<td>Cyclin A1</td>
<td>CCNA1</td>
<td>−9.62</td>
</tr>
<tr>
<td>NM_005311</td>
<td>Growth factor receptor–bound protein 10</td>
<td>GRB10</td>
<td>−6.45</td>
</tr>
<tr>
<td>NM_012280</td>
<td>FtsJ homolog 1 (Escherichia coli)</td>
<td>FTSJ1</td>
<td>−4.89</td>
</tr>
<tr>
<td>BI760915</td>
<td>E2F transcription factor 5, p130 binding</td>
<td>E2F5</td>
<td>−3.51</td>
</tr>
<tr>
<td>NM_000321</td>
<td>Retinoblastoma 1</td>
<td>RB1</td>
<td>−3.15</td>
</tr>
<tr>
<td>NM_019884</td>
<td>Glycogen synthase kinase 3 alpha</td>
<td>GSK3α</td>
<td>+5.23</td>
</tr>
<tr>
<td>AK092970</td>
<td>Protein (peptidyl-prolyl cis/trans isomerase) NIMA-interacting 1</td>
<td>PIN1</td>
<td>+2.487</td>
</tr>
<tr>
<td>NM_020239</td>
<td>Small protein effector 1 of Cdc42</td>
<td>SPEC1</td>
<td>+2.482</td>
</tr>
<tr>
<td>NM_004359</td>
<td>Dell division cycle 34</td>
<td>CDC34</td>
<td>+2.123</td>
</tr>
<tr>
<td>NM_001800</td>
<td>Cyclin-dependent kinase inhibitor 2D (p19, inhibits CDK4)</td>
<td>CDKN2D</td>
<td>+2.019</td>
</tr>
</tbody>
</table>

*Ratio of gene expression between resistant subline and parental cell line: + indicates an increase in gene expression in the resistant subline, and − indicates a decrease in gene expression in the resistant sublines.
Previous studies have demonstrated that inhibition of GSK-3α is sufficient to inhibit cell growth and induce apoptosis of myeloma cells, which lead to decreased pancreatic cancer cell proliferation and survival. It has also been reported that GSK-3α suppression sensitizes prostate cancer cells to TNF-related apoptosis-inducing ligand-induced apoptosis and GSK-3α activation may be responsible for TNF-related apoptosis-inducing ligand resistance in prostate cancer cells. In addition, GSK-3β inhibition promotes adriamycin/5-FU-induced apoptosis in human colorectal cancer cells in a p53-dependent manner. Furthermore, inhibition of GSK-3β facilitates Fas-induced apoptosis activation in leukemia Jurkat cells, and depletion of GSK-3β potentiates death receptor 5-mediated apoptosis specifically in Myc-expressing cells. Other studies also demonstrated that inhibition of the function of GSK-3β attenuated cell proliferation and survival in ovarian cancer, thyroid cancer, leukemia, and glioma cells. These studies suggest that GSK-3β activity is important for the proliferation and survival of some cancer cells, and GSK-3β is possibly an antiapoptotic molecule.

Surprisingly, several lines of evidence support GSK-3β as a proapoptotic protein. Inhibition of GSK-3β by lithium significantly reduced etoposide- and camptothecin-induced apoptosis in HepG2 hepatoma cells. Activation of GSK-3β by LY294002 sensitizes hepatoma cells to chemotherapy-induced apoptosis. Similarly, GSK-3β inhibition may confer resistance to cisplatin in ovarian carcinoma cell line A2780. In addition, selective inhibition of GSK-3β attenuated histone deacetylase inhibitor trichostatin A–induced cytotoxicity in MCF-7 breast cancer cells. Therefore, it is likely that the most influential of these complex actions of GSK-3β on apoptosis vary depending on the cellular insult and the cell type.

The precise mechanism by which increased GSK-3 expression may lead to paclitaxel resistance is not yet fully understood. The paradoxical proapoptotic and antiapoptotic actions of GSK-3 are known to be due to GSK-3 oppositely regulating the 2 major apoptotic signaling pathways. Glycogen synthase kinase–3 promotes cell death caused by the mitochondrial intrinsic apoptotic pathway but inhibits the death receptor-mediated extrinsic apoptotic signaling pathway. When extrinsic apoptotic signaling pathway is predominant in cells exposed to some stimuli, increased GSK-3 levels may prevent cell death occurring by inhibiting the death receptor–mediated apoptosis. In our opinion, death receptor–mediated signaling pathways may play a predominant role in these cell models, although the exact mechanism of paclitaxel-induced cell apoptosis is not yet known. On the other hand, GSK-3 interacts with the cell cycle through degrading cyclin D1, which plays a critical role in G1 progression by activating cyclin-dependent kinases 4 and 6, leading to phosphorylation of the tumor suppressor protein.

**FIGURE 3.** Comparison of expression levels of GSK-3α mRNA shown by Ct values in SKOV3 and SKOV3-TR30 cells. The expression of GSK-3α mRNA was normalized to the expression of the housekeeping gene (β2-microglobulin). Threshold cycle values were negatively correlated with expression level. Results represented the average of 3 independent experiments; bars, ±SE. *P < 0.01 versus control, ANOVA.

**FIGURE 4.** Western blot analysis of GSK-3 protein expression in SKOV3 and SKOV3-TR30 cells. Cells were harvested and lysed. The protein concentration was measured by the Bradford method. Protein (30 μg) was analyzed, and the relative amounts of each protein were quantified as ratios to β-actin. Results represented the average of 3 independent experiments; bars, ±SD. #P < 0.05 versus control, *P < 0.01 versus control, ANOVA.
Retinoblastoma protein, with repression of E2F-mediated transcription.  

A previous study revealed that inhibition of GSK-3 triggers the melanoma cells to move from the S and G2/M phase to the G0/G1 phase of the cell cycle. In our study, we found that the ratio of G0/G1 phase in SKOV3-TR30 was significantly lower than that in SKOV3. Tumor cells with slower cell cycle progression caused by the elevated GSK-3 may exhibit the lower paclitaxel sensitivity. Continuous exposure to paclitaxel may therefore select for paclitaxel-resistant tumor cells that express increased levels of GSK-3.

In addition to GSK-3, cell cycle cDNA microarray analysis revealed several other genes with expression that may be altered in paclitaxel-resistant ovarian carcinoma cell line (Table 2). These include decreased expression of cyclin A1, GRB10, FTSJ1, and others and increased expression of CDC34, CDKN2D, and others, which would affect cell cycle regulation and the consequent apoptosis in cells attacked by paclitaxel. However, these changes remain to be validated at the mRNA and protein levels.

The results from this study were obtained using focused cDNA microarrays based on cell cycle pathway that are believed to be involved in tumor progression and drug action. There are other more comprehensive cDNA microarray methods available, which produce copious amounts of data. However, our experimental approach provides a quicker, less complicated and more focused way to identify possible candidate genes. To the best of our knowledge, this is the first finding that GSK-3 protein expression is up-regulated in ovarian carcinoma cells with acquired paclitaxel resistance. Overexpression of GSK-3 probably plays a role in paclitaxel resistance in ovarian cancer. However, we acknowledge that the presentation of our results was limited because of only one cell line model. Further studies of other cell models or clinical samples with chemoresistance might support our hypothesis.

REFERENCES


