Molecular Description of Evolving Paclitaxel Resistance in the SKOV-3 Human Ovarian Carcinoma Cell Line

Diana E. Lamendola, Zhenfeng Duan, Rushdia Z. Yusuf, and Michael V. Seiden

Division of Hematology/Oncology, Massachusetts General Hospital Boston, Massachusetts 02114

ABSTRACT

Ovarian cancer is currently the most lethal gynecological malignancy in the United States. Although effective therapies exist, the acquisition of multidrug resistance within persisting tumor cells renders curative therapies elusive for the majority of women with ovarian cancer. In an attempt to better define the evolution of paclitaxel resistance, three SKOV-3 sublines were selected during successive rounds of exposure to increasing paclitaxel concentrations. The sublines were selected to represent early (0.003 µM), intermediate (0.03 µM), and late (0.3 µM) paclitaxel resistance. RNA from these cell lines, SKOV-3,003STR, SKOV-3,03STR, and SKOV-3,3STR, as well as the parent cell line SKOV-3, was analyzed by cDNA array to evaluate transcript expression profiles. Arrays were performed using Affymetrix HG-U95Av2 arrays, which contain probes for ~9600 known human genes. Signal intensities were calculated by Microarray Suite 5.0 (Affymetrix, Santa Clara, CA). Expression patterns were analyzed by Affymetrix Data Mining Tool 3.0 with filtering of expression patterns for fold change in expression (maximum divided by minimum expression value/gene) and for variation of expression (maximum minus minimum expression value/gene). This analysis disclosed ~11,000 of ~12,000 expression patterns. The remaining ~1000 expression patterns were normalized and segregated into 20 partitions of a self-organizing map (SOM). The resulting SOM discriminates between genes, which are differentially expressed in early versus intermediate versus late paclitaxel resistance. For example, multidrug resistance 1 transcript expression is not elevated in SKOV-3,003STR as compared with parental SKOV-3 but demonstrates elevated expression in SKOV-3,03STR and SKOV-3,3STR. In contrast, SOM analysis demonstrates early (SKOV-3,003STR) transcriptional changes in a wide variety of genes, including gene families involved in cell growth/maintenance, cell structure, signal transduction, and inflammatory response. The use of array analysis with SOMs in sublines with progressive paclitaxel resistance can successfully define an evolution of resistance. Such an analysis may be useful at defining candidate gene families involved in the early-drug resistance phenotype.

INTRODUCTION

Ovarian cancer is currently the leading cause of death from gynecological malignancies in the United States; approximately 80% of patients succumb to the disease within 5 years of diagnosis. Paclitaxel, originally isolated from Taxus brevifolia (Pacific yew), is a microtubule stabilizing chemotherapeutic used to treat ovarian, breast, and non-small cell lung cancers. Unfortunately, paclitaxel therapy is often hindered by the development of drug resistance. The majority of in vitro drug resistance studies aimed at identifying mechanisms of paclitaxel resistance have compared drug naive cell lines to subclones demonstrating highly resistant phenotypes. Although this approach has identified several genetic changes in drug-resistant cell lines, it does not allow the identification of the earliest genetic changes underlying the drug resistance phenotype. To improve therapeutic strategies for women with ovarian cancer, it is necessary to distinguish early from late genetic changes in evolving paclitaxel resistance.

cDNA array analysis is an efficient technology that allows a global view of gene expression. Combined with mathematical models for pattern recognition and similarity clustering, this global expression can be organized into a manageable dataset (1). A SOM, first described by Kohonen (2), is an unsupervised learning algorithm useful in pattern recognition, which can describe changes over time in a single sample or related samples (3). SOMs do not require a learning or test dataset, allowing pattern recognition without prior knowledge of the correct outcome (4). cDNA array coupled with SOM analysis has recently been applied to various biological questions, including the yeast cell cycle, hematopoietic differentiation (5), and inflammatory bowel disease (6). Hierarchical clustering, either as an unsupervised or supervised learning algorithm, can identify patterns of similarity/difference between unrelated samples or individual genes (4). Recent studies have used hierarchical clustering as well as other clustering analyses as a tool to genetically classify tumors (7), predict chemosensitivity (9–12), and describe drug resistance (13–15). A combination of SOM analysis and hierarchical clustering can identify patterns of change as a sample acquires a particular phenotype, in this case drug resistance, and can identify statistically significant clusterings of protein families, chromosomal locations, and molecular functions.

In this experiment, a paclitaxel-sensitive, ovarian carcinoma cell line, SKOV-3, was exposed to incrementally increasing concentrations of paclitaxel, resulting in the establishment of three paclitaxel-resistant sublines. The expression profile of each of the four cell lines (SKOV-3 and the three resistant lines) was determined by Affymetrix cDNA array of ~9600 known human genes. The expression profiles were then used to generate an SOM capable of differentiating patterns of change in gene expression across the paclitaxel-resistant sublines. The individual SOM partitions were then examined by hierarchical clustering to identify protein families significantly enriched in the given partition.

MATERIALS AND METHODS

Cell Line Generation and Maintenance. The SKOV-3 cell line was obtained from the American Type Culture Collection (Manassas, VA). SKOV-3 was exposed to incrementally increasing paclitaxel concentrations to generate three SKOV-3 sublines with varying degrees of resistance to paclitaxel. In an attempt to limit the founder effects associated with cloning, resistant cell lines are not strictly clonal; all surviving colonies were pooled after paclitaxel selection to create the respective resistant cell line. Cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin (all reagents purchased from Life Technologies, Inc., Grand Island, NY). Resistant sublines were continuously cultured in paclitaxel, which was purchased from a commercial source, to ensure the drug-resistant phenotype.

RNA Extraction. Total RNA was isolated from SKOV-3, SKOV-3,003STR, SKOV-3,03STR, and SKOV-3,3STR using Trizol Reagent (Life Technologies, Inc.) according to the manufacturer’s instructions. RNA was isolated from...
the parental SKOV-3 occurred. This increase in variance can be
in the variation of gene expression between the resistant sublines and
SKOV-3 cell line progressed to a paclitaxel resistant state, an increase
Subsequently, the ASI was used in all additional analyses. As the
arrays were hybridized, scanned, and assessed for quality. Indi-
defined by four ASIs obtained from the Microarray Suite 5.0 analysis, one/cell
neighborhood size of 5, and final neighborhood size of 0.2.
expression vectors, which passed filtering, were normalized with a mean of
zero and a variance of one. A four row by five column SOM was generated by
expression and early, unsustained increases (clusters 11 and 16) in
gene expression. It distinguishes early from intermediate (cluster 15)
in from late (cluster 10) increases in gene expression. As important to the
drug resistance phenotype are early decreases in gene expression,
visualized in clusters 1, 2, and 3. In essence, the SOM arbitrarily
defined 20 expression patterns (the number of partitions is user
visualized in clusters 1, 2, and 3. In essence, the SOM arbitrarily
defined 20 expression patterns (the number of partitions is user
transcriptional changes that directly induce a paclitaxel resistance
phenotype, this analysis has identified these transcriptional changes as
being associated with the emerging paclitaxel resistance phenotype.
A SOM Algorithm Can Successfully Define an Evolution of
Paclitaxel Resistance. As the R^2 values suggest, an evolution of
paclitaxel resistance exists in the SKOV-3 sublines. To define this
evolution, a 20-partition SOM was constructed (Fig. 2). The SOM
distinguishes between early, sustained increases (cluster 20) in gene
expression and early, unsustained increases (clusters 11 and 16) in
gene expression. It distinguishes early from intermediate (cluster 15)
in from late (cluster 10) increases in gene expression. As important to the
drug resistance phenotype are early decreases in gene expression,
visualized in clusters 1, 2, and 3. In essence, the SOM arbitrarily
defined 20 expression patterns (the number of partitions is user
defined) within the 1319 expression profiles, which passed initial
filtering.

A Literature Review Identifying Genes Known to be Involved in Ovarian Cancer and/or Drug Resistance Validates the Generated SOM. Genes previously identified as being involved in ovarian cancer and/or drug resistance is listed in Table 1. The genes were located within the SOM or identified as having been filtered from the analysis. As expected, MDR-1 (18) transcripts passed filtering and were partitioned to cluster 15 (MDR-1 was represented by three independent probes on each array). In contrast, MRP-1, which is implicated in doxorubicin, daunorubicin, and vincristine resistance but not associated with paclitaxel resistance (19), has been filtered from the analysis. Interestingly, the balance of apoptotic molecules has been recently implicated in the drug resistance phenotype (20–
22). This analysis demonstrates that as SKOV-3 acquires a paclitaxel
resistance phenotype, the expression level of antiapoptotic Bcl-X_l
(23, 24) is unchanged. However, the expression level of the apoptosis
regulator of Bcl-X_l (BAK, a proapoptotic molecule, which interacts
with Bcl-X_l; Ref. 25) decreases with progressive paclitaxel resistance.
This ratio of anti- to proapoptotic molecules favoring survival may be
responsible for overcoming a paclitaxel-induced death signal. Table 1, listing additional genes with associated partitions, demonstrates that the SOM generated is consistent with previously reported molecular descriptions of paclitaxel resistance as seen in both cell lines and clinical samples. In addition, prior and ongoing northern studies in our laboratory of the SKOV-30.3TR cell line demonstrates overexpression of MDR-1 (26), IL-6 (27), IL-8 (27), MAGE genes (28), and human guanylate protein 1 (29) as compared with the SKOV-3 parental line (26–29). These genes are demonstrated to be overexpressed in the SOM and are represented in partitions 15, 5, 20, 20, 10, and 20, respectively. As such, this SOM may be useful in identifying novel genes associated with the early paclitaxel resistance phenotype.

Hierarchical Clustering Identifies Protein Families Significantly Enriched in Individual SOM Partitions. Although individual changes in gene expression, as reported above, may be important in elucidating the molecular mechanisms of paclitaxel resistance, analysis of protein families and pathways provides a more realistic interpretation of genetic events. In an attempt to identify protein families that may be responsible for the paclitaxel resistance phenotype, hierarchical clustering of individual SOM partitions was performed. The authors acknowledge that although multiple SOM clusters represent potentially important early increases (clusters 11, 16, and 20) in gene expression, as well as potentially important early decreases (clusters 1, 2, and 3) in gene expression, for the purpose of illustration, SOM cluster 20 will represent early paclitaxel resistance, cluster 15 will represent intermediate paclitaxel resistance, and cluster 10 will represent late paclitaxel resistance. With this understanding, the early paclitaxel resistance phenotype is characterized by a sustained increase in expression of multiple inflammatory proteins (Table 2). Intermediate paclitaxel resistance is associated with a significant number of extracellular genes, transport genes, and G1-S transition genes (Table 3). Finally, late paclitaxel resistance is associated with an increase in expression of a number of tumor antigen, signal transducer, and peripheral plasma membrane genes (Table 4).

Table 1 Genes involved in ovarian cancer and drug resistance

<table>
<thead>
<tr>
<th>GenBank accession no.</th>
<th>Gene name</th>
<th>Function</th>
<th>SOM cluster</th>
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<td>X58723</td>
<td>MDR-1</td>
<td>Efflux pump</td>
<td>15</td>
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<td>Efflux pump</td>
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<td>MRP-4</td>
<td>Efflux pump</td>
<td>2</td>
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<tr>
<td>U83661</td>
<td>MRP-5</td>
<td>Efflux pump</td>
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</tr>
<tr>
<td>HT2348&lt;sup&gt;a&lt;/sup&gt;</td>
<td>α Tubulin (consensus sequence)</td>
<td>Microtubule subunit</td>
<td>Filtered</td>
</tr>
<tr>
<td>HT4592&lt;sup&gt;a&lt;/sup&gt;</td>
<td>β Tubulin (consensus sequence)</td>
<td>Microtubule subunit</td>
<td>19/20</td>
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<tr>
<td>AF043105</td>
<td>Glutathione S-transferase</td>
<td>Chemoremediator</td>
<td>15</td>
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<tr>
<td>M14745</td>
<td>Bcl-2 (B cell lymphoma protein 2)</td>
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<td>Filtered</td>
</tr>
<tr>
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<td>Bcl-X&lt;sub&gt;L&lt;/sub&gt;</td>
<td>Antiapoptotic</td>
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<tr>
<td>L22473</td>
<td>BAX</td>
<td>Proapoptotic</td>
<td>Filtered</td>
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<td>U16811</td>
<td>BAK (apoptosis regulator of Bcl-X&lt;sub&gt;L&lt;/sub&gt;)</td>
<td>Proapoptotic</td>
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<tr>
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<td>IL-6</td>
<td>Growth and differentiation</td>
<td>5</td>
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<td>J04156</td>
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<td>5</td>
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<td>M28130</td>
<td>IL-8</td>
<td>Proangiogenic</td>
<td>20</td>
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<tr>
<td>AF024710</td>
<td>Vascular endothelial growth factor</td>
<td>Proangiogenic</td>
<td>6</td>
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</tbody>
</table>

<sup>a</sup> Filtered genes did not pass the initial SOM filter for fold change and variation.

<sup>b</sup> The Institute for Genomic Research human transcript reference number.
Additional Evaluation of Identified Genes and Protein Families. Additional validation of the data presented in the analysis using northern, western, or quantitative reverse transcription-PCR would be useful to verify the integrity of the array data but will not prove that the effects are related to paclitaxel resistance. Distinguishing between genetic effects associated with paclitaxel resistance and genes capable of inducing paclitaxel resistance is a much longer and detailed process. Our laboratory is currently evaluating several candidate genes and gene families implicated in this analysis. For example, several cancer tests antigens associated with paclitaxel resistance in various resistant cell lines are also capable of directly inducing paclitaxel resistance upon transfection into the ovarian cancer cell line OVCAR 8. More comprehensive analysis of the functional changes associated with the genes identified in this analysis is outside of the scope of this preliminary study.

DISCUSSION

Paclitaxel resistance is indeed a complex phenotype characterized by a continuum of changes in gene expression. This study describes the transcriptional changes that occur in the ovarian cancer cell line, SKOV-3, with a progressive paclitaxel resistance phenotype. SKOV-3 was cultured for 8 months in the presence of increasing concentrations of paclitaxel. At three time points, sublines were selected to represent early, intermediate, and late paclitaxel resistance. cDNA arrays, performed in triplicate for each cell line, defined a global expression pattern for each intermediate, and late paclitaxel resistance. The signal log ratio is reported as the log 2 ratio of the average change in expression. A log 2 of 1 is a fold change of 2.

This analysis demonstrated a broad range of transcriptional changes that satisfied our selection criteria for inclusion in the SOM with ~9% of the transcripts having greater than a 2-fold increase or decrease in expression between the highest and lowest expressing SKOV-3 cell line. Although, the concept that hundreds of genes might be involved in the evolution of drug resistance is not proven or generally accepted, several points should be emphasized. First, reversal of single genetic abnormalities (such as MDR-1) has not been successful at restoring drug sensitivity in the clinic. Second, the pathways associated with many genes are complex and alterations in transcription and associated protein expression may affect many genes downstream of the primary event. Thus, it is likely that only a small proportion of the genes identified in this analysis specifically induce paclitaxel resistance. Finally, the study of nontransformed, nonmalignant organisms has demonstrated surprisingly broad range of transcriptional changes with acquired resistance. For example, Candida albicans made resistant to the antifungal fluconazole demonstrates altered expression of 301 of 5000 open-reading frames representing 6% of the transcripts under analysis (30).

A comprehensive discussion of all identified genes, gene families, or even SOM clusters is not practical. Nevertheless, a selective overview of three SOM clusters illustrates the potential use of this technology. For example, early paclitaxel resistance as reported here is associated with a statistically significant increase in inflammatory proteins, namely IL-8 and Exodus 1, chemokines capable of attracting various lymphocytes (31, 32), N-formyl peptide receptor 1, a chemoattractant usually expressed on neutrophils (33), and adenosine A2a receptor, a regulator of stress response (34). The implication that chemokines and inflammatory molecules are involved in carcinogen-
esis and drug resistance is not novel, yet the significance of such expression has not been elucidated. Mantovani et al. (35) hypothesize that chemokines secreted from tumors recruit macrophages, which in turn promote tumor growth and progression. In fact, IL-8, as well as other chemokines and cytokines, is elevated in the serum of ovarian cancer patients (36) and its overexpression is correlated to disease aggressiveness (37). In addition to being chemoattractant and angiogenic, IL-8 induces haptotatic migration and proliferation of keratinocytes and melanoma cells (38, 39). Although, IL-8 transfection into paclitaxel-sensitive U-2OS cells does not induce a paclitaxel resistance phenotype, it does promote cell proliferation (40). Although Exodus 1/macrophage inflammatory protein 3α has not been implicated in the drug resistance phenotype, it has been shown to promote proliferation of the pancreatic cell line COLO-357 and to promote the migration of pancreatic cell line PANC-1 (41). Exodus 1 is under transcriptional control of NF-κB (42), which is activated by tumor necrosis factor α in response to numerous stimuli, including proinflammatory cytokines and chemotherapeutic agents (43). N-Formyl peptide receptor 1 ligand binding has also been shown to activate NF-κB (44) and to induce IL-8 secretion (44). The adenosine A2a receptor when bound by extracellular adenosine released from metabolically active cells is an anti-inflammatory mediator and immunosuppressor (45), which is also responsible for vasodilatation of surrounding blood vessels (46).

Intermediate paclitaxel resistance is represented in cluster 15. Hierarchical clustering of cluster 15, containing 119 probes, identified an enrichment of G1-S transition genes. Control of the cell cycle is essential not only for normal development but often is necessary for effective chemotherapy. Many chemotherapeutics rely on cell cycle checkpoints to recognize irreparably damaged cellular DNA leading to apoptosis. Indeed, paclitaxel arrests cells in mitosis by hyperstabilizing microtubules and interrupting normal chromosomal segregation (47–49). In cancers as in normal cells, this cell cycle block is thought to induce apoptosis. However, as previously stated, the balance of apoptotic molecules in the SKOV-3 paclitaxel resistant sublines may be abrogated because of down-regulation of BAK expression. A shift toward survival combined with an increase in G1-S transition genes may potentially overcome a paclitaxel-induced death signal, promote cell proliferation, and hence contribute to the drug resistance phenotype.

The G1-S transition genes implicated in intermediate, progressive paclitaxel resistance include the pro-S-phase molecules: activator of S-phase kinase; growth factor independent 1; and S-phase kinase-associated protein 2 (p545). Activator of S-phase kinase has been shown to be essential for S-phase entry (50), whereas growth factor independent 1 is thought to regulate gene expression during S phase (51). S-phase kinase-associated protein 2, previously described as a proto-oncogene (52), associates with p21, p19, and proliferating cell nuclear antigen to form the CDK2/cyclin A kinase necessary for S-phase entry (53).

For example, some of the late paclitaxel resistance is associated with overexpression of multiple peripheral plasma membrane genes, including glial cell line-derived neurotrophic factor receptor α2, interleukin 1 receptor-associated kinase 1, and guanine nucleotide binding protein γ-11. Glial cell line-derived neurotrophic factor receptor α2 when bound by neutrin (a glial cell line-derived neurotrophic factor family member) stimulates autophosphorylation of its co-receptor RET (arranged during transfection) proto-oncogene (54, 55). Constitutively active RET has been implicated in carcinogenesis because of germ-line mutations (multiple endocrine neoplasia types 2a and 2b; Ref. 56) and gene rearrangement (papillary carcinoma of the thyroid; 57). Interleukin 1 receptor-associated kinase 1 associates with the IL-1 receptor upon ligand binding and leads to NF-κB activation in response to inflammation and stress (58, 59). Guanine nucleotide binding protein γ-11 belongs to a multigene family, which encodes the γ subunit of heterotrimeric G proteins, which coupled with receptors, is responsible for transducing extracellular signals intercellularly (60). However, late paclitaxel resistance as described here within may not accurately reflect clinical drug resistance. SKOV-3-0.1TR is 100-fold more resistant to paclitaxel than parental SKOV-3 (data not shown), whereas clinically lethal disease is often characterized by much less dramatic changes in relative resistance. The SOM has distinguished these late genetic changes from those changes most likely to be involved in the early paclitaxel resistance of SKOV-3.

It is important to emphasize that the above description of transcriptional changes represents a somewhat arbitrary and selective review of a small subset of genes identified by filtering of the array data and subsequent SOM analysis. Nevertheless, this limited analysis demonstrates that the drug resistance phenotype may be more complex than anticipated, supporting data, which demonstrates that reversal of MDR-1 does not completely restore chemotherapeutic efficacy in tumors known to express MDR-1 (61). In addition, cell lines are not necessarily representative of clinical disease. Sawiris et al. (62) recently demonstrated via 516 gene Ovachip that human epithelial ovarian cancer has a distinct gene expression signature as compared with various cell lines. In fact, the ovarian and colon cell lines clustered together rather than with tumors from the same tissue of origin. This analysis of the evolution of paclitaxel resistance should be repeated in either human disease or an animal model to additionally elucidate the underlying mechanisms of clinical drug resistance.

In conclusion, array technology combined with mathematical algorithms for pattern recognition and similarity clustering may be useful in defining complex phenotypes. Paclitaxel resistance is not explainable by a single genetic event; rather, it is the accumulation of events promoting cell survival and proliferation. This evolution of paclitaxel resistance describes 1000 changes in gene expression as SKOV-3 acquires a paclitaxel resistance phenotype. The SOM combined with hierarchical clustering has identified inflammatory proteins, G1-S transition proteins, and peripheral plasma membrane proteins as potentially important in different stages of paclitaxel resistance. Progress in this field will require the coupling of knowledge gained from such transcriptional analysis with functional techniques, which allow the analysis of polygenic effects on cellular phenotype.

REFERENCES


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