Indoleamine 2,3-Dioxygenase Serves as a Marker of Poor Prognosis in Gene Expression Profiles of Serous Ovarian Cancer Cells

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Abstract

Purpose: We aimed to find key molecules associated with chemoresistance in ovarian cancer using gene expression profiling as a screening tool.

Experimental Design: Using two newly established paclitaxel-resistant ovarian cancer cell lines from an original paclitaxel-sensitive cell line and four supersensitive and four refractory surgical ovarian cancer specimens from paclitaxel-based chemotherapy, molecules associated with chemoresistance were screened with gene expression profiling arrays containing 39,000 genes. We further analyzed 44 genes that showed significantly different expressions between paclitaxel-sensitive samples and paclitaxel-resistant samples with permutation tests, which were common in cell lines and patients' tumors.

Results: Eight of these genes showed reproducible results with real-time reverse transcription-PCR, of which indoleamine 2,3-dioxygenase gene expression was the most prominent and consistent. Moreover, by immunohistochemical analysis using a total of 24 serous-type ovarian cancer surgical specimens (stage III, n = 21; stage IV, n = 7), excluding samples used for GeneChip analysis, the Kaplan-Meier survival curve showed a clear relationship between indoleamine 2,3-dioxygenase staining patterns and overall survival (log-rank test, P = 0.0001). All patients classified as negative survived without relapse. The 50% survival of patients classified as sporadic, focal, and diffuse was 41, 17, and 11 months, respectively.

Conclusion: The indoleamine 2,3-dioxygenase screened with the GeneChip was positively associated with paclitaxel resistance and with impaired survival in patients with serous-type ovarian cancer.

Ovarian cancer is one of the primary causes of death related to gynecologic malignancies (1). Nearly 65% of ovarian cancer patients die from their disease within 5 years (2). Although ovarian cancer is considered highly responsive to combination therapy with paclitaxel and carboplatin (3), cancer recurs rapidly in >50% of responsive patients, and in many cases, the recurring cancer cells develop chemoresistance (4). Therefore, countering chemoresistance is essential for ovarian cancer management.

Properties within tumor cells that may lead to drug resistance in ovarian cancer include multidrug resistance proteins and mismatched repair processes (e.g., alterations in the p53 pathway; refs. 5–7). In addition, various molecules have been documented as candidates for chemoresistance in ovarian cancer (8–12). However, molecular targeting to overcome chemoresistance has not yet been delineated in ovarian cancer.

The development of microarray methods for large-scale analysis of gene expression makes it possible to search systematically for key molecules that may be involved in chemoresistance (13). We have already applied this approach to ovarian cancer (14) as well as to other cancers (15, 16). In previous works on ovarian cancer, gene expression profiling was used to distinguish types of ovarian cancer (17), malignant transformation from normal tissue (18, 19), serous uterine from ovarian cancers (20), or metastatic from nonmetastatic disease (21). Although some advances have been seen in chemoresistance of childhood acute lymphoblastic leukemia as well as other types of cancers (22–24), the technology has not elucidated a set of genes associated with chemoresistance, a critical factor for improving prognosis in most cancers.

In this experiment, GeneChip was applied to screen molecules expressed differentially between chemoresistant and chemosensitive cell lines as well as cancer cells derived from patients who were either clinically sensitive or resistant to chemotherapy. The clinical significance of a prominent molecule was further confirmed with immunohistochemical analysis to predict recurrence after chemotherapy.
Materials and Methods

**Tumor specimens.** The Jikei University School of Medicine Ethics Review Committee approved the study protocol with informed consent from all patients. A total of 32 ovarian cancer surgical specimens were obtained at the Jikei University Hospitals. Tumors were histologically classified according to the WHO international system and staged according to the International Federation of Gynecology and Obstetrics (25). All of the 32 cases underwent debulking surgery, and the sizes of the residual tumors were <2 cm in all cases. All cases were serous cystadenocarcinomas. There were 25 stage III cases and 7 stage IV cases.

Among the 32 cases, 4 patients with stage IIIC were diagnosed as having achieved a pathologic complete response according to a second-look operation after six courses of chemotherapy, including paclitaxel; cancer did not recur in these patients for >1 year. These cases were termed “supersensitive.” In addition, we also selected four patients with stage IIIC who showed progressive disease during chemotherapy, including paclitaxel; these cases were termed “refractory.” Three of four supersensitive cases completed six courses of paclitaxel (180 mg/m²)-carboplatin (AUC 5), and one supersensitive case underwent six courses of paclitaxel only due to an allergic reaction to carboplatin in the first course. On the contrary, refractory cases underwent two to four courses of paclitaxel only due to an allergic reaction to carboplatin in the first course. The resistances of these original paclitaxel-sensitive clones and newly developed paclitaxel-resistant 2008 clones were evaluated according to established methods: in vivo 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenoxy)-5-(2,4-disulfonyl)-2H-tetrazolium assay (26) and murine model in vivo (27). Briefly, for the in vitro experiments, a single-cell suspension of 2008, 2008/PX2, or 2008/PX24 in DMEM supplemented with 10% fetal bovine serum was seeded to a 96-well plate at 3,000 cells per well. Then, the cells were treated with a range of concentrations of paclitaxel and cisplatin (carboplatin is a derivative of cisplatin) from 0.00019 to 50 μmol/L with a 2-fold serial dilution. After 4 days of incubation at 37°C in a humidified incubator containing 5% CO₂, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenoxy)-5-(2,4-disulfonyl)-2H-tetrazolium reagent (Cell Counting Kit-8, Dojindo Laboratories, Tokyo, Japan) was added to each well, and the plates were further incubated for a few hours at 37°C. Finally, the absorbance at 450 nm was measured, and the antiproliferating activity of each drug was calculated using the formula: (1 – T / C) × 100 (%), where T and C represent the mean difference in absorbance at 450 nm of the cells treated with drugs (T) and that of the untreated control cells (C). The IC₅₀ was obtained from three independent experiments (Table 1).

For in vivo experiments, a single-cell suspension of 2008, 2008/PX2, or 2008/PX24 (1 × 10⁷ cells per mouse) was s.c. inoculated into the right flank of five female mice (BALB/c nu/nu). The tumor volume was estimated by two-dimensional measurements using the equation: ab² / 2, where a and b represent tumor length and width, respectively. When the tumor volume reached 200 to 300 mm³, 40 mg/kg paclitaxel, 80 mg/kg paclitaxel, or vehicle was given i.v. once weekly for 3 weeks (vehicle: 10% Cremophore/10% ethanol/80% saline). The tumor volumes were measured 2 or 3 times per week using a caliper. When the tumor volumes reached 1,400 mm³, 40 mg/kg paclitaxel (PTX) or vehicle (C) was administered s.c. into the right side of the flanks of the mice (28).

**RNA extraction.** Cryostat sections containing >80% cancer cells were microdissected and prepared as tumor specimens. Total RNA from established methods: in situ 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenoxy)-5-(2,4-disulfonyl)-2H-tetrazolium assay (26) and murine model in vivo (27). Briefly, for the in vitro experiments, a single-cell suspension of 2008, 2008/PX2, or 2008/PX24 in DMEM supplemented with 10% fetal bovine serum was seeded to a 96-well plate at 3,000 cells per well. Then, the cells were treated with a range of concentrations of paclitaxel and cisplatin (carboplatin is a derivative of cisplatin) from 0.00019 to 50 μmol/L with a 2-fold serial dilution. After 4 days of incubation at 37°C in a humidified incubator containing 5% CO₂, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenoxy)-5-(2,4-disulfonyl)-2H-tetrazolium reagent (Cell Counting Kit-8, Dojindo Laboratories, Tokyo, Japan) was added to each well, and the plates were further incubated for a few hours at 37°C. Finally, the absorbance at 450 nm was measured, and the antiproliferating activity of each drug was calculated using the formula: (1 – T / C) × 100 (%), where T and C represent the mean difference in absorbance at 450 nm of the cells treated with drugs (T) and that of the untreated control cells (C). The IC₅₀ was obtained from three independent experiments (Table 1).

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**Table 1. In vitro sensitivity of ovarian cancer cell lines to paclitaxel and cisplatin**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC₅₀ for paclitaxel* (ratio)</th>
<th>IC₅₀ for cisplatin* (ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2008</td>
<td>2.2 ± 0.54 (1.0)</td>
<td>0.50 ± 0.14 (1.0)</td>
</tr>
<tr>
<td>2008/PX2</td>
<td>200 ± 58 (92)</td>
<td>0.50 ± 0.42 (1.0)</td>
</tr>
<tr>
<td>2008/PX24</td>
<td>120 ± 22 (57)</td>
<td>0.24 ± 0.07 (0.48)</td>
</tr>
</tbody>
</table>

*IC₅₀ presents mean ± SD obtained from three independent experiments.  
The numbers in parentheses indicate the ratio of IC₅₀.
ovarian tumors and cell lines were isolated using the hot phenol method (28). Total RNA was isolated from three different cultures of each cell line. We also scraped ovarian surface epithelium from three menopausal patients with leiomyoma of the uterus who underwent total hysterectomy and bilateral salpingo-oophorectomy with informed consent, and the ovarian surface epithelium was immortalized by SV40 T antigen alone and with SV40 T antigen/human telomerase reverse transcriptase. All six immortalized cell lines are nontumorigenic, and immunocytochemical analysis showed a similar staining pattern to normal ovarian surface epithelium.8 Total RNA isolated from these immortalized ovarian surface epithelial cells was used as the control for real-time RT-PCR.

**Microarray.** Human genome-wide gene expression was examined using the Human Genome U133 Array (HG-U133 Set: GeneChip), which contains ~45,000 probe sets, representing >39,000 transcripts derived from ~33,000 well-substantiated human genes (http://www.affymetrix.com/products/arrays/specific/hgu133.affx).

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8 In preparation.
Double-stranded cDNA was synthesized, and the cDNA was subjected to in vitro transcription in the presence of biotinylated nucleotide triphosphates. The biotinylated cRNA (10 μg) was hybridized with a probe array for 16 hours at 45°C, and the hybridized biotinylated cRNA was stained with streptavidin-phycoerythrin and then scanned with a Gene Array Scanner. The fluorescence intensity of each probe was quantified using a computer program, GeneChip Analysis Suite 5.0 (Affymetrix). The

Fig. 3. Gene expression profiles using data of 44 gene expressions that showed significance under permutation tests. A, hierarchical clustering of 16 samples of both cell lines and surgical ovarian tumors using normalized data of 44 gene expression profile. Computation clearly separated 44 genes (row) into two clusters: cluster A (blue), up-regulated in chemosensitive cell lines and supersensitive surgical tumors; cluster B (pink), up-regulated in chemoresistant cell lines and resistant surgical tumors. Sp, supersensitive patient’s sample; Sl, chemosensitive cell line; Rp, resistant patient’s sample; Rl, chemoresistant cell line. B, hierarchical clustering of eight samples of cell lines using normalized data of 44 gene expression profile. Computation clearly separated 44 genes (row) into two clusters: cluster A (blue), up-regulated in chemosensitive cell lines; cluster B (pink), up-regulated in chemoresistant cell lines.
expression level of a single RNA was determined as the average fluorescence intensity among the intensities obtained by 11-paired (perfect-matched and single nucleotide–mismatched) probes consisting of 25-mer oligonucleotides. If the intensities of mismatched probes were very high, gene expression was judged to be absent even if a high average fluorescence was obtained with the Microarray Analysis Suite 5.0 program. The data were processed with Affymetrix’s default variables, except for scaling (target intensity, 1,000), without normalization procedures to calculate the level of gene expression as the signal.

Quantitative real-time reverse transcription-PCR. Taqman reverse transcription reagents (Applied Biosystems, Foster City, CA) were applied for cDNA synthesis. The SYBR Green reagents kit (Applied Biosystems) was used for quantitative real-time RT-PCR analysis and done according to the manufacturer’s recommendations. During RT-PCR, reactions were continuously monitored with an ABI Prism 7700 Sequence Detector (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase messages were used as the internal control. Primers for indoleamine 2,3-dioxygenase (IDO) and glyceraldehyde-3-phosphate dehydrogenase were purchased from Applied Biosystems.

Immunohistochemical analysis. For the immunohistochemical study, formalin-fixed, paraffin-embedded sections were used. Immunostaining was done using the labeled streptavidin-biotin peroxidase complex method with the Ventana auto-immunostaining system (Ventana Japan, Yokoyama, Japan). Murine monoclonal antibody against human IDO (1:1,000; ref. 29) was used. The antigen retrieval procedure was done with a microwave oven in DAKO antigen retrieval solution for 10 minutes at 95°C to efficiently stain the sample. The sections (DAKO Cytomation, Glostrup, Denmark) were developed with 3,3′-diaminobenzidine with 0.3% H₂O₂ and counterstained with hematoxylin. We used surgical specimens that were analyzed with the GeneChip and real-time RT-PCR as positive and negative controls. All of them showed consistent expression of IDO as the results of mRNA expression by real-time RT-PCR. Positive and negative controls were run in parallel for every stain.

Statistics. Hierarchical clustering was analyzed with Spotfire software version 8.0 (Spotfire, Somerville, MA). The Z-score (i.e., the SD from the normal mean value of raw data transformed by log₂ in each gene) was used for normalization. First, all genes were included for hierarchical clustering. Second, to adjust the significant level to account for multiple testing in the data sets, permutation tests were applied for gene screening to detect differential expression between chemoresistant and chemosensitive cell lines and patients’ tumors. The distribution of maximum t statistics based on 10,000 random permutations was compared with the observed values to determine the P and its 95% confidence interval for each gene using Stata 8.0 (Stata Corp., College Station, TX). Finally, these screened genes were recomputed with hierarchical clustering under sample sets of cell lines and patients’ tumors, cell lines alone, and patients’ tumors alone.

The association between the stage of cancer and the staining pattern was analyzed with the χ² test. Survival curves of the patients were compared using the Kaplan-Meier method. These analyses were done by the log-rank test using Stata 8.0.

Results

Establishment of paclitaxel-resistant ovarian cancer cell lines. After 40 weeks of exposure to paclitaxel, the 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfonyl)-2H-tetrazoli-um assay confirmed the development of two cell lines resistant to paclitaxel but still sensitive to cisplatin as follows: the ratio of IC₅₀ for paclitaxel between 2008 and 2008/PX2 increased to 92, whereas that for cisplatin remained at 1.0; the ratio of IC₅₀ for paclitaxel between 2008 and 2008/PX24 was 57, whereas that for cisplatin was 0.48 (Table 1). Thus, the degree of resistance against paclitaxel was greater in 2008/PX2 than in 2008/PX24, whereas the sensitivity against cisplatin remained the same.

Next, the resistance to paclitaxel of these new cell lines was examined using a murine in vivo model and compared with that of the parental cell line, 2008 (Fig. 1). The growth of 2008 in mice was almost completely suppressed by treatment with paclitaxel at 40 and 80 mg/kg (left), whereas at the same doses of paclitaxel the growth of 2008/PX2 and 2008/PX24 was only partially suppressed (middle and right). Thus, the two new cell lines were more resistant to paclitaxel than 2008 both in vitro and in vivo.

Screening with gene expression profiling. All cell lines (2008, 2008/PX2, and 2008/PX24) and eight surgical tumors from patients (four supersensitive and four refractory) were simultaneously analyzed under hierarchical clustering using all of gene expression data (Fig. 2). Although the cell lines and surgical tumors were clearly differentiated, the nature of the chemosensitivity or chemoresistance was independent of the clusters created by the analysis.

Then, the permutation tests were applied at a cutoff point of 0.05 to screen genes that differentially expressed chemosensitivity and chemoresistance, including both cell lines and surgical tumors. As a result, 44 genes (P < 0.05) were selected as candidates associated with chemoresistance or chemosensitivity...
Table 2. Permutation analyses of 17 genes

<table>
<thead>
<tr>
<th>ID</th>
<th>Gene</th>
<th>P*</th>
<th>95% Confidence interval*</th>
<th>R/S ratio both¹</th>
<th>R/S ratio cell line²</th>
<th>R/S ratio patients’ tumor³</th>
</tr>
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<tbody>
<tr>
<td>200963.s.at</td>
<td>Ribosomal protein L31</td>
<td>0.0076</td>
<td>0.0060-0.0095</td>
<td>0.83</td>
<td>0.93</td>
<td>0.75</td>
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<td>200008.s.at</td>
<td>GDP dissociation inhibitor 2</td>
<td>0.0015</td>
<td>0.0008-0.0025</td>
<td>0.68</td>
<td>0.91</td>
<td>0.50</td>
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<td>200052.s.at</td>
<td>Interleukin enhancer binding factor 2, 45 kDa</td>
<td>0.0080</td>
<td>0.0063-0.0099</td>
<td>0.68</td>
<td>0.96</td>
<td>0.46</td>
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<td>200008.s.at</td>
<td>GDP dissociation inhibitor 2</td>
<td>0.0086</td>
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<td>0.67</td>
<td>0.89</td>
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<td>200036.s.at</td>
<td>Ribosomal protein L10a</td>
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<td>0.0292-0.0363</td>
<td>0.75</td>
<td>0.75</td>
<td>0.77</td>
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<td>201105.at</td>
<td>Lectin, galactoside binding, soluble, 1 (galectin 1)</td>
<td>0.0077</td>
<td>0.0061-0.0096</td>
<td>0.53</td>
<td>0.41</td>
<td>0.66</td>
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<tr>
<td>200741.at</td>
<td>Ribosomal protein S27 (metalloparastatin 1)</td>
<td>0.0318</td>
<td>0.0284-0.0354</td>
<td>0.78</td>
<td>0.92</td>
<td>0.69</td>
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<td>221729.at</td>
<td>Collagen, type V, x2</td>
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<td>0.0029-0.0054</td>
<td>0.37</td>
<td>0.50</td>
<td>0.36</td>
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<td>218350.at</td>
<td>Geminin, DNA replication inhibitor</td>
<td>0.0081</td>
<td>0.0064-0.0101</td>
<td>0.43</td>
<td>0.93</td>
<td>0.28</td>
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<td>204031.s.at</td>
<td>DKKZp654J157 protein</td>
<td>0.0213</td>
<td>0.0186-0.0243</td>
<td>0.69</td>
<td>0.79</td>
<td>0.60</td>
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<td>201524.x.at</td>
<td>Ubiquitin-conjugating enzyme E2N (UBC13 homologue, yeast)¹</td>
<td>0.0219</td>
<td>0.0191-0.0250</td>
<td>0.83</td>
<td>0.98</td>
<td>0.67</td>
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<td>200934.at</td>
<td>DEK oncogene (DNA binding)</td>
<td>0.0295</td>
<td>0.0263-0.0330</td>
<td>0.63</td>
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<td>0.52</td>
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<td>200081.s.at</td>
<td>Ribosomal protein S6</td>
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<td>208782.at</td>
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<td>0.79</td>
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<td>201054.at</td>
<td>Heterogeneous nuclear ribonucleoprotein A0</td>
<td>0.0229</td>
<td>0.0201-0.0260</td>
<td>0.69</td>
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<td>201518.at</td>
<td>Chromobox homologue 1 (HP1/ homologue Drosophila)</td>
<td>0.0390</td>
<td>0.0353-0.0430</td>
<td>0.63</td>
<td>0.88</td>
<td>0.43</td>
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Permutation analyses of 17 genes up-regulated in both chemoresistant cell lines and resistant tumor specimens

<table>
<thead>
<tr>
<th>ID</th>
<th>Gene</th>
<th>P*</th>
<th>95% Confidence interval*</th>
<th>R/S ratio both¹</th>
<th>R/S ratio cell line²</th>
<th>R/S ratio patients’ tumor³</th>
</tr>
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<tr>
<td>205812.s.at</td>
<td>Sulfotransferase family, cytosolic, 1C, member 2</td>
<td>0.0016</td>
<td>0.001-0.003</td>
<td>1.58</td>
<td>1.59</td>
<td>1.63</td>
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<tr>
<td>201358.s.at</td>
<td>Coatamer protein complex, subunit 1</td>
<td>0.0028</td>
<td>0.0019-0.0040</td>
<td>1.66</td>
<td>1.50</td>
<td>1.90</td>
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<tr>
<td>200063.s.at</td>
<td>Nucleophosmin (nucleolar phosphoprotein B23, numatrin)</td>
<td>0.0052</td>
<td>0.0039-0.0068</td>
<td>1.24</td>
<td>1.22</td>
<td>1.24</td>
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<tr>
<td>204386.s.at</td>
<td>Mitochondrial ribosomal protein 63</td>
<td>0.0111</td>
<td>0.0091-0.0134</td>
<td>1.30</td>
<td>1.21</td>
<td>1.41</td>
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<tr>
<td>224778.s.at</td>
<td>Homo sapiens, clone IMAGE:5259584, mRNA</td>
<td>0.0103</td>
<td>0.0084-0.0125</td>
<td>1.56</td>
<td>1.70</td>
<td>1.41</td>
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<td>200039.s.at</td>
<td>Proteasome (prosome, macropain) subunit, type 2²</td>
<td>0.0056</td>
<td>0.0042-0.0073</td>
<td>1.45</td>
<td>1.35</td>
<td>1.52</td>
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<td>201288.at</td>
<td>Rho-GDP dissociation inhibitor, type 1</td>
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<td>3.13</td>
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<td>225301.s.at</td>
<td>Myosin VB</td>
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<td>200055.at</td>
<td>TAF10 RNA polymerase II, TATA box binding protein-associated factor, 30 kDa¹</td>
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<td>1.55</td>
<td>1.61</td>
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<td>200055.at</td>
<td>TAF10 RNA polymerase II, TATA box binding protein-associated factor, 30 kDa¹</td>
<td>0.0148</td>
<td>0.0125-0.0174</td>
<td>1.59</td>
<td>1.73</td>
<td>1.52</td>
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1⁰ Permutation analyses of 17 genes down-regulated in both chemoresistant cell lines and resistant tumor specimens

1⁰ Mean raw data of chemoresistant cell lines plus resistant patients’ tumors/mean raw data of chemoresensitive cell lines plus supersensitive patients’ tumors.

1⁰ Mean raw data of chemoresistant cell lines/mean raw data of chemoresensitive cell lines.

1⁰ Mean raw data of resistant patients’ tumors/mean raw data of supersensitive patients’ tumors.

1 Reduced expression of the gene in chemoresistant cell lines and resistant patients’ tumors was confirmed with RT-PCR.

*Increased expression of the gene in chemoresistant cell lines and resistant patients’ tumors was confirmed with RT-PCR.
and reanalyzed with hierarchical clustering (Fig. 3A). The 44 genes were classified into major two clusters: 17 kinds of genes were down-regulated (Table 2A) representing cluster A in Fig. 3A, whereas 27 genes were up-regulated representing cluster B in Fig. 3A in both chemoresistant cell lines and resistant surgical tumors (Table 2B). Furthermore, we repeated hierarchical clustering restricted to either cell lines alone (Fig. 3B) or surgical tumors alone (Fig. 3C).

Firstly, we assigned priority to 27 genes among 44 genes by either being reported as genes associated with carcinogenesis or being associated with notable pathways. Then, we selected eight genes that showed reproducible results by real-time RT-PCR comparing with the results of GeneChip analysis (Table 3). In particular, IDO was highly and consistently expressed in both chemoresistant cell lines and tumors from refractory patients but not in chemosensitive cell lines and tumors (Fig. 4). This finding was most prominent among these eight genes.

Expression of indoleamine 2,3-dioxygenase protein in pathologic specimens. Expression of IDO protein was further confirmed using pathologic specimens obtained from 24 patients with stage III or IV serous ovarian cancer, excluding samples used for GeneChip analysis. The staining patterns were classified as negative \( (n = 7) \), sporadic \( (n = 12) \), focal \( (n = 3) \), or diffuse \( (n = 2) \). There was no association between stage of cancer and staining pattern using the \( \chi^2 \) test.

Indoleamine 2,3-dioxygenase protein expressions and relapse-free survival. First, overall survival was compared between patients with stage III disease \( (n = 17) \) and stage IV disease \( (n = 7) \) using the log-rank test; no significant difference was noted. Next, Kaplan-Meier survival curves were generated based on the IDO staining pattern (Fig. 6). In contrast to clinical stages, staining patterns of IDO impaired survival (log-rank test, \( P = 0.0001 \)). All patients classified as negative survived without relapse. The 50% survival of patients classified as sporadic, focal, and diffuse was 41, 17, and 11 months, respectively. We also established a scoring system considering both pattern and intensity, and statistical analysis showed significant differences among every score (data not shown).

Discussion

We screened and identified IDO from 39,000 transcripts as a strong prognostic factor expressed in serous ovarian cancer. Most previous works using gene expression profiling were able to identify a bulk of genes that were highly expressed or suppressed in clinical subgroups of patients, such as those with a differential prognosis (30) or a pathologic type (31). However, fewer studies have shown a single molecule that can be used to clinically distinguish specific subgroups of disease (32, 33). Although microarray technology may be powerful enough to enhance the predictive ability of the prognosis (34), the cost of this technology is still high. In this study, we used microarray technology as a screening tool to identify key molecules associated with chemoresistance in serous ovarian cancer.

Gene expression profiling of novel chemoresistant cell lines was compared with an original chemosensitive cell line to exclude individual differences. However, this approach may

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDO</td>
<td>Tryptophan degradation</td>
</tr>
<tr>
<td>Immunoglobulin heavy constant ( \mu )</td>
<td>Immunity</td>
</tr>
<tr>
<td>Proteasome</td>
<td>Cleaving peptides in an ATP/ubiquitin – dependent process</td>
</tr>
<tr>
<td>Ubiquitin-conjugating enzyme E2N</td>
<td>Ubiquitin-conjugating enzyme</td>
</tr>
<tr>
<td>Ubiquinol-cytochrome c reductase</td>
<td>Mitochondrial respiratory chain</td>
</tr>
<tr>
<td>TAF10</td>
<td>RNA polymerase II</td>
</tr>
<tr>
<td>NHP2 nonhistone chromosome protein 2-like 1</td>
<td>RNA binding protein</td>
</tr>
<tr>
<td>Follistatin-like1</td>
<td>Cell growth</td>
</tr>
</tbody>
</table>

Table 3. Genes showing reproducible results by real-time RT-PCR

![Fig. 4. RNA expression of IDO by real-time RT-PCR. IDO expression in all cell lines (2008, 2008/PX2, and 2008/PX24) and surgical ovarian tumors (supersensitive, \( n = 4 \); refractory, \( n = 4 \)) was measured by real-time RT-PCR. Columns, ratio to a mean of six immortalized ovarian surface epithelial cells for each patient and for each cell line.]
pick up genes associated not only with chemoresistant-specific molecules but also with the concurrent changes obtained during the 40 weeks of culture. In contrast, using differential expressions of genes using patients’ cells derived from a small sample size, it may be difficult to detect chemoresistant genes, although we carefully selected eight patients who were in the same clinical stage but had a clear contrast between chemosensitive disease and chemoresistant disease in clinical settings. Few previous articles attempted to validate the results obtained from cell lines in patients’ cells (35). In this study, a hierarchical clustering of gene expression profiling showed a prominent difference between cell lines and surgically resected patients’ tumors but not between chemosensitivity and chemoresistance. Therefore, the permutation tests were applied to abstract chemoresistance-associated genes common to both cell lines and patients’ cells. In the selected 27 genes, only 8 were confirmed with real-time RT-PCR, suggesting that the results of the GeneChip and permutation tests cutoff at 0.05 may include some false-positive information. Levels of up-regulation in IDO expression were more prominent in results of real-time RT-PCR than with the GeneChip, which may be due to differences in the methods used to quantify the amounts of RNA expression.

We were able to validate the clinical importance of IDO expression retrospectively using 24 clinical paraffin-embedded specimens, excluding cases used for GeneChip analyses. For patients with advanced serous ovarian cancer, staining patterns of IDO protein expression clearly differentiated between those with a good prognosis and those with a poor prognosis; these prognoses were not predicted by standard clinical staging. This evidence may provide credence to the strategy of starting with genome-wide screening with gene expression profiling using microarray technology, narrowing the number of genes, and ending up with a single gene to link to clinical end points.

IDO, which is a rate-limiting enzyme that catabolizes tryptophan to kynurenine, first attracted a great deal of attention because it could protect against fetal rejection due to immune surveillance (36–38). Recently, tumor cells were also shown to express IDO and to escape the immune surveillance of the host (39, 40) by degrading local tryptophan, which suppresses T cells (41, 42) and natural killer cell proliferation (43, 44). All patients who were negative for IDO survived without relapse, although the duration of survival was impaired depending on the

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**Fig. 5.** IDO protein expression in ovarian cancer lesions with immunohistochemical staining. Formalin-fixed, paraffin-embedded sections were stained using the murine monoclonal antibody against human IDO (1:1,000) with the labeled streptavidin-biotin peroxidase complex method and counterstained with hematoxylin. Positive and negative controls of IDO were shown as A and B, respectively. The staining patterns were classified into diffuse (C), focal (D) sporadic (E), or negative (F). Original magnification, ×400.
pattern of IDO expression. This finding may be explained by the suppression of antitumor immune activities via IDO expression. On the other hand, the recurrence-free survival rate of IDO-positive patients with hepatocellular carcinoma was shown to be significantly higher than that of IDO-negative patients (45). According to their report, IDO-positive cells were identified to be tumor-infiltrating cells, not tumor cells, by immunohistochemical analysis. Although we also examined the staining pattern of tumor-infiltrating cells in the ovarian cancer portion, few cells showed positive staining. On the contrary, positive staining of tumor cells was much more prominent than that of noncancerous cells in all sporadic, focal, and diffuse patterns. Thus, the clinical significance of IDO expression being associated with prognosis in patients with serous-type ovarian cancer may not be universal to all types of cancer.

In this study, greater expression of IDO was confirmed not only in tumors from chemoresistant patients but also in chemoresistant cell lines, suggesting that IDO may affect chemosensitivity through intracellular mechanisms. Recently, IDO expression was shown to be suppressed by nitric oxide, which is known to mediate chemosensitivity in tumor cells via scavenging the production of large quantities of cytosolic superoxide anions (46). On the contrary, hypoxia-induced drug resistance seems to result, in part, from the downstream suppression of endogenous nitric oxide production (47–49). Therefore, the expression of IDO may be a parallel phenomenon to other mechanisms for chemoresistance, such as nitric oxide production, and may not cause chemoresistance directly. Just recently, Muller et al. reported that IDO inhibition cooperated with diverse chemotherapeutic agents to effectively promote the regression of established breast tumors that are refractory to chemotherapy (50). They used MMTV-Neu mice, a well-accepted transgenic mouse model of breast cancer, and showed that combining the IDO inhibitor 1-methyl-DL-tryptophan with paclitaxel resulted in a significant tumor decrease compared with paclitaxel alone ($P = 0.0010$). Their report supports our data, indicating that IDO is positively associated with paclitaxel resistance and impaired survival. They also indicated that Bin1 loss elevated the signal transducers and activators of transcription 1– and nuclear factor-kB–dependent expression of IDO. Nuclear factor-kB activation suppresses the apoptotic potential of chemotherapeutic agents (51). We speculate that IDO might be positively associated with paclitaxel resistance through the suppression of the apoptotic potential of paclitaxel.

Three of four supersensitive cases underwent paclitaxel-carboplatin, and one case underwent paclitaxel alone due to the hypersensitivity reaction to carboplatin. Moreover, neither 2008/PX2 nor 2008/PX24 showed cross-resistance for cisplatin. Carboplatin is a derivative of cisplatin and is a platinum compound. Using both surgical specimens and cell lines, we purified genes associated with paclitaxel resistance to prevent genes associated with platinum resistance. However, we speculate that IDO not only plays a role in paclitaxel resistance but also has an indirect effect on platinum in vivo. The latter speculation is supported by Muller et al. (50). They also showed that 1-methyl-DL-tryptophan with cisplatin also resulted in a significant tumor decrease compared with cisplatin alone. Future study should focus on the functional insights regarding the IDO gene for chemoresistance to paclitaxel by gene knockdown, such as the RNA interference technique.

In conclusion, IDO screened with the GeneChip was positively associated with paclitaxel resistance and impaired survival in patients with serous-type ovarian cancer.

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Indoleamine 2,3-Dioxygenase Serves as a Marker of Poor Prognosis in Gene Expression Profiles of Serous Ovarian Cancer Cells

Aikou Okamoto, Takashi Nikaido, Kazunori Ochiai, et al.


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