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Advances in Brief

Identification by cDNA Microarray of Genes Involved in Ovarian Carcinogenesis[1]

Kenji Ono, Toshihiro Tanaka, Tatsuhiko Tsunoda, Osamu Kitahara, Chikashi Kihara, Aikou Okamoto, Kazunori Ochiai, Toshihisa Takagi, and Yusuke Nakamura[2]

Laboratories of Molecular Medicine [K. On., T. Tan., O. K., C. K., Y. N.] and Genome Database [T. Ts., T. Tak.], Human Genome Center, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan, and Department of Obstetrics and Gynecology, Jikei University School of Medicine, Tokyo, Japan [A. O., K. Oc.]

Abstract

To identify genes involved in the development or progression of ovarian cancer, we analyzed gene expression profiles of nine ovarian tumors using a DNA microarray consisting of 9121 genes. Comparison of expression patterns between carcinomas and the corresponding normal ovarian tissues enabled us to identify 55 genes that were commonly up-regulated and 48 genes that were down-regulated in the cancer specimens. When the five serous adenocarcinomas were analyzed separately from the four mucinous adenocarcinomas, we identified 115 genes that were expressed differently between the two types of tumor. Investigation of these genes should help to disclose the molecular mechanism(s) of ovarian carcinogenesis and define molecular separation of the two most common histological types of ovarian cancer.

Introduction

Ovarian carcinoma has the worst prognosis among gynecological malignancies because most cases are not diagnosed until the disease is at an advanced stage. Although various therapeutic approaches are followed in clinical practice, most of them are not lifesaving. Hence, the discovery of ways to diagnose ovarian cancer at an early stage and establish more effective therapies is a critical and urgent issue.

To achieve this goal, identification and characterization of key molecules that participate in ovarian carcinogenesis are essential steps. Like cancers in other tissues, ovarian carcinomas are considered to result from a serial accumulation of genetic changes in a cell lineage (1). Mutations of the *p53*, c-*erb*B-2, c-*myc*, and K-*ras* genes appear to play important roles in this disease (2). However, histopathological differences that are reflected as serous, mucinous, endometrioid, clear cell, or transitional cell types of ovarian cancer cannot be explained by the presence or absence of those particular genetic changes. We also have no good parameters for distinguishing a variety of biological behaviors such as metastatic ability, invasiveness, and chemosensitivity.

To better understand ovarian carcinogenesis, we need to obtain a large body of information regarding each type of cancer material. To this end, we have applied recently established cDNA microarray technology, which can reveal the expression profiles of thousands of genes simultaneously (3, 4). Studies of this kind have identified genes related to carcinomas of the cervix, colon, breast, and prostate (5–9). The successful molecular classification of such tumors on the basis of gene expression profiles revealed on cDNA microarrrays indicates that this technology is likely to become an essential resource for the development of personalized medical treatments in the future (10–12).

Here we report the identification of dozens of genes whose expression was up- or down-regulated in multiple specimens of ovarian carcinoma using the cDNA microarray technique coupled with T7-based RNA amplification. In addition, we found a number of genes that were expressed differently between two major histological types, serous and mucinous carcinomas of the ovary.

Materials and Methods

Tissue Specimens. Ovarian cancer tissues, along with noncancerous ovarian tissues from the same patients, were excised during surgery after obtaining informed preoperative consent from the patients. Five samples diagnosed as serous adenocarcinoma and four samples of mucinous adenocarcinoma were selected for this study. Each corresponding normal tissue was confirmed histopathologically to be free of cancer cells. Clinical stages were determined on the basis of criteria outlined in 1988 by the International Federation of Gynecology and Obstetrics (FIGO).

T7-based RNA Amplification. Total RNA was extracted from each specimen using Trizol (Life Technologies, Inc.) according to the manufacturer's instructions. After treatment with DNase I (Nippon Gene), T7-based RNA amplification was carried out as described previously (13), with a few modifications. Using 2 μg of total RNA from each tissue sample as starting material, we performed two rounds of amplification; the amount of each amplified aRNA[3] was measured by a spectrophotometer, and its quality was checked by agarose gel electrophoresis.

Preparation of Target DNA. We first selected known cancer-related genes to be spotted onto glass slides, followed by other genes including housekeeping genes from a list provided by the Laboratory of Cancer Genetics, National Center for Human Genome Research, NIH as well as ESTs and hybridization controls. In all, 9121 genes were chosen as target cDNAs, and their sequences were retrieved from the UniGene database (National Center for Biotechnology Information). Polyadenylated RNA isolated from the liver, spleen, thyroid, placenta, skeletal muscle, small intestine, brain, heart, fetal lung, fetal liver, fetal kidney, and fetal brain (Clontech) were used for target cDNA preparation. RNA was reverse transcribed using oligo(dT) primer and Superscript II reverse transcriptase (Life Technologies, Inc.). We amplified cDNA segments of 200-1100-bp long without repetitive or polyadenylated sequences. The PCR products were purified and spotted in duplicate on type 7 glass slides (Amersham Pharmacia Biotech) using a Microarray Spotter Generation III (Amersham).

Labeling, Hybridization, and Scanning. The cDNA probes were prepared from aRNA as described elsewhere (13). Five-μg aliquots of aRNA from normal ovarian tissues and the corresponding cancers were labeled with Cy5-dCTP and Cy3-dCTP (Amersham Pharmacia Biotech), respectively. Labeled probes were mixed with microarray hybridization solution version 2 (Amersham) and formamide (Sigma) to a final concentration of 50%. After hybridization for 14–16 h at 42°C, the slides were washed in 2× SSC and 1% SDS for 10 min at 55°C, washed in 0.2× SSC and 0.1% SDS for 10 min at 55°C, washed in 0.1× SSC for 1 min at room temperature, and then scanned using the Array Scanner Generation III (Amersham).

The intensity of each hybridization signal was evaluated photometrically by the ArrayVision computer program (Amersham) and normalized to the averaged signals of housekeeping genes. The Cy3:Cy5 ratio for each sample was calculated by averaging spots. A cutoff value for each expression level was

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[2] To whom requests for reprints should be addressed, at Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. Phone: 81-3-5449-5372; Fax: 81-3-5449-5433; E-mail: yusuke@ims.u-tokyo.ac.jp.

[3] The abbreviations used are: aRNA, antisense RNA; EST, expressed sequence tag; RT-PCR, reverse transcription-PCR.

Table 1 *Clinical characteristics of nine ovarian adenocarcinomas*

Case no.	Histopathological type	Stage at diagnosis[a]	Size of residual tumor
2	Serous	IIIb	<2 cm
12	Serous	IIIc	<2 cm
13	Serous	IIIc	<2 cm
4	Serous	IIIc	≧2 cm
14	Serous	IIIc	≧2 cm
15	Mucinous	IIa	Micro[b]
10	Mucinous	IIc	Micro
11	Mucinous	IIc	Micro
8	Mucinous	IIIb	<2 cm

[a] Tumors were staged according to International Federation of Gynecology and Obstetrics criteria.
[b] Micro, .

automatically calculated according to the background fluctuation. The fluctuation can be estimated as the variance of the log ratio of Cy3:Cy5 minus the variance of the log ratio of Cy3:Cy5 of highly expressed genes (the upper 30%, where the background fluctuation is so small that it could be ignored). We used genes with an expression level (above about 10^5) where the fluctuation is less than a critical value (1.0) because the other genes (those that have low expression) are embedded in the background fluctuation (see Fig. 1). For comparisons of expression levels, the relative expression of each gene was recorded in one of four categories: (*a*) up-regulated in ovarian cancer (Cy3:Cy5 signal ratio $>$ 2.0); (*b*) down-regulated in ovarian cancer (Cy3:Cy5 ratio $<$ 0.5); (*c*) unchanged in normal or cancer cells; or (*d*) not expressed in normal or cancer cells. Within each group, the number of items in each category was recorded. Finally, for each gene, we calculated the *u* values of the Mann-Whitney test, which measures how the sample distribution between the serous group and the mucinous adenocarcinoma group is overlapped. The number of samples within each group is counted within each category (up-regulated, unchanged, and down-regulated). According to the order of the category, the number of overlapped samples are piled up into the *u* value. A small *u* value shows that the sample distribution of the two groups is clearly separated, *e.g.*, commonly up-regulated in serous types and commonly down-regulated in mucinous types. We applied a hierarchical clustering algorithm to all of the selected genes using hamming distance (edit distance).

Semiquantitative RT-PCR. From each tissue sample, 2 μg of total RNA were reverse-transcribed using the same gene-specific primers as those chosen for constructing the microarray. The number of PCR cycles was optimized in each case to ensure that product intensity fell within the linear phase of amplification.

Results

Clinicopathological features of the tumors examined in this study are summarized in Table 1. All patients with tumors of the serous type were diagnosed at an advanced stage (stage III). Among the tumors diagnosed as mucinous, three were at stage II, and one was at stage III. Because analysis of expression profiles on microarrays requires several micrograms of mRNA for synthesizing probes, we applied T7-based RNA amplification to obtain sufficient quantities of RNA from our limited materials. Total RNAs isolated from freshly frozen normal and tumor specimens from all nine patients were amplified about 10^4-fold; the estimated product sizes ranged between 0.3 and 1.0 kb (data not shown). After reverse transcription, we labeled each cDNA probe with Cy3 (cancer cells)- or Cy5 (normal cells)-conjugated dyes and hybridized them to microarrayed cDNAs or ESTs representing 9121 genes. A representative scatter plot of microarray analysis is shown in Fig. 1. Scatter plots of the two fluorescent signals revealed various gene expression patterns in cancerous tissues.

When we analyzed changes in expression patterns between all nine adenocarcinomas and their corresponding noncancerous ovarian tissues, we identified 55 genes that were up-regulated in at least six of the tumors and 48 genes that were down-regulated in at least eight cases (Fig. 2). Genes showing up-regulation in all nine tumors included protein disulfide isomerase-related protein, Mac-2-binding protein, and a gene corresponding to an EST (accession number AA723859). On the other hand, expression of Golgi SNARE, transforming growth factor β IIR α, and CL100 protein tyrosine phosphatase as well as that of genes corresponding to KIAA0851, KIAA0438, and ESTs (accession number AI830013) was down-regulated in all nine cases examined.

We then searched for genes that were regulated differently between the two major histological types of ovarian adenocarcinoma (serous and mucinous), and we identified 115 genes that had significantly different expression patterns as indicated by a *u* value $\leq$3, where P = 10% (Fig. 3). Expression of protein kinase PKX1, ribosomal protein L8, complement B, and regulator of G protein signaling (RGS12) was induced in all five serous tumors examined but was down-regulated or unchanged in most of the mucinous carcinomas. On the other hand, expression of creatinine kinase B and myosin

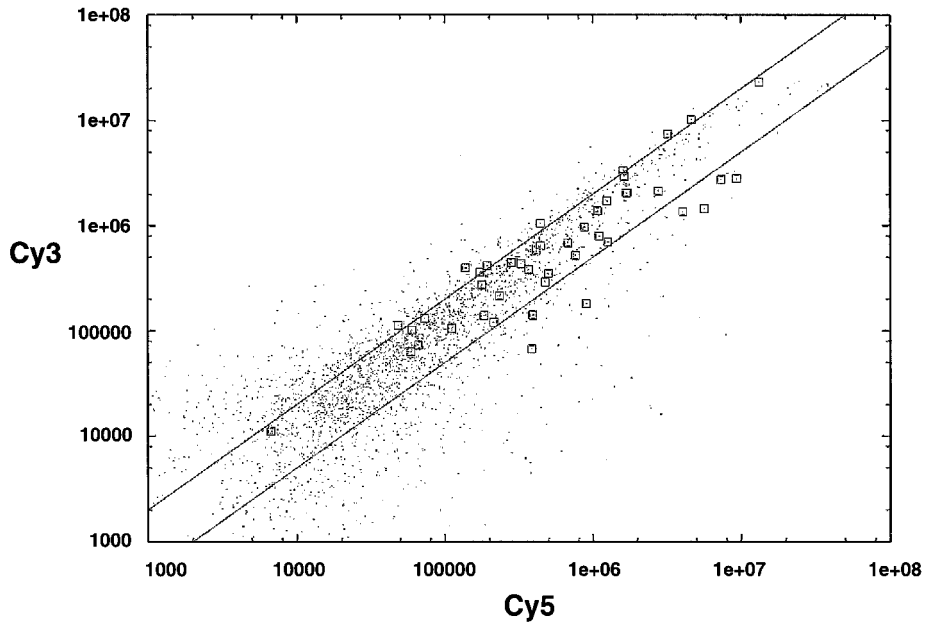

Fig. 1. Representative scatter plots of cDNA microarray analysis. Noncancerous ovary tissue (labeled with Cy5) and serous adenocarcinoma (labeled with Cy3) from case 10 were labeled and hybridized to the cDNA microarray. The *upper line* shows that Cy3 = 2 $\times$ Cy5, and the *lower line* shows that Cy3 = 0.5 $\times$ Cy5.

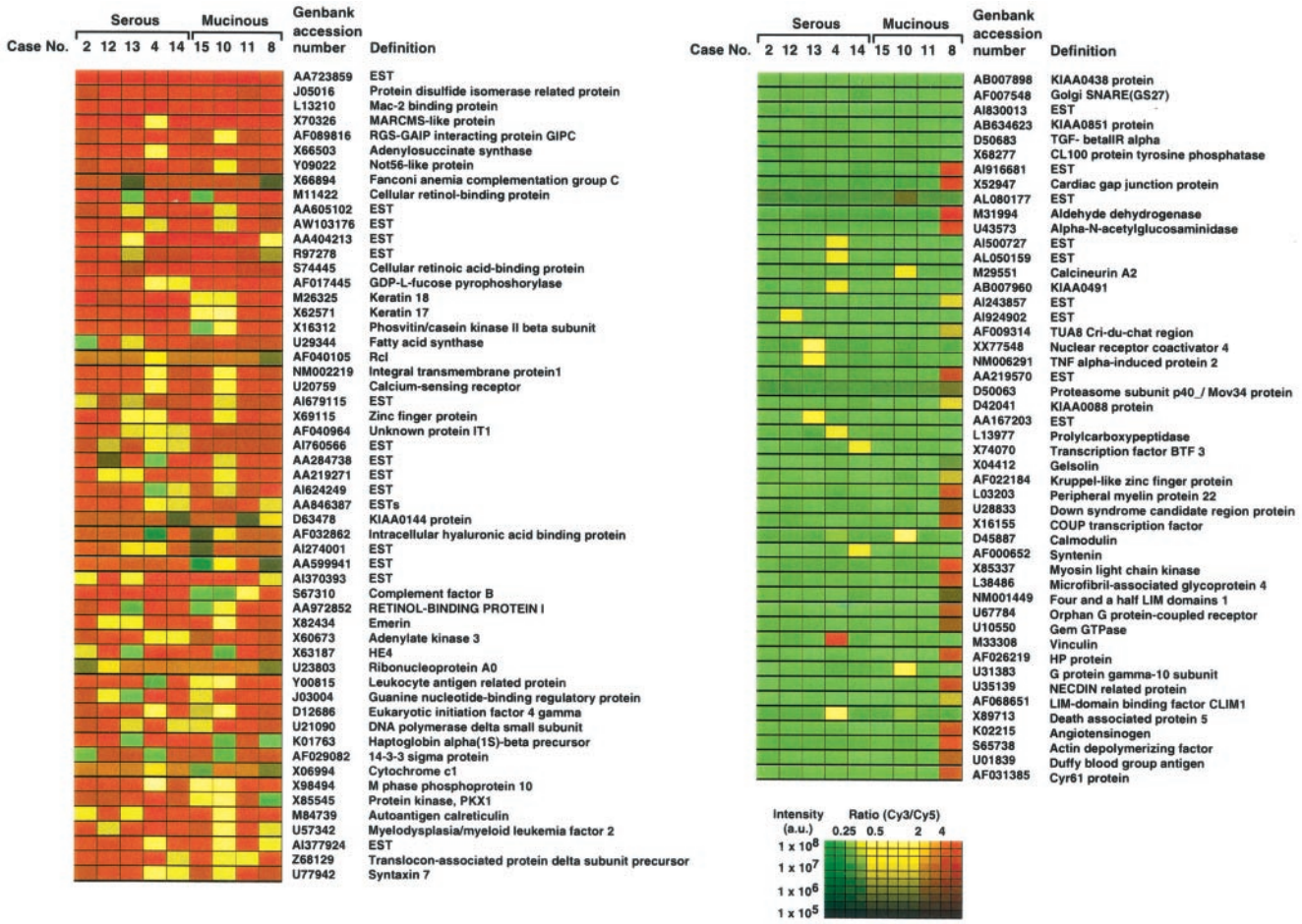

Fig. 2. Genes that were up-regulated (*left*) or down-regulated (*right*) in ovarian adenocarcinomas.

heavy polypeptide 11 was down-regulated in all of the serous adenocarcinomas but was unchanged or up-regulated in the mucinous tumors. Expression of A28-RGS14p was commonly up-regulated in the mucinous-type tumors, whereas genes encoding Gu-binding protein, D10S102, multiple membrane-spanning receptor TRC8, IFN-α14, and tropomodulin were down-regulated.

To investigate whether aRNA obtained by T7-based RNA amplification precisely reflected the original mRNAs in a proportional manner, we performed semiquantitative PCR experiments. As an example (Fig. 4), we used cDNA synthesized from nonamplified total RNA of one mucinous adenocarcinoma (case 15) as a template. RT-PCR experiments with several genes indicated that signals in the microarray analysis and the results of RT-PCR using aRNA reliably reflected the proportions present in the original total RNA.

Discussion

Because it is generally difficult to obtain sufficient quantities of RNA from clinical specimens to carry out the type of study reported here, we applied T7-based RNA amplification (14, 15) to obtain enough RNA from our limited starting materials to prepared cDNA probes for the microarray. Probes synthesized from aRNA generally reveal similar hybridization patterns as those from unamplified polyadenylated RNA (16). We compared the results of semiquantitative RT-PCR of unamplified total RNA isolated from clinical tissues with data obtained with aRNAs, and we confirmed that the data from microarray analysis reliably reflected the proportions in original mRNA populations.

Coupling the microarray with T7-based RNA amplification, we analyzed the expression profiles of nine pairs of normal and cancerous ovarian tissues and identified a set of genes whose expression was commonly altered in ovarian adenocarcinomas or in a specific type of tumor (serous or mucinous). The 55 genes that were often up-regulated in the nine adenocarcinomas examined (Fig. 2) represent candidates for stimulating cell growth and preventing apoptosis; some of them have already been implicated in carcinogenesis. For example, haptoglobin is an acute-phase reactant protein involved in regulation of the immune system, and a correlation between serum levels of this protein and epithelial cell marker CA125 among patients with ovarian carcinoma has been reported previously (17). Wang *et al.* (18) reported that HE4 protease inhibitor, the product of a gene expressed in the epithelial cells of the epididymal duct, was elevated in ovarian cancers. They performed a similar experiment and listed 30 genes that were up- or down-regulated in ovarian tumors (18). Nine of the 30 genes were also picked up by our experiments, but it is hard to compare the results of the two studies because no detailed data for genes examined or for histological data were provided in their report. Elevated expression of keratin has been observed in ovarian cancer cell lines, and overexpression of this protein may be a suitable marker for detection of disseminated ovarian cancer (19). Rcl, a c-myc-responsive gene, is thought to play a role in cellular proliferation and transformation; 14-3-3 σ protein tends to be overexpressed in pancreatic adenocarcinomas and in head and neck squamous cell carcinomas (20–22). In this study, we used normal ovarian tissue that was histopathologically confirmed to be free of cancer. However, it is

apparent that cell types other than epithelial cells were included in normal ovarian tissue. Thus, it is correct to state that the genes selected in our analysis were relatively up- or down-regulated compared with normal ovarian tissue. Hence, up-regulation or down-regulation of the selected genes may reflect the differences in cell populations. Ovarian tumors are derived mainly from the surface epithelium of the ovary and are classified into five major histopathological types: (*a*) serous; (*b*) mucinous; (*c*) endometrioid; (*d*) clear cell; and (*e*) transitional cell. Some of the molecular mechanisms of carcinogenesis among these types are assumed to be common to all of them, but some are likely to be different (23). Our results indicate similarities and differences among genes that were altered in the two major types examined. When we compared the gene expression patterns in five serous adenocarcinomas with those in four mucinous tumors, we identified 115 genes whose expression levels were significantly different. Some of these genes may be associated with carcinogenesis of both tumor types; indeed, some of them have already been implicated in cancers. For example, the multiple membrane-spanning receptor TRC8 gene, which was down-regulated in our group of mucinous tumors, has been identified as a segment polarity patched-related gene. In a series of sporadic renal carcinomas, an acquired TRC8 mutation has been identified (24). Expression of the A28-RGS14p gene was up-regulated in our mucinous adenocarcinomas; this gene has been identified as a novel p53 target gene that was induced in response to genotoxic stress and encodes a novel family member of regulators of G protein-signaling proteins with GTPase-activating protein activity (25).

We have demonstrated that cDNA microarrays represent a powerful approach to identify key molecules in the development and progression of ovarian cancer. A number of the candidates reported here should provide new markers that may contribute to the detection of tumor cells at an early stage. This information may also aid the development of new approaches to therapeutic intervention.

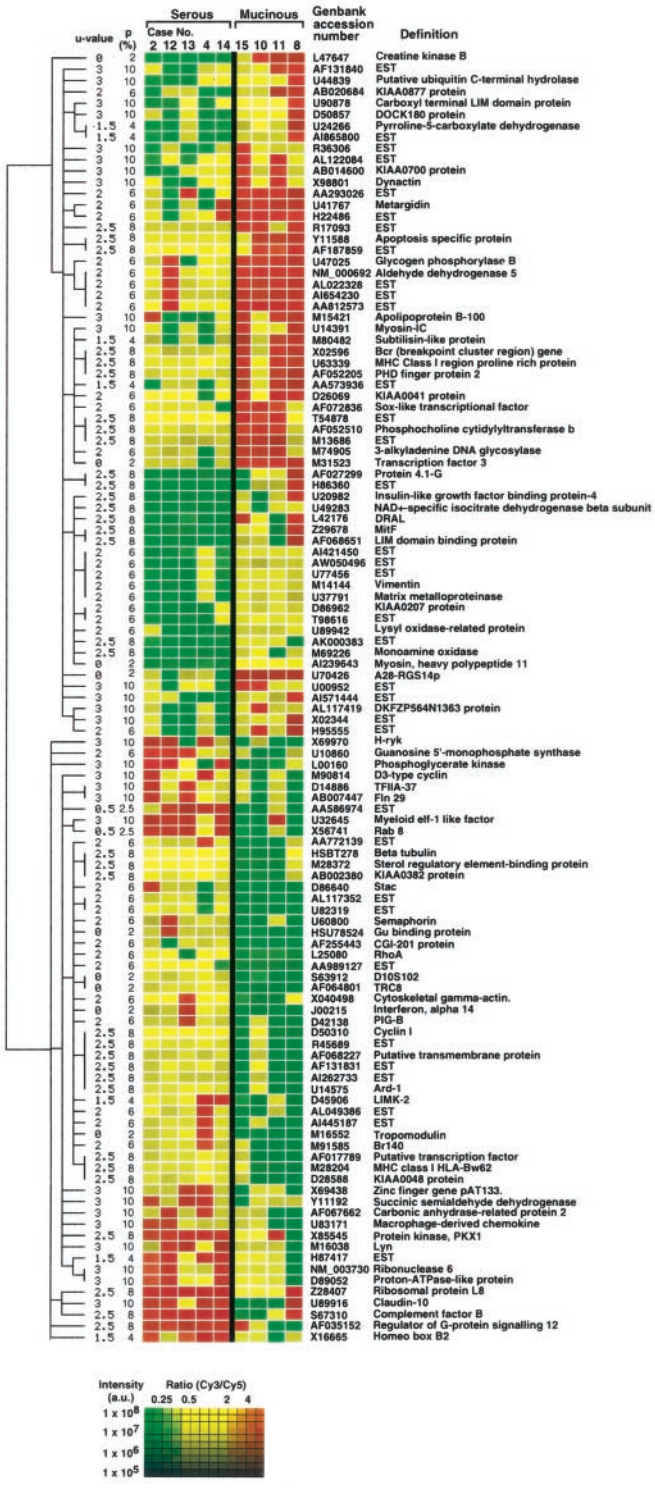

Fig. 3. Genes that were expressed differently in serous and mucinous adenocarcinomas.

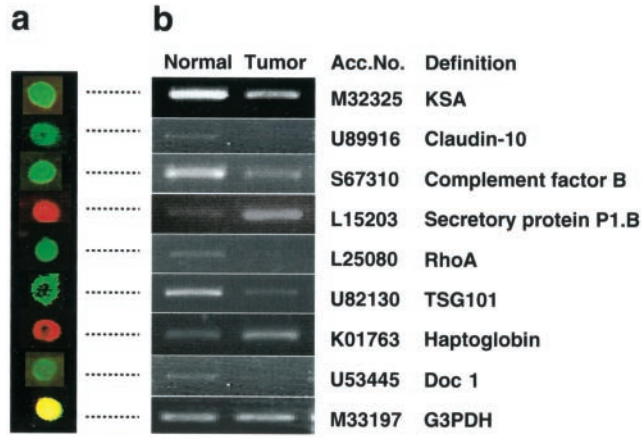

Fig. 4. Validation of microarray data by semiquantitative RT-PCR. *a*, aRNAs from normal ovarian tissue and mucinous adenocarcinoma from patient 15 were labeled with Cy5-dCTP (*green fluorescence*) and Cy3-dCTP (*red*), respectively. The labeled probes were mixed and then hybridized to the cDNA microarray. *b*, RT-PCR with unamplified total RNA from patient 15.

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