Product Application Focus

Identification of Differentially Expressed Genes from Ovarian Cancer Cells by MICROMAX™ cDNA Microarray System

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ABSTRACT

Using the MICROMAX™ cDNA microarray system, we were able to identify genes that are differentially overexpressed in ovarian cancer. A total of 30 putative genes, which are differentially overexpressed in ovarian cancer cell lines, were identified. The differential expression of some of these genes was further confirmed by real-time RT-PCR. Using this strategy, we have identified genes that either overexpress in all cancer cell lines or in only some cancer cell lines. Further characterization of these genes will allow them to be exploited in diagnosis, prognosis, anticancer therapy, and molecular classification of ovarian cancer.

INTRODUCTION

Ovarian cancer is the leading cause of death among women from gynecologic malignancies (1). Previously, we have successfully applied RAP-PCR (differential display) technology to identify differentially expressed genes in ovarian cancer, and only a few genes have been identified in the last few years (5–7).

cDNA microarray technology is a very sensitive method that allows one to analyze thousands of genes simultaneously (2,3,9). Several commercial human cDNA microarray systems are currently available. Here, we discuss the use of the MICROMAX™ system (NEN® Life Science Products, Boston, MA, USA) to identify genes that are differentially overexpressed in ovarian cancer cells. The use of an efficient tyramide signal amplification (TSA) system in the MICROMAX system enables us to use 20–100 times less total RNA than the currently used method. The use of a lower amount of RNA is very desirable in microarray analysis of precious RNA samples extracted from primary cell cultures or microdissected tissue samples.

MATERIALS AND METHODS

Cell Culture

Cultures of the normal human ovarian surface epithelial (HOSE) cells were established by scraping the HOSE cells from the ovary and growing in a mixture of Medium 199 and MCDB105 supplemented with 10% fetal calf serum (Sigma, St. Louis, MO, USA) as described previously (7). The seven HOSE cells used were HOSE17, HOSE636, HOSE642, HOSE695, HOSE697, HOSE713, and HOSE726. The ovarian cancer cell lines used were OVCA3, OVCA420, OVCA432, OVCA433, OVCA633, SKOV3, and ALST. All cell cultures and cell lines were established in the Laboratory of Gynecologic Oncology, Brigham and Women's Hospital, except SKOV3, which was purchased from ATCC (Manassas, VA, USA).

Microarray Probe and Hybridization

MICROMAX human cDNA microarray system, which contains 2400 known human cDNA on a 1 × 3 inch slide, was used in this study. Microarray probe and hybridization were performed as described in the instruction manual. In brief, biotin-labeled cDNA was generated from 3 µg total RNA, which was pooled from HOSE17, HOSE636, and HOSE642. Dinitrophenyl (DNP)-labeled cDNA was generated from 3 µg total RNA that was pooled from ovarian cancer cell lines OVCA 420, OVCA 433, and SKOV3. Before the cDNA reaction, an equal amount of RNA control was added to each batch of the RNA samples for normalization during data analysis. Biotin-labeled and DNP-labeled cDNA were mixed, dried, and resuspended in 20 µL hybridization buffer, which was added to the cDNA microarray and covered with a cover-

Table 1. List of Genes Differentially Overexpressed in Ovarian Cancer Cells More than Tenfold

Accession No.	Description	Before Extensive Washing (Cy3/Cy5)	After Extensive Washing (Cy3/Cy5)	Cy3 Signal Intensity
M33011	Carcinoma-associated antigen GA733-2	472	444	1249
J04765	Osteopontin	156	184	11851
L41351	Prostasin	44	170	3172
L19783	GPI-H	4	88	916
U96759	Von Hippel-Lindau binding protein (VBP-1)	60	59	1377
M57730	B61	20	49	5514
L33930	CD24 signal transducer and 3' region	24	47	26722
D55672	hnRNP D	45	44	950
U97188	Putative RNA binding protein KOC	223	38	3599
L19871	ATF3	9	37	3507
J04991	p18	15	34	9914
D00762	mRNA for proteasome subunit HC8	17	29	4703
U17989	Nuclear autoantigen GS2NA	5	28	721
U43148	Patched homolog (PTC)	10	28	4155
AF010312	Pig7 (PIG7)	13	23	17379
M80244	E16	18	21	4180
X99802	mRNA for ZYG homologue	14	21	2086
U05598	Dihydrodiol dehydrogenase	10	18	21595
L47647	Creatine kinase B.	7	18	787
M55284	Protein kinase C-L (PRKCL)	7	16	863
X15722	mRNA for glutathione reductase	23	14	794
S54005	Thymosin beta-10	6	13	1476
AB006965	mRNA for Dnm1p/Vps1p-like protein	7	13	4183
M83653	Cytoplasmic phosphotyrosyl protein phosphatase	6	13	2156
X12597	mRNA for high mobility group-1 protein (HMG-1)	7	12	2785
M18112	poly(ADP-ribose) polymerase	6	12	9277
U56816	Kinase Myt1 (Myt1)	4	11	1773
X06233	mRNA for calcium-binding protein in macrophages (MRP-14)	7	11	3007
D85181	mRNA for fungal sterol-C5-desaturase homolog	6	11	3571
M31627	X box binding protein-1 (XBP-1)	5	10	12151

slip. Hybridization was carried out overnight at 65°C inside a hybridization cassette (Telechem, Sunnyvale, CA, USA).

Posthybridization and Cyanine-3 (Cy3™) and Cyanine-5 (Cy5™) TSA

After hybridization, the microarray was washed with 30 mL 0.5× SSC, 0.01% SDS, and then with 30 mL 0.06× SSC, 0.01% SDS. Finally, the microarray was washed with 0.06× SSC. Hybridization signal from biotin-labeled cDNA was amplified with streptavidin-horseradish peroxidase (HRP) and Cy5-tyra-

mide, while hybridization signal from DNP-labeled cDNA was amplified with anti-DNP-HRP and Cy3 tyramide. After signal amplification and posthybridization wash, the cDNA microarray was air-dried and detected with a laser scanner.

Image Acquisition and Data Analysis

Cy3 signal was derived from ovarian cancer cells, and Cy5 signal was derived from HOSE cells. Laser detection of the Cy3 and Cy5 signal on the microarray was acquired with a confocal laser reader, ScanArray® 3000 (GSI Lumonics,

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Table 2. Cy3 versus Cy5 Ratio for a Set of Genes that Are Previously Shown to Express at Relatively Constant Level (2)

Accession No.	Description	Before Extensive Washing (Cy3/Cy5)	After Extensive Washing (Cy3/Cy5)
X06323	MRL3 mRNA for ribosomal protein L3 homologue	3.31	5.22
AF006043	3-phosphoglycerate dehydrogenase	3.81	4.8
M37400	Cytosolic aspartate aminotransferase	3.03	3.66
D30655	mRNA for eukaryotic initiation factor 4AII	4.17	3.48
J04208	inosine-5'-monophosphate dehydrogenase (IMP)	1.13	2.15
M17885	Acidic ribosomal phosphoprotein P0	2.74	2.09
X54326	mRNA for glutamyl-tRNA synthetase	1.17	2.01
J04973	Cytochrome bc-1 complex core protein II	0.98	1.6
D13900	mitochondrial short-chain enoyl-CoA hydratase	0.91	1.52
Z11531	mRNA for elongation factor-1-gamma	0.76	0.89
D78361	mRNA for ornithine decarboxylase antizyme	0.51	0.82
U13261	eIF-2-associated p67 homolog	0.41	0.82
X15183	mRNA for 90-kDa heat-shock protein	0.8	0.75
M36340	ADP-ribosylation factor 1 (ARF1)	0.5	0.66
X91257	mRNA for seryl-tRNA synthetase	0.75	0.52
AF047470	Malate dehydrogenase precursor (MDH) mRNA	0.41	0.51
D13748	mRNA for eukaryotic initiation factor 4AI	0.33	0.43
L36151	Phosphatidylinositol 4-kinase	0.26	0.38
X04297	mRNA for Na,K-ATPase alpha-subunit	0.27	0.34
X79535	mRNA for beta tubulin, clone nuk_278	0.18	0.28
J04173	Phosphoglycerate mutase (PGAM-B)	0.14	0.23

Watertown, MA, USA). Separate scans were taken for each fluor at a pixel size of 10 μm . cDNA derived from the control RNA hybridized to 12 specific spots within the microarray. Cy3 and Cy5 signals from these 12 spots should theoretically be equal and were used to normalize the different efficiencies in labeling and detection with the two fluors. The fluorescence signal intensities and the Cy3/Cy5 ratios for each of the 2400 cDNAs were analyzed by the software Imagene 3.0TM (Biodiscovery, Los Angeles, CA, USA).

Real-Time Quantitative RT-PCR

Real-time PCR was performed in duplicate using primers sets specific to GA733-2, osteopontin, prostasin, creatine kinase B, CEA, KOC, and a housekeeping gene, cyclosporin, in an ABI PRISMTM 5700 Sequence Detector (Applied Biosys-

tems, Foster City, CA, USA). RNA was first extracted from normal ovarian epithelial cell cultures (HOSE 695, 697, 713, and 726) and six ovarian carcinoma cell lines (OVCA3, OVCA432, OVCA433, OVCA633, SKOV3, and ALST). cDNA was generated from 1 μg total RNA using the TaqMan[®] reverse transcription reagents containing 1 $\times$ TaqMan reverse transcription buffer, 5.5 mM MgCl_2 , 500 μM dNTP, 2.5 μM random hexamer, 0.4 U/ μL RNase inhibitor, 1.25 U/ μL MultiScribeTM reverse transcriptase (Applied Biosystems) in 100 μL . The reaction was incubated at 25°C for 10 min, 48°C for 30 min, and finally at 95°C for 5 min. Briefly, 0.5 μL cDNA was used in a 20- μL PCR mixture containing 1 $\times$ SYBR[®] PCR buffer, 3 mM MgCl_2 , 0.8 mM dNTP, and 0.025 U/ μL AmpliTaq Gold[®] (Applied Biosystems). Amplification was then performed with denaturation for 10 min at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension

at 60°C for 1 min. The changes in fluorescence of SYBR Green I dye in every cycle was monitored by the ABI 5700 system software, and the threshold cycle (C_T) for each reaction was calculated. The relative amount of PCR products generated from each primer set was determined based on the C_T value. Cyclosporin was used for the normalization of quantity of RNA used. Its C_T value was then subtracted from that of each target gene to obtain a ΔC_T value. The difference ($\Delta\Delta C_T$) between the ΔC_T values of the samples for each gene target and the ΔC_T value of the calibrator (HOSE726) was determined. The relative quantitative value was expressed as $2^{-\Delta\Delta C_T}$.

RESULTS AND DISCUSSION

The MICROMAX System

The MICROMAX system allows the simultaneous analysis of the expression level of 2400 known genes. The use of TSA in the MICROMAX system after hybridization reduces the amount of total RNA needed to a few micrograms, which is about 20–100 times less than currently used method. The detail of TSA has been described previously for chromosome mapping of PCR-labeled probes less than 1 kb by FISH (10). In this study, we were able to identify 30 putative differentially overexpressed genes (excluding nine ribosomal genes) in ovarian cancer cell lines (Table 1). Using high-density cDNA array on membrane, Schummer et al. (11) have identified 32 known genes that exhibit tumor-to-HOSE ratios of more than 2.5-fold. Fourteen of these 32 genes were present in the MICROMAX cDNA microarray, but only five of them were more than threefold in our study. This difference may be due to the use of cancer cell lines in our study versus the use of bulk tumor tissues in the study by Schummer et al. (11).

In this study, biotin-labeled cDNA was made from ovarian cancer cell lines, while DNP-labeled cDNA was made from

HOSE cells. The differential TSA of the hybridization signal depends on the use of streptavidin-HRP conjugate or anti-DNP-HRP conjugate in a sequential step. At each step, Cy5-Tyramide or Cy3-Tyramide can be added, and the HRP will then catalyze the deposit of Cy3 or Cy5 onto the hybridized cDNA nonspecifically. As a result, we can choose to have either Cy3 or Cy5 signal for the cDNA derived from ovarian cancer cell lines, and vice versa for HOSE cells. Thus, we do not have to make two different sets of probes if we want to compare the effect of Cy3 or Cy5 fluorescence as a result of their differences in extinction coefficients and quantum yields. We also found that the Cy3 and Cy5 signals on the processed slides were stable for more than six months.

Normalization of Signals

The MICROMAX system has three nonhuman genes as internal controls. Each of the control genes has been spotted four times on the microarray. Equal amounts of polyA RNA derived from these control genes were spiked into the total RNA samples derived from both HOSE and ovarian cancer cell lines during cDNA synthesis. Thus, hybridization signals from these control genes in two RNA samples should theoretically be the same. The Cy3-to-Cy5 ratios for these control genes varied from 0.4 to 4.0, and the average ratio was 1.5 ± 1.1 (data not shown). From a prior microarray analysis of human cancer cells, 88 genes have been identified to express at relatively constant levels in many cell types (2). The MICROMAX microarray also contains 58 of these 88 genes and 21 of these genes with a signal-to-noise ratio more than threefold were analyzed (Table 2). The ratios varied from 0.23 to 5.22. The average ratio is 1.6 ± 1.5 . Thus, the result of internal control RNA for normalizing signal was similar to that of genes that express at a relatively constant level in different cell types. The set of nonhuman control genes will be useful as control for other custom-designed chips.

Figure 1. Effect of extensive washing on signal-to-noise ratios.

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Table 3. Real-Time Quantitative RT-PCR Analysis of a Few Selected Genes^a

	GA733-2	Osteopontin	KOC	Prostasin	Creatine kinase B	CEA	RGS
Normal ovarian cells							
HOSE695	4	21	5	28	0.4	5	32
HOSE697	1	1	2	4	0.4	3	18
HOSE713	1	20	7	5	1	16	25
HOSE726	1	1	1	1	1	1	1
Average (HOSE)	2	11	4	9	1	6	19
Ovarian cancer cell lines							
OVCA3	419	6	4	61	393	1	4
OVCA432	136	0	0	17	8	0	1
OVCA433	2048	0	52	57	12	1	4
OVCA633	2917	13777	3	228	4	15	27
SKOV3	2856	265	10	2	31	6	13
ALST	3875	6081	78	10	1	2	5
Average (OVCA)	2042	3355	24	62	75	4	9
OVCA/HOSE (average)	1361	310	6	7	103	1	0.5

^aEach gene was analyzed using an identical panel of 10 cDNA samples that were comprised of our normal ovarian surface epithelial cells and six ovarian cancer cell lines. The expression of each gene for each cDNA sample was normalized against cyclosporin. Duplicated reactions were performed for each of the genes, and similar results were obtained.

Effect of Background Signal on the Identification of Differentially Expressed Genes

In our study, 1357 of the 2400 genes on the microarray have Cy3 signals (from ovarian cancer cell lines) that were at least twofold higher than the background, or 740 genes have Cy3 signals that were at least threefold higher than the background. After posthybridization washes, there was still significant background intensity for the Cy3 signal (Figure 1) but very low background for Cy5 (data not shown). Subsequently, we washed the microarray again in 30 mL TNT buffer at 42°C for 20 min instead of at room temperature, followed by 30 mL 0.006× SSC for 1 min. The washed microarray was then dried and rescanned. This process was repeated several times until the number of genes with signal-to-noise ratios at least threefold remained the same. The extensive washing steps decreased the background intensity significantly, but there were no obvious changes in the signal intensity. As a result, the number of genes with at least threefold signal-to-noise ratios has increased from 740 to 791 genes. Moreover, the differential expression ratios, in general, have increased as shown in both Table 1 and Table 2. More importantly, after the extensive washing, we were able to detect the differential expression of two weakly expressed genes, thiol-specific antioxidant protein (4.5-fold) and elongation factor-1-β (9.7-

fold), which were previously identified by Schummer et al. (11). Thus, we suggest that extensive posthybridization washing and rescanning of signals may be necessary to decrease background signal, especially in the case of differentially expressed genes with low expression level.

Confirmation of Differential Expression by Real-Time Quantitative PCR

To further validate the differential expression, we chose five interesting genes, GA733-2, osteopontin, *koc*, prostasin, and creatine kinase B, for real-time PCR analysis. All of these genes are either surface antigens or secreted proteins. Thus, they may be useful as tumor markers for ovarian cancer. GA733-2 is a cell surface 40-kDa glycoprotein associated with human carcinomas of various origins (13). Osteopontin is a secreted glycoprotein with a conserved Arg-Gly-Asp (RGD) integrin-binding motif and is expressed predominantly in bone but has been found in breast cancer and thyroid carcinoma with enhanced invasiveness (12,15). Prostasin is a novel secreted serine proteinase that was originally identified in seminal fluid (16). The *koc* transcript is highly overexpressed in pancreatic cancer cell lines and in pancreatic cancer. It is speculated that *koc* may assume a role in the regulation of tumor cell proliferation by interfering with tran-

scriptional and or posttranscriptional processes (8). Creatine kinase B is a serum marker associated with renal carcinoma and lung cancer (4,14). Moreover, two randomly selected genes, CEA and RGS, were used as negative controls.

The results (Table 3) showed that all the tested ovarian cancer cell lines expressed higher levels of GA733-2. However, osteopontin, prostaticin, KOC, and creatine kinase B were overexpressed in only some of the cancer cell lines. Since we were using pools of RNA, the differential expression that we have observed is an average of the gene expression from three independent HOSE cells or three different cancer cell lines. This strategy allows us to capture genes that overexpress in either some or all of the cell lines. Genes that only overexpress in some of the ovarian cancer cell lines may be useful for molecular classification of ovarian cancer cells. Since as little as 10 pg cDNA is enough for real-time quantitative RT-PCR, RNA extracted from microdissected tissue would be enough for thousands of such real-time quantitative RT-PCR analyses.

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