Analysis of gene expression profiles in normal and neoplastic ovarian tissue samples identifies candidate molecular markers of epithelial ovarian cancer

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Communicated by Peter G. Schultz, Genomics Institute of the Novartis Research Foundation, La Jolla, CA, December 1, 2000 (received for review November 2, 2000)

Epithelial ovarian cancer is the leading cause of death from gynecologic cancer, in part because of the lack of effective early detection methods. Although alterations of several genes, such as c-erb-B2, c-myc, and p53, have been identified in a significant fraction of ovarian cancers, none of these mutations are diagnostic of malignancy or predictive of tumor behavior over time. Here, we used oligonucleotide microarrays with probe sets complementary to >6,000 human genes to identify genes whose expression correlated with epithelial ovarian cancer. We extended current microarray technology by simultaneously hybridizing ovarian RNA samples in a highly parallel manner to a single glass wafer containing 49 individual oligonucleotide arrays separated by gaskets within a custom-built chamber (termed “array-of-arrays”). Hierarchical clustering of the expression data revealed distinct groups of samples. Normal tissues were readily distinguished from tumor tissues, and tumors could be further subdivided into major groupings that correlated both to histological and clinical observations, as well as cell type-specific gene expression. A metric was devised to identify genes whose expression could be considered ideal for molecular determination of epithelial ovarian malignancies. The list of genes generated by this method was highly enriched for known markers of several epithelial malignancies, including ovarian cancer. This study demonstrates the rapidity with which large amounts of expression data can be generated. The results highlight important molecular features of human ovarian cancer and identify new genes as candidate molecular markers.

Epithelial ovarian cancer has one of the worst prognoses among gynecologic malignancies. The majority of early-stage cancers are asymptomatic, and over three-quarters of the diagnoses are made at a time when the disease has already established regional or distant metastases. Whereas the 5-yr survival is favorable for women with an early diagnosis (~90%), survival with distant metastases over the same time period is less than 20% (1). Most ovarian cancers arise from the surface (coelomic) epithelium that covers the ovary and are thought to progress through premalignant phases before becoming frankly invasive (2). The only validated marker in current use for the management of ovarian cancer is CA125, which can be detected in the serum of more than 80% of women with ovarian carcinomas (3). However, CA125 is thought to be robust only in following the response or progression of the disease, but not as a diagnostic or prognostic marker (4). Thus, there is an important need for additional diagnostic and prognostic markers for this disease.

Molecular genetic analyses of ovarian cancers have uncovered genetic alterations of several genes, such as c-erb-B2, c-myc, and p53, in a significant fraction of tumors (5). Global studies of genomic rearrangements suggest that additional genes involved in ovarian tumor progression correlate to a variable extent with clinical parameters (6). Previously, spotted cDNA filters and microarrays have been used to profile limited numbers of epithelial ovarian adenocarcinomas (7, 8). These studies identified several genes, such as HE4, that may be diagnostic of ovarian cancer and other genes that correlate with serous or mucinous histology. Despite this progress, however, relatively little is known about the cellular changes that correlate with, or determine the different biological properties and diverse behavior of, individual ovarian epithelial tumors. For example, it is not known what underlying molecular properties distinguish borderline from invasive tumors.

We used oligonucleotide microarrays with probe-sets complementary to more than 6,000 human genes to monitor the levels of expression within aggregate normal and malignant ovarian tissues. To help interpret the observed patterns of gene expression, we selectively macrodissected normal samples into epithelial and stromal fractions. We also hybridized RNAs from endothelial and activated B cells to help discern patterns of gene expression consistent with the presence of blood vessels and/or infiltrating immune cells. A new, high-throughput protocol allowed simultaneous hybridization of 49 samples to individual microarrays on a single glass wafer (P.P.Z., J. K. Mainquist, M. Zamora, D. Stern, J.B.W., L.M.S., G.M.H., and D.J.L., unpublished data). Data obtained from normal and malignant ovarian tissues allowed us to distinguish these two classes of tissue based on quantitative expression levels, and to propose several genes as candidate diagnostic markers. Expression profiles of different tumors demonstrate significant heterogeneity, but it is nonetheless possible to identify groups of genes whose altered expression may influence their clinical behavior.

Materials and Methods

Tissues and Cell Lines. Twenty-seven flash-frozen serous papillary adenocarcinomas of the ovary and three normal samples of whole ovarian tissue were made available through the Cooperative Human Tissue Network (CHTN Midwestern Division, Columbus, OH). Institutional Review Board approvals were granted before accession of the tissues. Frozen sections were taken from each tissue block before removing tissue for expres-
sion profiling. Each tumor was then histologically analyzed by one of us (C.A.B.). Clinical and pathologic details of the samples are tabulated in supplementary Table 1, which is published as supplemental data on the PNAS web site, www.pnas.org. Ovarian cancer-derived cell lines, SK-OV-3 [American Type Culture Collection (ATCC) HTB-77], MDAH-2774 (ATCC CRL-10303), and CAOV-3 (ATCC HTB-75), were obtained from the ATCC and grown in DMEM (Life Technologies, Rockville, MD), supplemented with 10% (vol/vol) FCS and penicillin/streptomycin. RNAs from human umbilical vein endothelial cells (HUVECs) and human activated B cells were gifts from Drs. Akira Kawamura (The Scripps Research Institute, San Diego, CA) and Michael Cooke (Genomics Institute of the Novartis Research Foundation), respectively. RNA from normal human ovarian tissue was purchased from BioChain Institute (Hayward, CA). RNAs from heart, lung, liver, thymus, testis, and spleen were purchased from CLONTECH.

Tissue Processing and Preparation of RNA. Fragments of normal and malignant ovarian tissue were sharp dissected and homogenized with a rotary homogenizer (Omi International, Warrenton, VA) in RNEasy lysis buffer (Qiagen, Chatsworth, CA). For each of the normal tissues (case nos. 102, 233, and 278), surface epithelium was selectively procured by macrodissection. In one case (no. 278), two samples were prepared, one of which was enriched for epithelium and one for stroma. RNA from each of the tissue samples was prepared by using the RNEasy Mini Kit (Qiagen) and used at a final concentration of 2 mg/ml.

Hybridization of RNAs to Oligonucleotide “Array of Arrays.” Labeling of samples, hybridization to oligonucleotide arrays, and scanning were performed essentially as described (9), with modifications to allow simultaneous processing of samples in 96-well plates. Hybridizations were performed simultaneously in a custom-built chamber. Briefly, we obtained a glass wafer bearing 49 identical HuGeneFL arrays before division and packaging as individual chips (Affymetrix, Santa Clara, CA). Gaskets allowed sample separation during hybridization on the glass wafer. The separation between arrays was removed for staining, washing, and scanning steps. Arrays on the wafer were scanned in series by using a custom-made confocal laser scanner with a specially constructed translation stage. The quality and reproducibility of the data derived from the glass wafer array-of-arrays are high and will be described elsewhere (P.P.Z., J. K. Mainquist, M. Zamora, D. Stern, J.B.W., L.M.S., G.M.H., and D.J.L., unpublished data). Three samples (tumor 1, MDAH 2774, and CA-OV-3) were hybridized in duplicate, and the data from the two cell lines were averaged. The data from duplicate hybridizations of tumor 1 were kept separate to assess the reproducibility of the data through cluster analysis (see below). RNA from six normal tissues (lung, liver, heart, spleen, thymus, and testis) was pooled before labeling and hybridization. RNA from activated B cells and HUVECs was hybridized separately to microarrays containing ~12,000 human genes (Affymetrix; U95A). To include data from B cells and HUVECs in the analysis, we excluded most of the information from the U95A arrays and used only the hybridization intensities of genes that were also represented on the HuGeneFL arrays (see supplementary data).

Data Analysis. Scanned image files were visually inspected for artifacts and analyzed with GENECHIP 3.1 (Affymetrix). Each image was then scaled to an average hybridization intensity of 200, which corresponds to ~3–5 transcripts per cell (9). The expression level (average difference) for each gene was determined by calculating the average of differences in intensity (perfect match-mismatch) between its probe pairs (9–11). Genes with average hybridization intensities <0 across all samples were excluded from further analysis. CLUSTER and TREEVIEW (12) were used to select, group, and visualize genes whose expression varied across the samples with SD $\geq$ 250.

To identify potential tumor markers, the hybridization intensity of each gene in normal and malignant samples was compared, and three different estimates for population differences (difference of means, fold change, and unpaired t test) were applied in parallel. The genes were ranked according to each metric, and the sum of the metrics was used to derive a semiquantitative estimate of the differential abundance of each transcript.

Reverse Transcription (RT)-PCR. One microgram of total RNA from selected normal and tumor tissue samples (see legend to Fig. 3) was reverse transcribed with random hexamers and thermostable TaqGold polymerase by using the Thermoscript RT-PCR System (Life Technologies). One microliter of RT product was amplified by primer pairs specific for HE4, CD24, and L (BCAM). Primers specific for 18s ribosomal RNA and empirically determined ratios of 18s competitors (Ambion, Austin, TX) were used to control for the amounts of cDNA generated from each sample. Amplification was carried out by using 1 unit of polymerase in a final volume of 20 μl containing 2.5 mM MgCl2. TaqGold was activated by incubation at 96°C for 12 min, and the reactions were cycled 26–30 times at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a final extension at 72°C for 10 min. PCR products were visualized on a% agarose gels stained with ethidium bromide, and images were captured by an Ultraviolet Products Image Analysis System.

Results

To identify patterns of gene expression in normal and malignant ovarian tissue, we performed cluster analysis (12) on hybridization intensity values for each gene. We chose 1,243 genes with average hybridization intensities >0 that varied most across the samples (SD $\geq$ 250), to select for genes with strong differential expression. Cluster analysis for these genes is shown in Fig. 1 and supplementary Fig. 4. The complete dataset is available at our web site (http://www.snf.org/cancer/ovary).

Identification of Sample Groups by Hierarchical Clustering. The dendrogram illustrated in Fig. 1a showed a major division in the distribution of samples. In the leftmost columns (colored blue), the 5 normal ovarian tissue samples and a subset of 7 ovarian tumors were grouped together. Within this branch, the normal tissues were found to cluster tightly together on a subbranch, and the independent hybridizations of tumor 1 correlated well with each other, as expected. The remaining samples formed 2 groups (black and red branches of the dendrogram). One group (red branches) contained all of the ovarian cancer-derived cell lines, activated B cells, and endothelial cells, as well as a subset of 8 of the ovarian tumors. Samples in the middle columns (black) included 12 ovarian tissue samples.

Clusters of Genes Expressed in Normal and Malignant Tissues. The tight clustering of the normal tissues was largely “driven” by two distinct profiles of gene expression. The first was represented by a group of about 100 genes that were highly expressed in normal tissue and underexpressed in the tumors and cell lines [some of which are highlighted in Fig. 1c (Normal)]. Many of these are immediate early genes (e.g., c-fos, jun-B, and EGR-1) and genes whose expression is stimulated by estrogen in breast cancer cell lines (e.g., the family 4 nuclear hormone receptors 1, 2, and 3; unpublished data). The second profile was represented by several smaller clusters of genes that were underexpressed in normal tissue as compared with ovarian tumors and cell lines (see below). One of these clusters is expanded in Fig. 1c (Tumor) and includes genes highly expressed in a large fraction of the tumors, such as HE4 and the preferentially expressed antigen of
Fig. 1. Legend on next page.
melanoma gene (PRAME). These two genes were highly expressed in 14/27 and 15/27 of the tumors, respectively. Other similar clusters contained genes known to be amplified in ovarian cancer, such as c-erb-B2, which was elevated in about 30% of the tumors (see supplementary data), consistent with previous reports (13).

Coclustering of normal and tumor tissues within the leftmost branch of the dendrogram (colored blue) was due, in part, to high expression of a very large group of ribosomal genes, 17 of which are shown in detail in Fig. 1c (Ribosomal cluster). Expression of these genes was not entirely confined to this subset of tumors, however, because other tumors within the middle branch (black) of the dendrogram (e.g., tumors 15, 24, and 31) also expressed some of these genes at high levels. Eight of the ovarian tumor samples were found to cluster close to the group of cell lines grown in vitro. This group, which is illustrated by the Proliferative cluster (Fig. 1c), showed coordinated expression of multiple genes associated with cell cycle, such as cdc28 protein kinases 1 and 2, cdc25B, and cdc20. These results suggested that this subset of tumors contained a high S-phase fraction (SPF), which is thought to be predictive of aggressive tumor behavior (14).

Identification of Cell Type-Specific Gene Expression. Selective enrichment of the normal ovarian tissues into epithelial (102, 233, and 278-E) and stromal (278-S) fractions facilitated identification of genes expressed within one or both types of cells. For example, genes encoding members 1 and 2 of the family 4 nuclear hormone receptors, the zinc-finger transcription factor AREB6, and the early growth response protein hEGR3 (PILOT) were sought genes whose expression pattern could be considered diagnostic of epithelial ovarian cancer. Good tumor markers fulfill several criteria, including low expression in normal tissue and high expression in neoplastic tissue. Markers should also exhibit a clear cutoff in expression levels between normal and neoplastic tissues to unambiguously resolve the two diagnostic conditions. As illustrated above, some of the tumor samples exhibited strong signals from genes characteristic of stroma and/or infiltrating immune cells. This observation suggested that they contained a low fraction of epithelial cells, which was confirmed by histology. Because we sought genes whose expression was diagnostic of malignant epithelium, 14 of the 27 tumors were excluded from this analysis based on their “non-epithelial” behavior (14).

Candidate Molecular Markers of Epithelial Ovarian Cancer. We next sought genes whose expression pattern could be considered diagnostic of epithelial ovarian cancer. Good tumor markers fulfill several criteria, including low expression in normal tissue and high expression in neoplastic tissue. Markers should also exhibit a clear cutoff in expression levels between normal and neoplastic tissues to unambiguously resolve the two diagnostic conditions. As illustrated above, some of the tumor samples exhibited strong signals from genes characteristic of stroma and/or infiltrating immune cells. This observation suggested that they contained a low fraction of epithelial cells, which was confirmed by histology. Because we sought genes whose expression was diagnostic of malignant epithelium, 14 of the 27 tumors were excluded from this analysis based on their “non-epithelial” behavior (14).
expression levels of cancers, we used oligonucleotide microarrays to monitor the
identification of candidate molecular markers of epithelial ovarian

Discussion

breast lesions and in a primary medulloblastoma.
cancer cell line OVCA432-2.
primary ovarian tumors, respectively, as well as in the ovarian
tissues.

Methods
and expression patterns (see Discussion). To estimate how well a
transcript level might correlate with malignancy, we compared
the groups of normal (n = 4) and tumor samples (n = 13) with
respect to the differences and fold changes in hybridization
intensities. The significance of the differential expression was
measured by using the Student unpaired t test. These parameters
were ranked, and each gene was then given a score based on the
sum of the ranks. The full list of genes is provided on our web
site, and the expression levels of the thirty most highly scoring
genes are shown in Fig. 2. Each gene’s expression level in a pool
of six normal tissues is also shown for comparison. The genes
shown in Fig. 2 were all strongly differentially expressed, with P
scores ranging from 2.5 × 10⁻⁴ (MUC-1) to 6.5 × 10⁻⁹ (TAC-
STD1/GA733-2).

Validation of Tumor-Specific Gene Expression. We validated differen-
tial expression of genes discovered by the ranking method in
two distinct ways. First, fragments of CD24, HE4, and LU were
amplified by RT-PCR from the RNAs of three epithelial-

tissues. The results of these experiments demonstrated overexpression of three genes in
tumor tissues relative to normal ovarian epithelium (Fig. 3). Second, we queried the National Center for Biotechnology
expression patterns of these same three genes in tumor cells and
tissues. LU and HE4 were highly expressed in 4/4 and 2/3
primary ovarian tumors, respectively, as well as in the ovarian
cancer cell line OVCA432-2. CD24 was highly up-regulated in
only one of three ovarian tumors, but also highly expressed in two
breast lesions and in a primary medulloblastoma.

Discussion

To identify candidate molecular markers of epithelial ovarian
cancers, we used oligonucleotide microarrays to monitor the
expression levels of >6,000 human genes in 27 tumor and four
normal tissue samples. Whereas microarrays have now become
an established tool with which to study gene expression patterns
in human cancer (15), the process of carrying out array exper-
iments one sample at a time is limiting. Here, we addressed this
issue by performing all of the sample hybridizations simulta-
neously on a single glass wafer containing 49 individual human
HuGeneFL oligonucleotide arrays in a custom-built chamber.
The physical process of tissue preparation, isolation of RNAs,
labeling, hybridization, washing, and reading the arrays was
completed in under 1 wk. Correlation coefficients between
samples hybridized in duplicate on the array-of-arrays and those
hybridized in duplicate on single arrays were better in many
instances, most likely because all of the chips on the glass wafer
were handled identically (1). This method minimized some of the
variation inherent in microarray experiments.

Hierarchical clustering of the samples and gene expression
levels within the samples led to the unambiguous separation of
normal and malignant tissues, as well as the identification of
three subsets of ovarian tumor tissue samples. One group of
tumors segregated with normal tissues and conversely on a single glass wafer
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excluding 14 tumors based on high expression of genes within non-epithelial cells, the list of genes was highly “enriched” for known or suspected makers of epithelial malignancy (Fig. 2). The most highly ranked gene, CD24, was first proposed as a therapeutic target for lung cancer (17, 18), and more recently as a diagnostic marker for breast cancer (19). This gene has been reported to have a role in increasing the motility of breast cancer cells (20). Another highly ranked gene, HE4, a secreted extracellular protease inhibitor, has been shown to be overexpressed in a significant fraction of ovarian cancers and lowly expressed in other tissues (7, 8). The product of this gene has been proposed as a diagnostic marker of ovarian cancers (8). CD9, which was also found to be overexpressed in ovarian tumors, is similarly overexpressed in carcinomas of the colon (21) and lung (22), and may participate in tumor invasion (23). Other highly ranked genes included the tumor-associated antigen GA733-2 (TACSTD1), cytokeratins 7, 8, 18, and 19, and MUC-1 (Fig. 2).

Several of these have been reported as highly expressed in a variety of epithelial cancers, including ovarian carcinoma. The expression of the highest-ranked genes was also assessed in a pool of six normal human tissues to determine levels of expression in tissues other than ovary (depicted by the green lines in Fig. 2). The results suggest that the expression of many of these genes is low in normal tissue and confirms the restricted expression in tissues other than ovary (depicted by the green lines in Fig. 2). The list of genes was highly “enriched” for gene clusters can be used to determine tumor behavior over outcomes is required to identify whether any of these genes or gene clusters can be used to determine tumor behavior over time. We are also aware that changes in mRNA abundance may not always correlate with changes in protein levels or activity, and we have not investigated whether the protein levels of CD24 or HE4, for example, are high in ovarian tumors. However, many of the genes identified as overexpressed in this study have been used as immunohistochemical markers in other tumor types, and thus correlations between changes in transcript and protein levels may exist for other genes that we identified.

An important aspect of the work presented here is the identification of multiple genes of interest, which was afforded by the highly parallel monitoring of gene expression levels in a sufficiently large number of cancers. This enabled stringent criteria to be imposed to identify “idealized” candidates. The identification of many candidates by this approach increases the probability that we can reduce to practice the clinical utility of one or more gene products in ovarian cancers. In general, we believe that this rapid and systematic approach toward marker identification will be useful in finding genes of interest from many cancer types and that knowledge of the function of the gene products is likely to provide significant insight into neoplasia.

We found a tentative molecular distinction between less aggressive, borderline tumors and rapidly dividing malignancies identified by differential expression of ribosomal and cell-cycle genes. However, profiling a larger cohort of tumors with defined outcomes is required to identify whether any of these genes or gene clusters can be used to determine tumor behavior over time. We are also aware that changes in mRNA abundance may not always correlate with changes in protein levels or activity, and we have not investigated whether the protein levels of CD24 or HE4, for example, are high in ovarian tumors. However, many of the genes identified as overexpressed in this study have been used as immunohistochemical markers in other tumor types, and thus correlations between changes in transcript and protein levels may exist for other genes that we identified.

We thank the Cooperative Human Tissue Network for tissue samples, Drs. Michael Cooke and Akira Kawamura for data and valuable discussions, and Don Bambico for excellent technical assistance.
