Parallel Analysis of Sporadic Primary Ovarian Carcinomas by Spectral Karyotyping, Comparative Genomic Hybridization, and Expression Microarrays¹,²

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ABSTRACT

Analysis of ovarian carcinomas has shown that karyotypes are often highly abnormal and cannot be identified with certainty by conventional cytogenetic methods. In this study, 17 tumors derived from 13 patients were analyzed by a combination of spectral karyotyping (SKY), comparative genomic hybridization (CGH), and expression microarrays. Within the study group, a total of 396 chromosomal rearrangements could be identified by SKY and CGH analysis. When the distribution of aberrations was normalized with respect to relative genomic length, chromosomes 3, 8, 11, 17, and 21 had the highest frequencies. Parallel microarray expression studies of 1718 human cDNAs were used to analyze expression profiles and to determine whether correlating gene expression with chromosomal rearrangement would identify smaller subsets of differentially expressed genes. Within the entire set of samples, microarray expression analysis grouped together poorly differentiated tumors irrespective of histological subtype. For three patients, a comparison between genomic alterations and gene expression pattern was performed on samples of primary and metastatic tumors. Their common origin was demonstrated by the close relationship of both the SKY and CGH karyotypes and the observed profiles of gene expression. In agreement with the pattern of genomic imbalance observed for chromosome 3 in ovarian cancer, the relative expression profile with respect to a normal ovary exhibited a contiguous pattern of reduced expression of genes mapping to the 3p25.5–3p21.31 and increased expression of genes from 3q13.33–3q28. This study demonstrates that SKY, CGH, and microarray analysis can in combination identify significantly smaller subsets of differentially expressed genes for future studies.

INTRODUCTION

Ovarian cancer is the leading cause of death from a gynecological malignancy and the fourth leading cause of cancer death among North American women. Response to treatment is generally poor, and the 5-year survival rate for all cases, at best, is only 50%. This poor outcome is because most ovarian cancers are asymptomatic, not detected until the disease has metastasized beyond the ovary and has a high cytological grade. Although the mechanisms of the disease are not well understood, there is a causal association with mutations of BRCA¹ and BRCA² in familial disease (1). Sporadic ovarian cancer, which makes up >85% of all cases, rarely shows mutations in BRCA¹ and BRCA² (1). Acquired mutations of p53 have been frequently identified among the serous subtype (2), whereas mutations of PTEN occur in the endometrioid subtypes (3), suggesting that they arise through distinct developmental pathways.

Cytogenetic analyses of ovarian carcinomas show both simple numerical and structural changes and complex aberrant changes (4–11). A cytogenetic survey of 244 ovarian carcinomas identified a clustering of chromosomal translocation breakpoints occurring in the regions 1p1*, 1q1*, 1p2*, 1q2*, 1p3*, 1q3, 3p1*, 1q4*, 6q1*, 6p2, 6q2, 7p1, 7p2*, 11p1*, 11q2*, 12p1, 12q2*, 13p1, and 19q1 (where the asterisk (*) denotes the most commonly involved regions of chromosomal rearrangement) (12–14). The presence of translocations in the regions shown above was associated with reduced patient survival. Chromosomal alterations in the regions 1p1 and 3p1 were found to confer an independent deleterious effect. One disadvantage of this study was that individual breakpoints were assigned to very large chromosomal regions (containing several hundred genes), making specific recurrent structural aberration identification difficult.

In recent years, molecular cytogenetic studies such as CGH⁶ (15) and SKY (16) have demonstrated their power in identifying recurrent chromosomal aberrations. To date, CGH has been widely used (17–25) in studying ovarian carcinomas and has identified increased copy numbers at 1q32, 3q26, 8q24.1-q24.2, 20q13.2-qter and frequent chromosomal losses identified at 5q21, 9q, 17p, 17q12-q21, 4q26-q31, 16q, and 22q. Sites of amplification have been identified at 7q36, 8q24.1-q24.2, 3q26.3, 17q25, 19q13.1-q13.2, and 20q13.2-qter by CGH.

Microarray analysis is a relatively new technique (26, 27), which allows the simultaneous expression analysis of thousands of genes from a single sample. Profiling and clustering of data from solid tumors have suggested new molecular classifications in lymphoma and breast cancer and generated hypotheses for metastatic markers in melanoma. Similar approaches have identified deregulated genes in ovarian cancer (13, 28–32); however, the relationship between these candidates and prognosis or classification is not yet clear. Microarray studies on nonovarian malignancies show that cancers with identical histological phenotype have marked differences in global expression patterns. Given this variability, we hypothesized that correlating expression analysis with chromosomal dosage change or rearrangement would allow better identification of key genetic changes in ovarian cancer.

In this study, we have performed parallel analysis using SKY, CGH, and microarray methods on a small cohort of ovarian carcinomas. Our objective was to identify a correlation between deviations in gene expression and chromosomal abnormalities identified by SKY.

¹The abbreviations used are: CGH, comparative genomic hybridization; SKY, spectral karyotyping; EST, expressed sequence tag; OPN, osteopontin; DAPI, 4',6-diamidino-2-phenylindole; RT-PCR, reverse transcription-PCR; IGFBP, insulin-like growth factor binding protein; VEGF, vascular endothelial growth factor.

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and CGH, in combination with a pilot microarray expression profiling of ovarian carcinoma.

**MATERIALS AND METHODS**

**Ovarian Tissue and Tumor Samples**

Seventeen tumors from 13 patients were obtained at the time of surgery and promptly processed for DNA and RNA extraction and, where possible, short-term culture for cytogenetic preparation. The study group consisted of 9 serous tumors (including 1 serous carcinoma), 6 endometrioid tumors, 1 clear cell tumor, and 1 mucinous tumor of low malignant potential as determined by standard histopathological classification. In 4 patients, two distinct tumor samples were obtained. OCA3A/OCA3B, OCA13A/OCA13B, OCA21A/OCA21B are paired primary/metastasis samples. OCA15A/B tumors were obtained from the right and left ovaries, respectively. The histopathological classification, age, stage, and grade of each tumor are summarized in Table 1. In all cases, except for OCA6 and OCA13A/B, the tumors obtained were from the first surgery and were untreated. Some patients received treatment at other institutions, and for others follow-up data are unavailable.

**DNA and RNA Extraction**

Seventeen tumor specimens were processed for DNA and RNA extraction. DNA extraction was performed using standard phenol:chloroform extract methods (33). Frozen tumor samples were rapidly minced on a −70°C stage, homogenized, and RNA was extracted using standard methods (34). Additional purification was performed using RNeasy columns (Qiagen, Mississauga, Ontario, Canada) following the manufacturer’s protocol. RNA quality was evaluated by spectrophotometry and by electrophoresis on a 1% denaturing formaldehyde-agarose gel. RNA from 14 tumor samples derived from 9 ovarian patients (OCA2, OCA3A/B, OCA5, OCA9, OCA13A/B, OCA15A/B, OCA17, OCA19, and OCA21A/B) was found to be suitable for microarray expression analysis. DNA and RNA specimens were stored at −70°C until ready for use. RNA derived from nine cell lines, HL-60, K562, H226, COLO 205, OVCAR-3, Caki-1, PC-3, MCF7, and Hs 578T (American Type Culture Collection, Rockville, MA), were extracted, pooled in equal amounts, and used as the reference RNA for initial microarray expression analysis (35). Two reference normal ovarian RNA samples were used for a subset of microarray expression experiments. One RNA sample (Stratagene, La Jolla, CA) was derived from bulk-extracted normal margin to hemorrhagic tumor.

**Table 1** Summary of tumor specimens analyzed and CGH results*

<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Histology</th>
<th>Grade</th>
<th>Stage</th>
<th>Status</th>
<th>CGH results</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCA1</td>
<td>55</td>
<td>Papillary serous adenocarcinoma</td>
<td>3/3</td>
<td>IV</td>
<td>First surgery</td>
<td>Untreated</td>
</tr>
<tr>
<td>OCA2</td>
<td>56</td>
<td>Endometroid adenocarcinoma</td>
<td>2/3</td>
<td>III</td>
<td>First surgery</td>
<td>Untreated</td>
</tr>
<tr>
<td>OCA3A-primary</td>
<td>81</td>
<td>Papillary serous cystadenocarcinoma</td>
<td>3/3</td>
<td>III</td>
<td>First surgery</td>
<td>Untreated</td>
</tr>
<tr>
<td>OCA3B-metastasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OCA5</td>
<td>66</td>
<td>Clear cell adenocarcinoma</td>
<td>NA</td>
<td>I</td>
<td>First surgery</td>
<td>Untreated</td>
</tr>
<tr>
<td>OCA6</td>
<td>55</td>
<td>Papillary serous adenocarcinoma</td>
<td>ND</td>
<td>IC</td>
<td>Second surgery</td>
<td>3 courses of treatment prior to surgery</td>
</tr>
<tr>
<td>OCA8</td>
<td>55</td>
<td>Papillary serous adenocarcinoma</td>
<td>2/3</td>
<td>II</td>
<td>First surgery</td>
<td>Untreated</td>
</tr>
<tr>
<td>OCA9</td>
<td>64</td>
<td>Endometroid adenocarcinoma</td>
<td>2/3</td>
<td>I</td>
<td>First surgery</td>
<td>Untreated</td>
</tr>
<tr>
<td>OCA13A-primary</td>
<td>79</td>
<td>Endometroid adenocarcinoma</td>
<td>Poorly differentiated</td>
<td>3/3</td>
<td>III</td>
<td>Second surgery</td>
</tr>
<tr>
<td>OCA13B-metastasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OCA15A-right ovary</td>
<td>43</td>
<td>Endometroid adenocarcinoma</td>
<td>Poorly differentiated</td>
<td>3/3</td>
<td>III</td>
<td>First surgery</td>
</tr>
<tr>
<td>OCA15B-left ovary</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OCA16</td>
<td>35</td>
<td>Mucinous Low malignant potential</td>
<td>3</td>
<td>NA</td>
<td>First surgery</td>
<td>Untreated</td>
</tr>
<tr>
<td>OCA17</td>
<td>70</td>
<td>Papillary serous adenocarcinoma</td>
<td>Moderately differentiated with some endometrioid components</td>
<td>2</td>
<td>III</td>
<td>First surgery</td>
</tr>
<tr>
<td>OCA19</td>
<td>57</td>
<td>Carcinoma serous Undifferentiated with mesenchymal components</td>
<td>NA</td>
<td>IB or II</td>
<td>First surgery</td>
<td>Untreated</td>
</tr>
<tr>
<td>OCA21A-primary</td>
<td>50</td>
<td>Serous adenocarcinoma Poorly differentiated</td>
<td>NA</td>
<td>III</td>
<td>First surgery</td>
<td>Untreated</td>
</tr>
<tr>
<td>OCA21B-metastasis</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

* ND, not done; NA, not available.
number changes detected along the length of a given chromosome. In determining the mean number of breakpoints present between endometrioid and serous subtypes, the full karyotypes (i.e., stemline and unique breakpoints) were averaged giving a total of 443 breakpoints for tumors OCA2, OCA3A, OCA8, OCA13A/B, OCA15A/B, and OCA21A/B.

CGH

Metaphase spreads from normal human lymphocytes were prepared using standard protocols (38). Ten to 12 images were captured using a Nikon Labophor-2 microscope equipped with an automatic filter wheel and an 83,000 frame per second camera (Packard BioScience) dual laser scanner and quantified with GenePix Pro 3.0 software (Axon). Features that were not quantified because they were flagged as bad or absent by GenePix Pro were manually reviewed for accuracy. Filtering, normalization of the raw data, and complete data analysis of the data sets were carried out with an algorithm developed in house that will be independently normalized by equalizing the Cy3 intensities, with respect to the Cy5 intensities.

Microarray Analysis with a Nine Cell Line Reference RNA. For the microarray analysis of 13 tumor samples against RNA derived from a pool of nine cell lines, the RNA samples were labeled using amino-allyl indirect labeling using a 2:1 ratio of amino-allyl dUTP/dTTP. Tumor RNAs were labeled with Cy5, and the pooled reference RNA was labeled with Cy3, followed by column purification (High Pure PCR Product Purification kit; Roche). The eluates were lyophilized and stored at −70°C. Duplicate arrays were hybridized for each tumor sample. For a subset of 4 tumor samples, there was sufficient RNA for comparisons to two normal ovarian reference RNAs. In these experiments, all RNAs were labeled using direct labeling. In one set of arrays, all 4 tumor RNAs and the Stratagene normal ovary RNA were labeled with Cy3, and the reference normal ovary RNA (Ambion) was labeled with Cy5.

Microarray Analysis Using a Normal Ovarian RNA Reference. In a second set of arrays, all tumor RNAs and the Stratagene normal ovary RNA were labeled with Cy3, and the reference RNA (Ambion) was labeled with Cy5 (dye switches) to account for possible labeling bias. Slides were scanned using either an Axon GenePix 4000A (Axon, Foster City, CA) or ScanArray 4000 scanner (Packard BioScience) dual laser scanner and quantified with GenePix Pro 3.0 software (Axon). Features that were not quantified because they were flagged bad or absent by GenePix Pro were manually reviewed for accuracy. Filtering, normalization of the raw data, and complete data analysis of the data sets were carried out with an algorithm developed in house that will be published elsewhere. Briefly, each of the 16 subgrids on each array was independently normalized by equalizing the Cy3 intensities, with respect to the Cy5 intensities, while excluding features flagged bad or absent by GenePix Pro.
Pro. Other features excluded by the algorithm included saturated spots and spots with foreground:background intensity ratio < 2. The Cy5:Cy3 normalized intensity ratio was determined for each spot, and these values from the duplicate spots within each array were averaged. Subsequently, Cy5:Cy3 ratios for the same spots between replicate experiments of each sample were averaged together as a single project file. Finally, the project files representing each sample and its replicates were combined into a single text file for processing by the Treeview software for hierarchical clustering, and the Treeview software was used for generating the graphical visualization of the clustering (39).11 Clustering was performed on genes showing expression values present in ≥80% of samples. For the first series of microarray experiments, we used median centering of the genes and tumors to emphasize differences between tumors rather than changes with respect to the standard comparator RNA (39, 40). The agglomerative hierarchical clustering algorithm used in the Cluster software successively joins gene expression profiles to form a dendrogram based on their pairwise similarities. The same procedure is followed when clustering by experiment, i.e., the similarity between each experiment is determined over the total number of genes as an average, and experiments with similar averages are grouped together (41). Two-dimensional hierarchical clustering firstorders genes and then reordered tumors based on similarities of gene expression between samples. The datasets used for hierarchical clustering generated by this study are available as tab-delimited format text files.12 Custom software was also developed to automate retrieval of chromosome localizations of microarray cdNAs from the UniGene database (at present, build 144). This allowed for arrangement of cdNAs into sequential order in megabases along each chromosome. The mapping distribution of the 1718 human cdNAs (1.7K2 and 1.7K3) used in this study, as well as the software, to carry out retrieval of chromosomal localizations are available.13 Confirmation of Microarray Results by Semi-quantitative RT-PCR. cdDNA was prepared from 20 ng of total RNA extracted from samples OCA17, OCA19, OCA21A, and OCA21B by reverse transcriptase-PCR using specific primers for the following genes using the Sigma One-Step RT-PCR kit (Sigma-Aldrich, St. Louis, MO): OPN (sense: 5′ ACAGACATCCTGGGACCACGAGA3′, antisense: 5′ CATCATTCAACTCTACCACCC3′); decorin: sense: 5′ CAGGTTATGTCTATTGGTGGCTGAC3′, antisense: 5′ CGTAAAGGAAAGGAGGAAGACC3′); and ribosomal 60S (sense: CGGCCTAAACAGGAGCCGAAAGT3′, antisense: 5′ ACCCGCGGTCTTTGTTTCC3′). RT-PCR reactions were also run in parallel with PCR primer pairs for the 18S rRNA (Ambion) as an internal standard. The following reaction/cycling conditions were used: 45 min at 55°C; 3 min at 94°C; 30 cycles of 45 s at 94°C, 30 s at 55°C, and 1 min at 72°C; and 5 min at 72°C. An aliquot of each reaction was run onto a 1% agarose gel, and the gel was stained with ethidium bromide. The intensities of each band were quantified using a Gel Documentation Alphalmager System (Alpha Innotech, San Leandro, CA) and were normalized using the intensities obtained for the 18S internal standard. The results are displayed in Fig. A.2,14 RESULTS Overall SKY Analysis of Ovarian Carcinoma Study Group. Examples of SKY analysis are shown in Fig. 1, and a detailed summary of both SKY breakpoint distribution and CGH imbalance profiles is presented using international system for human cytogenetic nomenclature as supplementary material to this article and in Fig. 2. Of the 292 breakpoints shown in Fig. 2, 229 (78.4%) could be mapped nomenclature as supplementary material to this article and in Fig. 2. Of the 292 breakpoints shown in Fig. 2, 229 (78.4%) could be mapped to infer the probable region of genomic imbalance. These data are 40 clonal.
Fig. 1. SKY and CGH analysis of ovarian tumors. A, SKY and CGH analysis of OCA13A. Shown are the results of SKY and CGH analysis of a primary endometrioid tumor. The SKY karyogram illustrates the pseudocolored classified colors enabling better identification of regions of chromosomal transition. Illustrated beside the karyogram is the corresponding CGH profile identifying net chromosomal gains and losses as indicated by green bars and red bars, respectively. B and C, SKY analysis of OCA21A and OCA21B, respectively. Shown are the SKY karyograms of endometrioid tumors derived from the same patient, one the primary (OCA21A) and the second the metastasis (OCA21B). In these karyotypes, both the classified and inverted DAPI banding images are shown. The panel below illustrates the RGB (Red-Green-Blue) and inverted DAPI banding images of the metaphases karyotyped above. D, integrated CGH and SKY analysis for chromosome 3. Shown are the tumor specimens possessing net genomic changes on chromosome 3 (denoted by ‘*’ and ‘**’) that correlate
Fig. 2. Distribution of SKY and CGH aberrations based on the analysis of nine ovarian carcinoma patients. SKY results are summarized where circles to the left of the ideogram indicate a site of clonal rearrangement. Circles to the right of the ideogram indicate a probable site of rearrangement based on limited banding information, and numbers in parentheses above the ideogram indicate the additional number of times that chromosome was involved in a clonal chromosomal rearrangement but where a breakpoint locus could not be readily identified. CGH results are summarized with color-coded bars denoting the histopathological subtype as described in the legend. Bars to the right of the ideogram (left ideogram of each pair) indicates a net gain of chromosomal material, whereas bars to the left of the ideogram indicates a net loss of chromosomal material. Because the paired samples (OCA13, OCA15, and OCA21) showed similar CGH results, these results were counted only once. “+” denotes the CGH changes that are concordant with the location of unbalanced structural chromosomal rearrangements identified by SKY analysis.

A. Profiles from 19(t;8;19) (?p11;q13), +der(19)(9pter→p13::19p13→q13::8?q11→q?), (33.3%), and 6p (25%). The most frequent losses were detected in chromosomes 4 (58.3%), 3p (41.6%), 6q (33.3%), and 18q (33.3%). The minimal common regions of chromosomal gains were identified at 1q21-q44, 3q13.3-q29, 6p21.2-p23, 7q32-q36, 8q23-q24.3, and 20p, whereas the minimal region of common loss was identified as 3p14-p26, 4q, 6q21-q27, and 18q21-q22. To determine an integrated overall chromosomal distribution of genomic rearrangements as identified by both SKY and CGH, the total number of aberrations per chromosome shown in Fig. 2 was enumerated, and 396 rearrangements were identified in the study group. When the distribution was normalized with respect to relative genomic length, chromosomes 3, 8, 11, 17, and 21 had the highest frequencies of structural and numerical aberrations identified by SKY and CGH. Analysis of these combined CGH and SKY data demonstrates that regions of chromosomal imbalance (as detected by CGH) were concordant with the locations of chromosomal breakpoints (as identified by SKY), particularly for chromosomes 3 and 8. Consistent losses distal to 3p21 and gains of 3q (detailed in Fig. 1D) in tumors OCA2, OCA3A, OCA15A/B, and OCA19 were observed, whereas tumors OCA3, OCA5, OCA19, and OCA21A/B all had both SKY and CGH aberrations of the 8q23-q24 regions (Fig. 1E).

Microarray Expression Analysis of Study Group. To determine whether patterns of gene expression could be associated with the chromosomal features associated with the study group and to explore expression profiling of the tumor samples, two series of microarray experiments were carried out. At the time we began this study, normal ovary RNA was not available to us (commercially or otherwise). Therefore, in a first series of experiments, 13 ovarian tumors were analyzed by microarray using a pool of RNA derived from nine cell lines as an internal standard. When used in conjunction with two-dimensional average linkage unsupervised clustering, this approach allows the determination of relative gene expression profiles in multiple samples and is commonly used in microarray studies (40, 43–45). The results of the two-dimensional hierarchical clustering for this first series of experiments is presented in Fig. 3A. Profiles from primary tumor and metastasis isolated from the same patient, irrespective of biopsy time and anatomical site, showed the greatest similarity and always clustered close together as shown previously (27, 40, 46–50), indicating the robustness of the clustering algorithm. Consistent grouping of poorly differentiated tumors within this tumor set was seen independent of histological subtypes. Surprisingly, OCA15A/B, also a poorly differentiated tumor, clustered with the to breakpoints as determined by SKY analysis. For each specimen, the classified SKY image is displayed for each chromosomal aberration that contributes to the net gain or loss of material from chromosome 3. E. Analyzed by microarray using a pool of RNA derived from nine cell lines as an internal standard. Therefore, in a first series of experiments, 13 ovarian tumors were analyzed by microarray using a pool of RNA derived from nine cell lines as an internal standard. When used in conjunction with two-dimensional average linkage unsupervised clustering, this approach allows the determination of relative gene expression profiles in multiple samples and is commonly used in microarray studies (40, 43–45). The results of the two-dimensional hierarchical clustering for this first series of experiments is presented in Fig. 3A. Profiles from primary tumor and metastasis isolated from the same patient, irrespective of biopsy time and anatomical site, showed the greatest similarity and always clustered close together as shown previously (27, 40, 46–50), indicating the robustness of the clustering algorithm. Consistent grouping of poorly differentiated tumors within this tumor set was seen independent of histological subtypes. Surprisingly, OCA15A/B, also a poorly differentiated tumor, clustered with the
well-differentiated tumors. In addition, the three tumor pairs of karyotypically related tumors (OCA13A/B, OCA15A/B, and OCA21A/B) exhibited similar profiles and each tumor pair clustered together.

In a second series of microarray experiments, we studied the expression profiles of a subset of 4 ovarian tumor samples OCA17, OCA19, OCA21A, and OCA21B, as well as 1 normal ovary RNA sample (Stratagene RNA). We added to this subset of tumor samples a normal ovary RNA sample to assess variability between normal ovary RNA samples from different individuals. For these 5 samples, we used normal ovary RNA from a second commercial source (Ambion RNA) as a common reference, which allowed us to explore gene expression differences at the gene level between normal ovarian tissue and ovarian tumor tissue and to attempt a better correlation with the SKY and CGH results. Two-dimensional hierarchical clustering of the
The microarray data showed that, as observed in the first series of microarray experiments, profiles from primary tumor and metastasis (OCA21A and OCA21B) obtained from the same patient showed the greatest similarity. Sample OCA19 showed an expression profile closely related to the one of OCA21A/B, similarly to what was observed when a pool of cell lines was used as a reference. Taken together, these results provide additional validation of the approach of using a pool of cell lines as an internal standard when studying global gene expression differences within a set of biological samples. Sample OCA17, which was not included in the first series of microarray experiments, shows a profile of expression slightly different from the 3 other tumor samples. As expected, the Stratagene normal ovary sample showed a gene expression profile very distinct from the 4 tumor samples and showed the least changes of gene expression as compared with the Ambion normal ovary RNA used as a reference. This result is illustrated in Fig. 4.

Two main clusters of gene expression were observed, designated I and II (Fig. 3B). A total of 194 genes showed expression levels that changed by more than a factor of two across the 4 tumor samples with 93 genes showing up-regulation and 101 genes showing down-regulation. We are currently exploring a number of these differentially expressed genes and chose to focus on a subset in the present publication.

Among the genes that showed up-regulation in the tumor samples (cluster I) was the gene encoding the OPN precursor, which showed a 53-fold increase in expression in sample OCA17 and a 2-fold increase in the other tumor samples. Five genes encoding metallothionein showed up-regulation (average ratios between tumor samples between 7.01 for MT-II and 2.87 for MT-IH), as well as gene encoding for the VEGF receptor 2 precursor (average ratio, 3.17), caspase-8 precursor (average ratio, 3.13), MYB-related protein (average ratio, 2.57), and six HLA class II histocompatibility antigens (average ratios between 3.66 and 2.23). Among the genes that showed down-regulation in the tumor samples (cluster II), there were 19 transcripts encoding 60S and 40S ribosomal proteins (average ratios between 0.49 and 0.23), IGFBP-3 (average ratio, 0.34), IGFBP-4 precursor (average ratio of 0.28), tumor necrosis factor α-induced protein 1 (average ratio, 0.26), G2/mitotic specific cyclin 2 (average ratio, 0.25), and decorin (average ratio, 0.15). The expression level of a subset of the differentially expressed genes was analyzed by semi-quantitative RT-PCR. For OPN, decorin, and the ribosomal 60S L19 gene, the levels of overexpression or underexpression relative to a normal ovary were consistent with the levels determined by microarray expression analysis.

Microarray Expression Analysis of cDNAs Mapping to Chromosomes 3 and 8. Because both the literature and our parallel SKY and CGH analysis have identified elevated frequencies of rearrangement and imbalance on chromosomes 3 and 8, we performed a detailed analysis of the results of ovarian cancer expression levels relative to a normal ovary for serous-derived tumors OCA17, OCA19, and OCA21A. A total of 94 and 51 targets represented genes with specific Blast and UniGene chromosomal band assignments on chromosomes 3 and 8, respectively, on the 1.7K3 cDNA array (Table 3). There was a contiguous distribution of reduced expression of seven genes mapping to 3p25.5–3p21.3 interval and increased expression of four genes from 3q13.33–3q28. These expression data were consistent with the observed pattern of CGH loss in distal 3p and gain in 3q observed both in this study and in the literature. Similarly, CGH data have consistently showed frequent gain and amplification in the 8q23–24 region in ovarian cancer, and the Exostosin Type I gene mapping to 8q23 exhibited increased expression in all three tumors.

DISCUSSION

This is the first cytogenetic study of short-term cultured ovarian chromosomal preparations using SKY and CGH methods (Table A). The combined findings of this study (Fig. 2; Table 2) identified chromosomes 3, 8, 11, 17, and 21 as having the highest frequencies of structural and numerical aberrations identified by SKY and CGH. More evident were concordant CGH and SKY aberrations leading to losses distal to 3p21 and gains of 3q and of the region 8q23-24 arising as a consequence of unbalanced structural rearrangements. In addition, other regions of rearrangement on chromosomes 1, 7, 12, 13, and 19 previously identified by G-banded chromosomal analysis (12, 13) were also noted. One-third more structural chromosomal aberrations were identified in the endometrioid tumors than the serous tumors. The poorly differentiated tumors, irrespective of histological subtype, showed twice as many chromosomal aberrations as the more well-differentiated tumors.

Previous conventional cytogenetic analysis has identified frequent breakage at 1p1, 1q1, 1p2, 1q2, 1p3, 1q3, 3p1, 1q4, 6q1, 6p2, 6q2, 7p1, 7p2, 11p1, 11q2, 12p1, 12q2, 13p1, and 19q (12). Similarly, loss of heterozygosity studies by Launonen et al. (51) have identified an association between loss of sequences at 3p14.2, 11p15.5, 11q23.2-q24, 16q24.3, and 17p13.1 and adverse outcome. The resulting loss of heterozygosity in these and other regions can result from simple deletions or deletions as a result of translocations. Because SKY can identify cryptic translocations and poorly resolved “marker chromosomes” with greater certainty, a much more accurate picture of overall cytogenetic change is possible. Many chromosomal translocations were centromeric or pericentromeric, suggesting the involvement of repetitive centromeric sequences or Alu repeats that may facilitate rearrangements leading to chromosomal imbalance (52). Chromosomal breakage at 1p1 and 3q4 occur in ovarian cancer (5, 7, 11) and confers a poor outcome (13).

15 Table A to be available as supplementary on the web edition. Full description of SKY and CGH results.

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Ovarian cancer often presents in more than one site or has metastasized at the time of surgery. We studied the chromosomal and gene expression patterns in tumors arising in two sites to determine their common origin. In three instances, two separate tumors were available for simultaneous analysis of patterns of gene expression and karyotypes. In all three pairs, there was a marked similarity in the chromosomal constitution of the tumors and each tumor pair clustered together on the dendrogram obtained with microarray analysis. One tumor pair presented an interesting opportunity to determine cytogenetic changes during metastasis. OCA21 possessed a tetraploid primary tumor and a diploid karyotype in the metastatic tumor, suggesting that either a reduction in chromosomal ploidy occurred in the primary tumor before metastasis or that the reduction occurred at the metastatic site. The cytogenetic description of the metastatic biopsy is similar to components of structural alterations observed in the primary tumor, suggesting that a selection of a dominant clone with specific structural abnormalities arose in the metastatic tumor.

The minimal regions of chromosomal imbalance identified by CGH were gains at 1q21-q44, 3q13.3-q29, 6p21.2-p23, 7q22-q36, and 8q23-24.3 and losses at 3p14-p26, 4q, 6q21-q27, and 18q12-q27. Previous CGH studies (17–24) have identified a similar pattern of genomic change, and attempts have been made to correlate these data with clinical endometrioid tumors. The high percentage of chromosome 4 involvement in advanced staged tumors (18). The expression clustering of genes/pathways may elucidate those critical genes that promote both a more aggressive disease course and worse outcome and may provide a marker for treatment resistance. The first series of microarray experiments described here consisted of analyzing 13 tumor samples using a pool of nine cell lines as a common reference. Two-dimensional hierarchical clustering identified a grouping of predominantly poorly differentiated subtypes (OCA13A/B, OCA19, and OCA21A/B), which were independent of histological grouping. The only discrepancy was the tumor OCA15 that failed to cluster with the other poorly differentiated tumors in the study group. It is possible that histological heterogeneity or the lower quantity of RNA obtained for this particular tumor sample resulted in this grouping by obtaining clustered analysis.

The second series of microarray experiments carried out on a subset of tumor samples and using normal ovarian tissue as a common reference identified 194 genes differentially expressed at a level of at least 2 (up or down) between tumor samples and the reference normal sample. Two-dimensional hierarchical clustering and statistical analysis revealed a very similar expression profile for all 4 tumor samples, with only 13% of genes showing a SD > 1 across all tumor samples, consistent with their similar serous histology (OCA17, OCA19, and OCA21A/B). As expected, the normal ovary sample used as a negative control displayed very few significantly differentially expressed genes when compared with the normal reference (Fig. 4).

One of the objectives of this study was to identify correlations between gene expression deviations observed by microarray analysis and chromosomal imbalances identified by CGH. Because chromosomes 3 and 8 have been previously shown to be subject to rearrangement and copy number change (12–14) and were found to be commonly involved in rearrangements by SKY and/or CGH, we performed a more detailed expression evaluation of cDNA mapping to these chromosomes. We identified a contiguous distribution of reduced expression of seven genes mapping to 3p25-3p22 and increased expression of four genes from 3q13.33–3q28 that were consistent with loss of 3p and gain of 3q in this and other CGH studies of ovarian cancer (17–25). Within the 3q13.33–3q28 interval, it is noteworthy that there was elevated expression of the calcium-sensing receptor (55) that has been previously shown to induce human ovarian surface epithelial cells to modulate extracellular calcium and induce proliferation.

Two-dimensional hierarchical clustering revealed two large clusters of expression profiles (I and II, Fig. 3B). The first cluster consists of up-regulated genes and most striking is the very high value (ratio of 53) obtained for the OPN precursor in sample OCA17. This gene is also overexpressed in the other tumor samples but to a much lesser
extent. OPN is a noncollagenous bone-related protein that has been detected by immunohistochemical and in situ hybridization in calcified psammona bodies, which frequently occur in ovarian serous papillary tumors (56). This protein may be causally related with the calcification of the psammona bodies of the ovarian serous papillary tumors such as OCA17. Five genes encoding metallocitonein are also up-regulated in all 4 tumor samples. Metallocitonein is a key component of platinum resistance in epithelial ovarian cancer and is thought to be of prognostic significance (57).

Cluster I also contains five genes encoding HLA class II histocompatibility antigens. All five genes are located in the 6p region, which was involved in genomic imbalance and structural rearrangement, so it was of interest that MHC class II genes of the leukocyte antigen (HLA) complex emerges as a strong cluster of expression. This observation is in agreement with a recent high-density filter array analysis of ovarian cancer, which also reported differential expression of immune response mediators (58). Previously, an association between the T-cell response and alterations to MHC expression has been observed in ovarian cancer (59, 60). However, it is conceivable that infiltrating lymphocyte may also contribute to overexpression of immune response genes.

The VEGF receptor 2 precursor is up-regulated in all tumor samples, and this is consistent with a previous cDNA microarray study (58) on four poorly differentiated serous papillary tumors that showed an overall increase in angiogenesis-related markers, including VEGF.

Among the down-regulated genes, we identified 19 genes encoding ribosomal proteins. Ribosomal proteins have been found to be up-regulated in some tumor tissues (61). However, a recent study (62) using a data mining tool called Digital Differential Display has shown that distinct ribosomal proteins were found to be up-regulated in breast and prostate carcinoma-derived libraries, whereas others such as L9, L23, and L37A were down-regulated in ovarian cancer-derived libraries. Our present findings are consistent with these results. IGFBP-3 precursor showed down-regulation in all tumor samples as observed by others (63). Finally, the gene encoding a decorin precursor showed the highest level of down-regulation. Decorin is a small proteoglycan protein, which is part of the cellular matrix and has been shown to inhibit the growth of two ovarian cell lines in vitro (64). Decorin inhibits transforming growth factor β and induces p21, resulting in inhibition of proliferation (65).

A marked decrease in the endogenous level of decorin precursor might therefore result in an increase in cell proliferation in tumor cells.

A more complete cytogenetic and microarray analysis of a larger collection of ovarian tumor and normal samples and using cDNA microarrays spotted with 19,200 genes and ETSs is under way in our laboratories that will allow us to uncover additional differentially expressed genes and to further explore the ones identified in the present study, as well as additional regions of chromosomal instability associated with ovarian cancer. In this study, we performed a comparative analysis using SKY. CGH, and microarrays on a small cohort of ovarian carcinomas. We showed that chromosomes 3, 8, 11, 17, and 21 had the highest overall frequencies of structural and numerical aberrations and losses distal to 3p21, and consistent gains of 3q were associated with a pattern of reduced expression of genes mapping to 3p25.5–3p21.3 and increased expression of genes from 3q13.33–3q28. Thus, although there is inherently limited resolution of all cytogenetically based techniques, we have shown that a parallel strategy of genomic and expression analysis can facilitate the identification of smaller subsets of genes pertinent to ovarian cancer.

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REFERENCES

Parallel Analysis of Sporadic Primary Ovarian Carcinomas by Spectral Karyotyping, Comparative Genomic Hybridization, and Expression Microarrays

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