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Genetic Analysis of Early- *versus* Late-Stage Ovarian Tumors[1]

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ABSTRACT

In the United States, ovarian cancer is the fourth most common cause of cancer-related deaths among women. The most important prognostic factor for this cancer is tumor stage, or extent of disease at diagnosis. Although women with low-stage tumors have a relatively good prognosis, most women diagnosed with late-stage disease eventually succumb to their cancer. In an attempt to understand early events in ovarian carcinogenesis, and to explore steps in its progression, we have applied multiple molecular genetic techniques to the analysis of 21 early-stage (stage I/II) and 17 advanced-stage (stage III/IV) ovarian tumors. These techniques included expression profiling with cDNA microarrays containing approximately 18,000 expressed sequences, and comparative genomic hybridization to address the chromosomal locations of copy number gains as well as losses. Results from the analysis indicate that early-stage ovarian cancers exhibit profound alterations in gene expression, many of which are similar to those identified in late-stage tumors. However, differences observed at the genomic level suggest differences between the early- and late-stage tumors and provide support for a progression model for ovarian cancer development.

INTRODUCTION

Of the cancers unique to women, ovarian cancer has the highest mortality rate. Over 26,000 women are diagnosed with this disease in the United States annually, and 60% of those diagnosed will die of the disease (1). There has been little change in ovarian cancer incidence and mortality over the past 5 decades and unfortunately there are a number of significant barriers to progress in its treatment. These include poor understanding of the underlying biology of this disease, inadequate screening tools as well as few early warning signs. The 5-year survival for patients with stage I disease can exceed 90%, but it is less than 25% for advanced-stage disease (2). These statistics underscore the need for better tools for the screening and staging of ovarian cancer.

Like other solid tumors, ovarian cancer is thought to result from an accumulation of genetic alterations. These events lead to changes in expression of many genes. Alterations in tumor suppressor genes such as *p53* (3, 4), *pRB* (3, 5), *NOEY2* (6), *BRCA1* (7), and oncogenes such as *K-ras* (8), *c-myc* (9) and *c-erbB-2* (10) have been shown to play an important role in ovarian carcinogenesis. There is very little information, however, on the sequence of genetic changes that are associated with progression of disease from early to advanced stage.

Examining tumors for alterations in gene expression is a potentially useful approach to identifying molecular differences between early- and late-stage ovarian carcinomas. Although there are several approaches to investigate differential gene expression in tumors, here we have used cDNA microarrays to develop expression profiles of early- and late-stage ovarian cancer (11–13). To extend our understanding of any stepwise genetic alterations that may underlie the assumed clinical progression from early- to late-stage ovarian cancer, CGH[3] (14) was used to identify regions of genome loss and gain in the same series of tumors.

MATERIALS AND METHODS

Tissue Processing and Tumor Selection. Surgically removed ovarian tumors were snap-frozen in the surgical pathology unit of the Mayo Clinic. The tumor content of the specimens was assessed by H&E-stained sections. Only high-grade specimens containing more than 75% tumor were used for these experiments. Twenty normal OSEs from patients without cancer were used as normal controls. The epithelial nature of these brushings was verified by cytokeratin staining. The majority of these patients were between 45 and 65 years old, undergoing incidental oophorectomy at the time of pelvic surgery for other indications. All of the ovaries were examined pathologically and found to be benign or they were excluded. RNA from five such pooled brushings were profiled on cDNA microarrays along with five stage I, two stage II, and seven stage III ovarian tumors. The histology, grade, and stage of each tumor used in cDNA microarray, semiquantitative RT-PCR, and CGH studies are listed in Tables 1 and 5, respectively. Tumors were staged according to International Federation of Gynecology and Obstetrics criteria.

Ovarian Cell Lines. Five of seven ovarian-carcinoma cell lines (OV 167, OV 177, OV 202, OV 207, and OV 266) were low-passage primary lines established at the Mayo Clinic (15). OVCAR-5 is a NIH cell line (16) and SKOV-3 was purchased from American Type Culture Collection (Manassas, VA). All of the cells were grown according to the provider's recommendations.

cDNA Microarrays. The cDNA microarray consisted of 25,000 elements from the Unigene set from Research Genetics Inc. (Huntsville, AL), which included 10,000 known genes; 13,000 ESTs; and 2,000 elements made up of control genes, dyes, bacterial genes, and water controls. After consolidating genes or ESTs belonging to the same Unigene clusters, we estimated that there were a total of 18,304 unique genes or ESTs. All of the genes and ESTs on the array were sequence verified before being arrayed on a high-precision robotics platform. Each 3- × 5-inch membrane contained up to 6,000 elements and each probe was hybridized to five sets of membranes in duplicate.

Total RNA Isolation and Labeling. Total RNA was extracted from specific tumor samples using Trizol (Life Technologies, Inc., Rockville, MD) as recommended by the manufacturer after estimating the tumor content by standard histological methods. The integrity of the RNA was assessed by ethidium bromide staining after agarose gel electrophoresis. Total RNA was labeled with [α-^{33}P]CTP after reverse transcription with oligo(dT)$_{30}$. Fifteen μg of total RNA was annealed to 3 μg of oligo-dT$_{30}$ primer in 13 μl of total volume and incubated at 70°C for 2 min in a PCR machine and held at 4°C.

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[3] The abbreviations used are: CGH, comparative genomic hybridization; OSE, ovarian epithelial cell brushing; EST, expressed sequence tag; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table 1 *Tumor cohort*

Histology	Stage	Grade	cDNA microarray	RT-PCR
Cl cell OV 106	I	3	+[b]	+
Cl cell OV 267	I	3		+
Cl cell OV 496	I	3	+	+
Endo OV 51	I	3		+
Endo OV 78	I	3		+
Endo OV 88	I	3		+
Endo OV 105	I	3		+
Endo OV 338	I	3		+
Endo OV 647	I	3	+	+
Serous OV 6	I	3		+
Serous OV 17	I	3	+	+
Serous OV 20	I	3		+
Serous OV 90	I	3		+
Serous OV 234	I	3		+
Serous OV 363	I	3		+
Serous OV 526	I	3	+	+
Cl cell OV 102	II	3		+
Endo OV 296	II	3		+
Serous OV 149	II	3		+
Serous OV 354	II	3		+
Serous OV 401	II	3	+	+
Serous OV 402	II	3	+	+
Serous OV 414	II	3		+
Cl cell OV 176	III	3		+
Endo OV 93	III	3		+
Endo OV 110	III	3		+
Endo OV 259	III	3		+
Serous OV 4	III	3	+	+
Serous OV 11	III	3	+	+
Serous OV 13	III	3	+	+
Serous OV 16	III	3	+	+
Serous OV 29	III	3	+	+
Serous OV 150	III	3	+	+
Serous OV 167	III	3		+
Serous OV 206	III	3		+
Serous OV 208	III	3		+
Serous OV 461	III	3		+
Serous OV 472	III	3	+	+
Serous OV 97	IV	3		+

[a] Cl, clear; OV, ovarian; Endo, endometrioid.
[b] +, the tumors used in each analysis.

Reverse transcription of this annealed template was carried out in a final volume of 50 μl, 1× first strand buffer (DTT, 40 units of Rnase inhibitor, 400 units of Superscript II reverse transcriptase, 2 μl of 10 mM dNTP mix with dCTP at 0.1 mM concentration, 100 μCi of [α-^{33}P] dCTP at 42°C for 60 min. The reaction was stopped by consecutive addition of the following reagents: 4 μl of 50 mM EDTA, 4 μl of 0.5 M NaOH, and 2 μl of diethylpyrocarbonate water. Heating at 65°C for 10 min degrades the RNA template in the reaction. Unincorporated nucleotides were removed from the labeled probe using a Clontech (Palo Alto, CA) Chroma Spin TE-30 spin column. The labeled probe with an estimated specific activity of 1×10^6 cpm/μl was preannealed to 100 mg of COT1 DNA to block repetitive sequences in a final volume of 100 μl. The blocked probe was heated for 5 min at 95°C followed by 65°C for 20 min and then added to the duplicate filters along with 5 μg/ml of denatured salmon sperm DNA. The filters were hybridized overnight at 65°C in a Hybaid hybridization oven. Each probe was hybridized to duplicate filters. The next day, the filters were washed for 15 min at 65°C with 4% SDS solution in 20 mM sodium phosphate (pH 7.2) with 1 mM EDTA followed by 1% SDS solution in the same buffer with three changes at 65°C. After the wash, the filters were dried between whatman papers and baked at 80°C for 1 h. The filters were imaged on a storm 840 phosphoimager after 24–48 h exposure.

Image Analysis and Data Recovery. The ArrayVision program (Imaging Research Inc., St. Catharines, ON, Canada) was used to quantify the spot intensities of the scanned images for each membrane. For each membrane, the spot intensities were normalized by the median intensity for the entire array, resulting in a new median intensity of 1.0. Spots that were <0.1 after normalization were thresholded to 0.1 to account for the level of background noise. After normalization and thresholding, spot intensities were averaged across the duplicate filters for each tissue sample. Fold-regulation for each tumor, at each gene, was determined by calculating the ratio of tumor expression:average expression for the five (pooled) control normal OSEs.

Semiquantitative RT-PCR. Reverse transcribed cDNAs (50–100 ng) were used in a multiplex reaction with gene-specific primers and GAPDH, forward primer (5′-ACCACAGTCCATGCCATCAC-3′) and reverse primer (5′-TCCACCACCCTGTTGCTTGTA-3′) yielding a 450-bp product. The PCR reaction mixtures consist of 50 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 400 μM concentration of each primer for the specific gene to be analyzed (50 μM for the GAPDH primers), and 0.5 units of Taq polymerase (Promega, Madison, WI), in a 12.5-μl reaction volume. The conditions for amplification were as follows: 94°C for 3 min, then 29 cycles of 94°C for 30 s, 50–62°C for

Table 2 *Differential gene expression in ovarian tumors: number of sequences with expression changes >2-fold, 5-fold, 10-fold, and 20-fold*

No. of tumors	Down-regulated				Up-regulated			
	2-fold	5-fold	10-fold	20-fold	2-fold	5-fold	10-fold	20-fold
14	148	24	9	2	11	1	0	0
13	262	48	16	6	30	6	1	0
12	409	56	19	6	43	9	2	1
11	565	68	21	7	67	11	5	1
10	719	87	24	8	108	12	5	1
9	945	108	27	11	168	15	6	1
8	1261	135	34	12	265	25	8	1
7	1750	179	46	12	435	36	12	3
6	2346	248	56	18	794	51	18	5
5	3115	409	115	42	1417	72	27	7
4	4159	565	144	52	2510	116	32	12
3	5718	781	187	65	4303	198	51	25
2	8245	1159	279	81	7051	388	105	42
1	12106	2940	1044	414	11178	1552	480	183

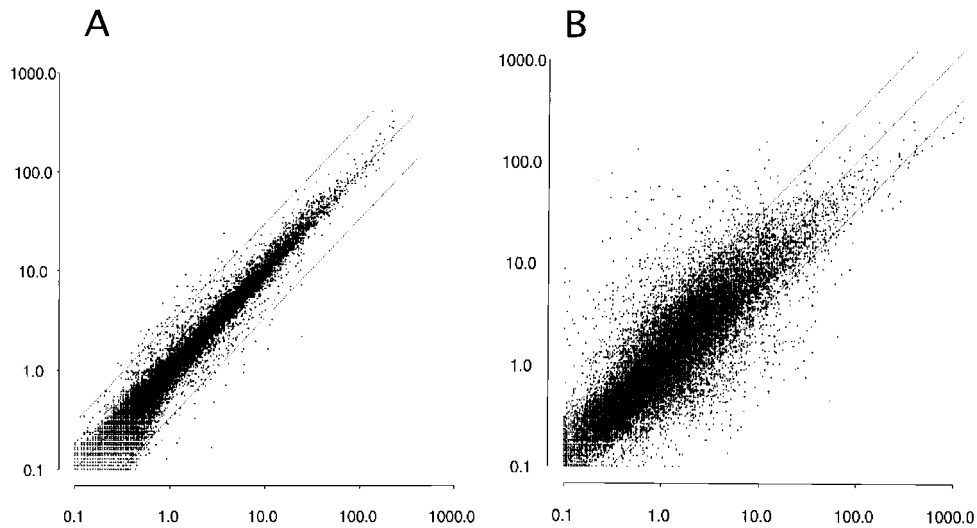

Fig. 1. Representative scatter plots of cDNA microarray analysis. *A*, duplicate filters of OV 106 hybridization (Pearson correlation, 0.97). *B*, scatter plot of an early-stage tumor (OV106) *versus* one of the pooled normal epithelial brushings (Pearson correlation coefficient, 0.76). The *lines*, 2-fold differences in expression levels between samples.

Table 3 *Differential gene expression as a function of tumor stage*

	Stage I/II down				Stage III/IV down				Stage I/II up				Stage III/IV up			
No. of tumors	2-fold	5-fold	10-fold	20-fold	2-fold	5-fold	10-fold	20-fold	2-fold	5-fold	10-fold	20-fold	2-fold	5-fold	10-fold	20-fold
7	200	35	11	2	406	56	18	6	19	2	0	0	48	10	3	0
6	413	62	19	6	739	91	22	9	64	8	2	1	99	12	4	1
5	759	86	26	8	1393	154	36	11	185	19	7	1	239	16	5	1
4	1322	152	38	13	2360	321	99	42	498	39	15	3	714	27	8	2
3	2500	275	65	16	3365	508	127	47	1311	92	36	9	1615	65	17	8

30 s depending on the gene-specific primers being tested, and 72°C for 30 s in a Perkin-Elmer-Cetus (Norwalk, CT) 9600 Gene-Amp PCR system. The products of the reactions were resolved on a 1.6% agarose gel. Band intensities were quantified using the Gel Doc 1000 photo-documentation system (Bio-Rad, Hercules, CA) and its associated software. Gene specific primers were as follows: for *Gas 1*, forward, CGC GCC TCG TCT CCT TTC CC, and reverse, GGC GCG TGG GCT AAA AGA GC; for *PAI1*, forward AAT CGC AAG GCA CCT CTG AG, and reverse, GAT CTG GTT TAC CAT CTT TT; for *StAR*, forward, GAC CCC ACC ACT GCC ACA TT, and reverse, GAT CTT AGA CTT GCA GGC TT; for *AREG*, forward, CCG CTG CGA AGG ACC AAT GA, reverse, CTA TGA CTT GGC AGT CAG TC; for *FGF7*, forward, TAA TGC ACA AAT GGA TAC, and reverse, ATT GCC ATA GGA AGA AAG; for *HPR6*, forward, TGC CTA GCG CGG CCC AAC, and reverse, CAG ACT GGA CTG TTA CAA ATG; for *ITM2A*, forward, CGC AGC CCG AAG ATT CAC TAT G, and reverse, R-CTT ATT ACC AAG GAC ACT CTA TCT; and for *decorin*, forward, CCT GGT TGT GAA AAT ACA TGA, and reverse, TGA CAT TAA CAA GAT TTT GCC.

CGH. Metaphase spreads from normal human lymphocytes were prepared using standard protocols (17). The slides were aged for 2–3 days prior to denaturation at 70°C by 70% formamide in 2× SSC, followed by dehydration in a series of ethanol-water mixtures. The slides were treated with proteinase K, at a concentration of 0.1 μg/ml in 20 mM Tris (pH 7.5)-2 mM $CaCl_2$, prior to hybridization. The CGH procedure was similar to published standard protocols (14, 17). Twenty images were captured using a Nikon Labophot-2

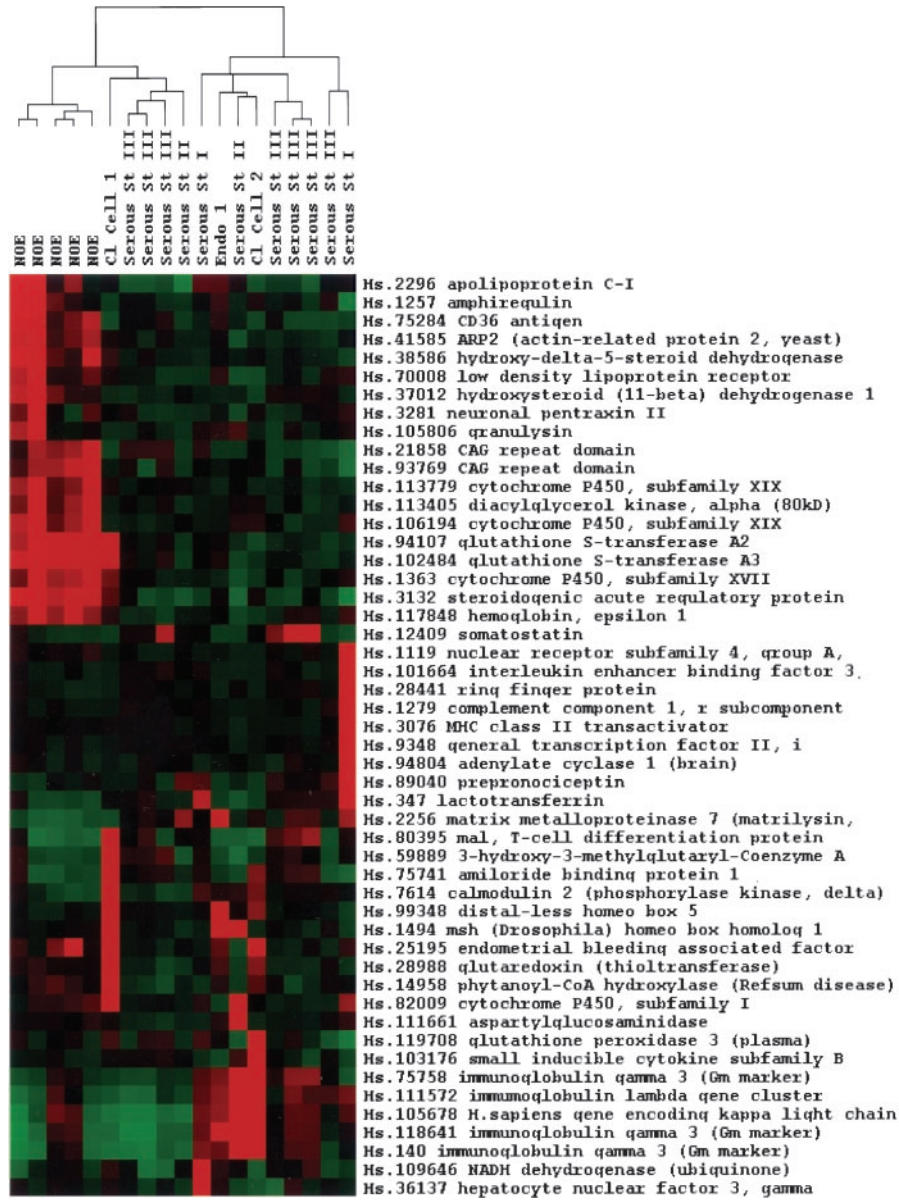

Fig. 2. Hierarchical clustering of 19 ovarian epithelial samples, using Eisen's Stanford clustering package. Eighteen thousand expressed sequences were profiled against five normal ovarian epithelial (NOE), two early-stage clear cell, one early-stage endometrioid, four early-stage serous, and seven late-stage (stage-III) serous samples. The 50 annotated clones with maximal normalized expression variance (SD divided by mean of expression) are shown. Data were log-transformed after addition of a baseline noise compensation term and were clustered using centered Pearson correlation. Whereas the normal samples neatly cluster together, separation between early- and late-stage samples is less compelling.

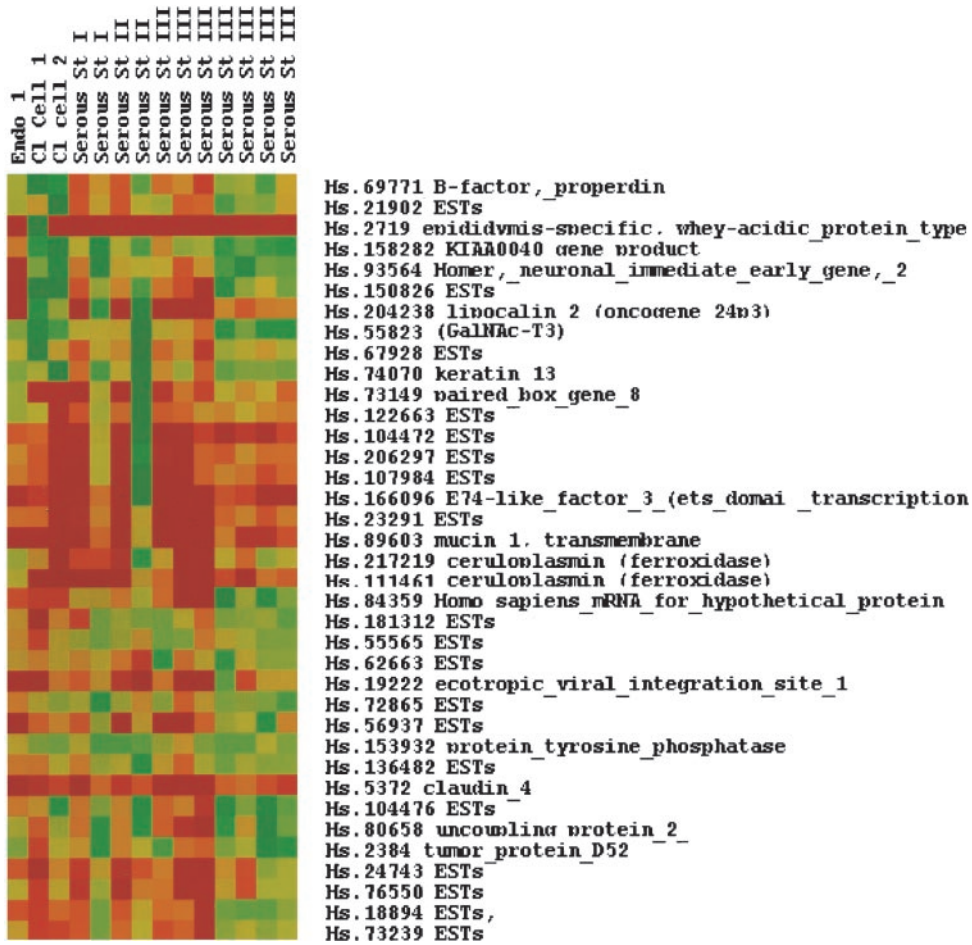

Fig. 3. Hierarchical clustering of 14 ovarian tumor samples, using Eisen's Stanford clustering package. Data were log-transformed and clustered by centered Pearson correlation. Cluster tree analysis of five times or more up-regulated genes in early- and late-stage tumors. *Red*, fold up-regulation of five times or more. *Green boxes*, fold regulation between 1 and 2. *Different shades of green*, values above 2 but less than 3.

microscope equipped with an automatic filter wheel and an 83,000 filter set (Chroma, Brattleboro, VT) with single band pass exciter filters for UV/FITC (490 nm), 4′,6-diamidino-2-phenylindole (360 nm), and rhodamine (570 nm), and were analyzed using the QUIPS CGH software version 3.12 (Vysis Inc., IL). Using this software, the ratio of rhodamine:FITC signal is expressed as a red:green ratio, with deviations from a 1:1 ratio indicative of gain or loss of chromosome material. The lower and upper limits for gain and loss were established by performing control CGH experiments with a well-characterized tumor cell line, IMR32 (18), and DNA derived from male or female normal tissue. On the basis of these findings, a 95% confidence interval for gain and loss was set at 1.20 and 0.80, respectively, with gene amplification defined as gain >1.5.

Cluster Analyses. Hierarchical cluster analyses were performed using the Cluster software package (19). Genes were clustered using the Pearson correlation coefficient as the distance metric, and clusters were agglomerated using the average linkage criterion. Graphical displays of our cluster analyses were obtained using the TreeView program (19).

RESULTS

Gene Expression Patterns of Early-Stage (I/II) and Late-Stage (III/IV) Tumors. We examined gene expression in seven early- and seven late-stage ovarian tumors (Table 1) using cDNA microarray filters containing approximately 25,000 members of the Unigene set and control probes. Each tumor was examined in duplicate, with results indicating excellent reproducibility (Pearson correlation, >0.97 in all cases). The overall degree of similarity in gene expression between duplicate microarray experiments and among different tissue specimens was assessed using scatter plots. A representative scatter plot for two duplicate filters is shown in Fig. 1*A* (Pearson correlation, 0.97), and Fig. 1*B* displays the scatter plot of an early-stage tumor (OV106) *versus* one of the pooled normal epithelial brushings (Pearson correlation coefficient, 0.76).

Analysis of the Differential Expression in Early- and Late-Stage Ovarian Tumors. Table 2 displays the number of genes for which we found varying levels of differential expression relative to normal ovarian epithelial cell brushings. Generally speaking, more genes were determined to be down-regulated than up-regulated. For example, 46 genes are at least 10-fold down-regulated in at least seven of the tumors that were expression-profiled. The number of genes that are 10-fold up-regulated in at least seven tumors is only 12. A similar trend was evident when the tumors were grouped by stage (Table 3). Heirarchical clustering for a set of genes selected for maximal variance (without prejudice toward differential expression) are shown in Fig. 2. The dendrogram in Fig. 2 shows the tight clustering of normal samples, whereas separation between early- and late-stage samples is far less compelling. Figs. 3 and 4 display heirarchical clustering for a set of genes that are $\geq$5-fold up- and down-regulated in a significant number of early- and late-stage tumors, respectively, relative to the pooled epithelial cell brushings. It is evident from these analyses that several of the genes are aberrantly regulated to the same extent in both early- and late-stage tumors.

To assess the extent to which genes in specific functional categories are differentially expressed in both early- and late-stage tumors, expressed sequences showing five times the expression changes in a majority of early-and late-stage tumors were grouped in one of four categories: cell-cell interactions; intermediate filament markers; cell cycle and growth regulators; and genes involved in invasion and metastasis (Fig. 5). This analysis revealed that genes involved in cell-cell interactions such as *cadherin 11* (20), *cadherin 2* (21), and

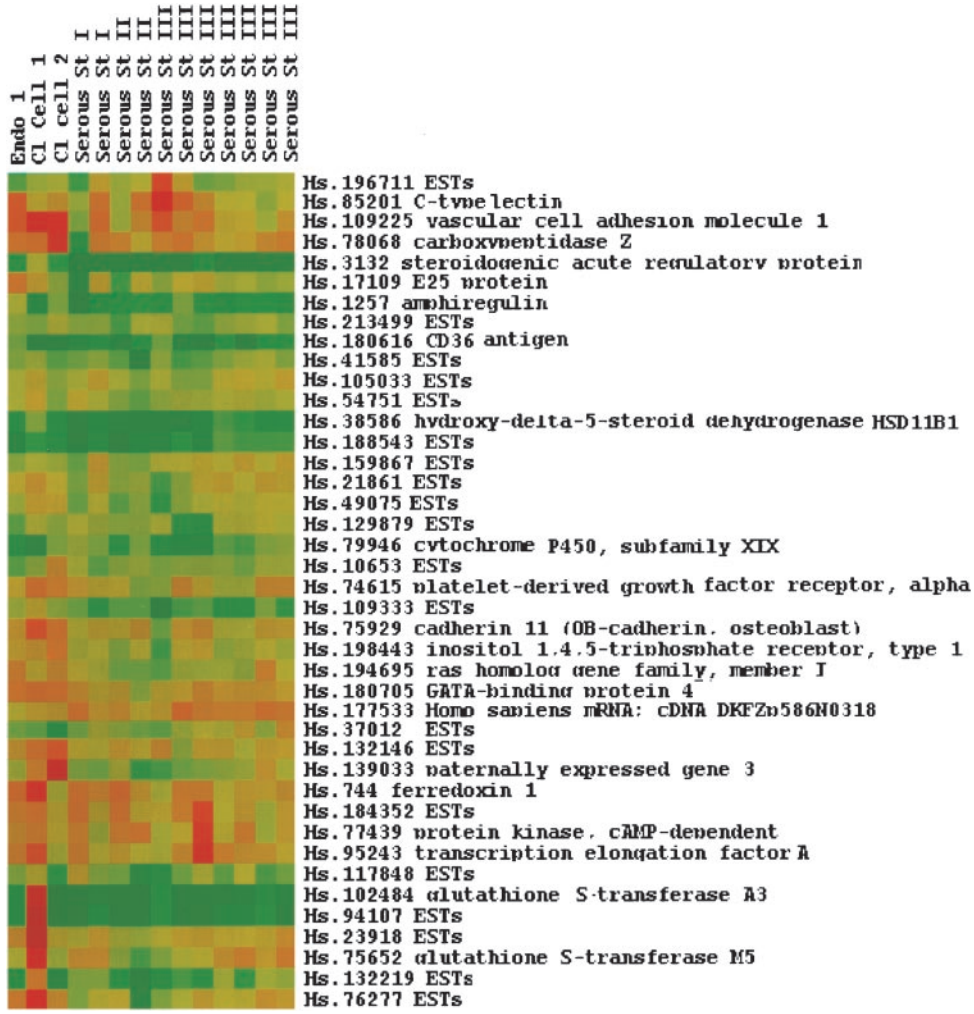

Fig. 4. Hierarchical clustering of 14 ovarian tumor samples, using Eisen's Stanford clustering package. Data were log-transformed and clustered by centered Pearson correlation. Cluster tree analysis of five times or more down-regulated genes in early- and late-stage tumors. *Green boxes*, fold down-regulation of five times or more. *Red boxes*, fold regulation between 1 and 2. *Different shades of red*, values above 2 but less than 3.

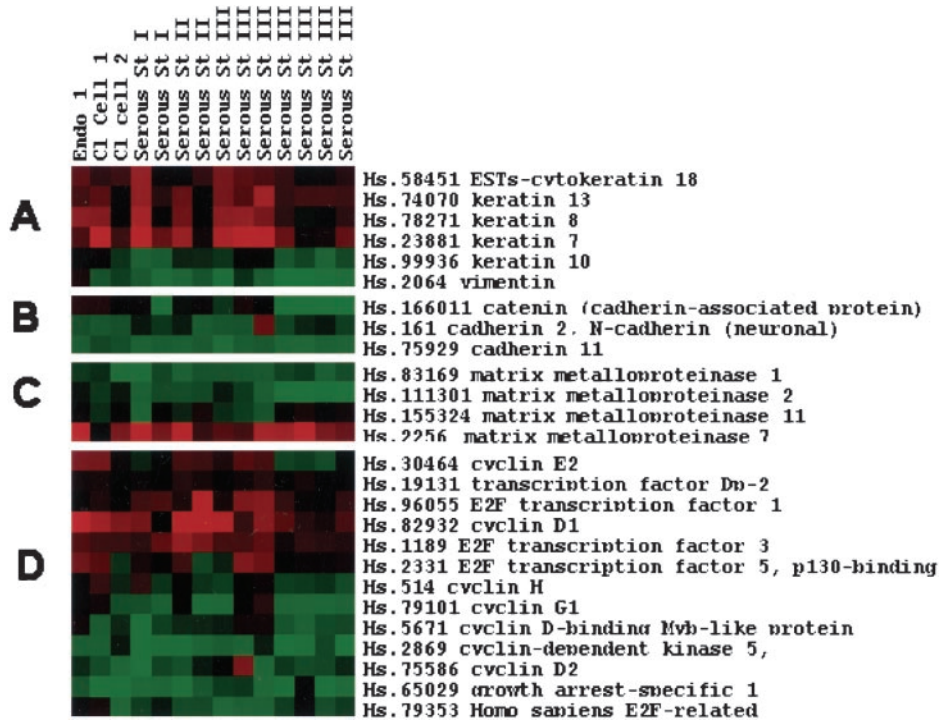

Fig. 5. Cluster tree analysis of gene categories. five times or more differentially regulated genes in early- and late-stage tumors. *Green boxes*, fold down-regulation of five times or more. *Red boxes*, fold up-regulation. *A*, intermediate filament genes; *B*, cell-cell interactions; *C*, cell invasion and metastasis; and *D*, cell cycle and growth regulators.

Table 4 *Genes with expression level changes >10-fold in both early- and late-stage tumors*

Cluster identification	Cluster title
10-fold down-regulated genes in early- and late-stage tumors	
Hs.[a]38586	*Hydroxy-δ-5-steroid dehydrogenase, 3 β- and steroid δ-isomerase 1*
Hs.213499	*ESTs*
Hs.49075	*Human DNA sequence from clone 22D12 on chromosome Xq21.1-21.33*
Hs.188543	*ESTs*
Hs.129879	*ESTs*
Hs.79946	*Cytochrome P-450, subfamily XIX (aromatization of androgens)*
Hs.180616	*CD36 antigen (collagen type I receptor, thrombospondin receptor)-like 1*
Hs.159867	*ESTs*
Hs.1275	*Amphiregulin (schwannoma-derived growth factor)*
Hs.41585	*ESTs*
Hs.94107	*ESTs*
Hs.3132	*Steroidogenic acute regulatory protein*
Hs.105033	*ESTs*
Hs.109333	*ESTs*
Hs.117848	*ESTs*
Hs.102488	*Glutathione S-transferase A3*
Hs.54751	*ESTs*
Hs.17109	*Integral membrane protein 2A*
Hs.21858	*CAG repeat containing (glia-derived nexin I α)*
Hs.54751	*ESTs*
Hs.9914	*Follistatin*
Hs.139033	*Paternally expressed gene 3*
Hs.105806	*Granulysin*
Hs.169228	*δ (Drosophila)-like 1*
Hs.37012	*Homo sapiens DNA sequence from PAC 434O14 on chromosome 1q32.3-41*
Hs.148493	*Cathepsin B*
10-fold up-regulated genes in early and late stage tumors	
Hs.5372	*Claudin 4*
Hs.2719	*Epididymis-specific, whey-acidic protein type, HE 4*
Hs.16696	*E74-like factor 3 (ets domain transcription factor)*
Hs.19222	*Ecotropic viral integration site 1*
Hs.896603	*Mucin 1, transmembrane*
Hs.24743	*ESTs*
Hs.111461	*Ceruloplasmin (ferroxidase)*
Hs.206297	*ESTs*
Hs.104472	*ESTs*
Hs.23291	*ESTs*
Hs.73149	*Paired box gene 8*
Hs.2256	*Matrix metalloproteinase 7 (matrilysin, uterine)*
Hs.204238	*Lipocalin 2 (oncogene 24p3)*
Hs.76550	*ESTs*
Hs.36451	*Glutamate receptor, ionotropic, N-methyl D-aspartate 2C*
Hs.155097	*Carbonic anhydrase II*

[a] Hs., unigene cluster IDs.

nidogen (*enactin*; Ref. 22) were all down-regulated in a majority of tumors, whereas genes involved in invasion and metastasis, including *matrilysin* (*MMP 7*; Refs. 23, 24), *gelatinase* (*MMP 9*; Ref. 25), *matrix metalloproteinase 10* and *12* (Ref. 23) were up-regulated in a majority of tumors. Genes belonging to the intermediate filament category, such as *vimentin* (26) and *keratin 10* (27) were down-regulated in both early- and late-stage tumors, whereas *keratins 8* (28), *13* (29), and *18* (28) were up-regulated in both stages. For the cell cycle/growth regulators, *cyclin D2* (30, 31), *cyclin dependent kinase 5*, *growth arrest-specific 1* (32), and the *E2F*-related transcription factor *DP1* (33) were all down-regulated. Other genes in this category, such as *cyclin E2* (34), proliferating cell nuclear antigen (35), and transcription factor *E2F5* (36), were up-regulated in both early and late-stage tumors.

The genes with an average-fold change of at least 10-fold change across all tumors are listed in Table 4. Several of these genes, such as *mucin 1* (37), *ceruloplasmin ferroxidase* (38), *claudin 4*, and *HE4* (39, 40) have been previously identified as up-regulated in ovarian tumors.

Validation of Microarray Results by Semiquantitative RT-PCR. To validate the expression levels of genes from the profiling analysis, we performed semiquantitative RT-PCR, with GAPDH as a control in seven ovarian tumor cell lines, on 20 early (I/II)- and 16 late (III/IV)-stage tumors (Table 1) with a set of genes that showed 5-fold differential regulation in at least 50% of the tumors. Fig. 6*A* shows the expression levels of a subset of the genes in ovarian tumor cell lines compared with short-term cultures of ovarian epithelial cells. Fig. 6*B* shows the expression levels of four of these genes in primary ovarian tumors compared with normal epithelial cell brushings. Among the genes with the most striking difference in expression in tumors relative to normal epithelial cell brushings, *FGF7* showed complete loss of expression in all of both early- and late-stage serous tumors and in six of the seven ovarian tumor cell lines. *Decorin*, a small proteoglycan involved in the activation of *EGFR* (41) pathway showed lower levels of expression in 22 of 31 tumors and in five of seven cell lines. The expression of plasminogen activator inhibitor 1 (*PAI1*; Ref. 42), a well-characterized serine protease inhibitor, was also lost in most (25 of 36) of the tumors tested. Among the genes showing frequent up-regulation by microarray analysis, *PUMP-1* (24, 43) and *HE4*, an epididymis-specific whey acidic protein (40), showed overexpression in almost all of the tumors tested (data not shown).

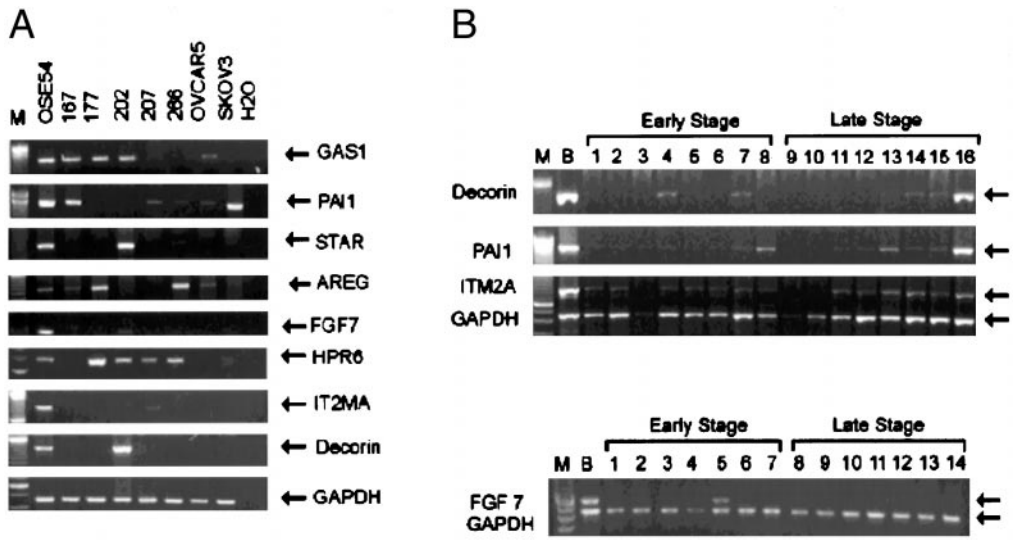

Fig. 6. *A*, agarose gel showing the products of semiquantitative RT-PCR in the ovarian cell lines. *M*, 100-bp ladder; *Lane 1*, short-term cultures of normal ovarian epithelial cells (*OSE54*); *Lane 2*, OV 167; *Lane 3*, OV 177; *Lane 4*, OV 202; *Lane 5*, OV 207; *Lane 6*, OV 266; *Lane 7*, OVCAR 5; *Lane 8*, SKOV; *Lane 9*, water control (*H2O*). Probes: *panel 1*, growth arrest-specific gene 1 (*GAS1*); *panel 2*, plasminogen activator inhibitor 1 (*PAI1*); *panel 3*, steroidogenic acute regulatory protein (*STAR*); *panel 4*, amphiregulin (*AREG*); *panel 5*, fibroblast growth factor 7 (*FGF 7*); *panel 6*, human progesterone binding protein (*HPR6*); *panel 7*, integral membrane protein 2A (*ITM2A*), *panel 8*, decorin; *panel 9*, GAPDH. *B*, agarose gel showing the products of the result of semiquantitative RT-PCR resolved on a 1.6% agarose gel. *On top of the figure*, sample numbers; *on top of the tumor numbers*, the staging information for these tumors. *M*, 100-bp ladder; *Lane 1*, normal epithelial cell brushings (*B*). *Panel 1*, decorin; *panel 2*, plasminogen activator inhibitor 1 (*PAI1*); *panel 3*, integral membrane protein 2A (*ITM2A*); *panel 4*, fibroblast growth factor 7 (*FGF 7*) and GAPDH.

Table 5 *CGH results*

Patient no. (histology; stage)	CGH changes
OV106 (Clear Cell; I)	−1p32-pter;+1q;+**3q22-26**[a];−4q;−6q24-27;+8q;+12p;−13q21-qter;−18q12-21;−Xq21-23
OV496 (Clear Cell; I)	+1p32-pter;−2q21-qter;−3;−4q12-28;+5p;−5q;−6;−12q;−13q21-33;+16;+18;+19;−Xq
OV51 (Endo; I)	NAD
OV105 (Endo; I)	+1q31-42;−2q22-24;−4q13-24;−5q12-23;−6q21-23;+11q22-24;+12q23-qter;−13q21-31;−15q21-24;+16p12-13;+19;+20q;−21
OV338 (Endo; I)	+1q21-qter;+6;+8;−9p;−21q
OV647 (Endo; I)	−4q;−5q;+6q24-27;−7q21-31;+16p12-13.2;+17;−18q;+20
OV6 (Serous; I)	+1p35-qter;−1p21-31;−2q23-32;−4q21-31.3;−5q14-32;+8q;−13q13-33;+19;+20q;+22q;−Xq21-qter
OV90 (Serous; I)	−1q24-31;+3q24-qter;−4q13-25;−5q21-23;−6;+8q23-qter;−9p13-23;+12p11.2-qter;−13q21-qter;−15q21;+17q24-25;−18q12-21;+20q12-qter;+22q
OV234 (Serous; I)	−1p33-35;+2q22;−4q22-31;−5q14-21;−6q14-16;−10p;+12p12-13;+17q;+20q
OV102 (Clear Cell; II)	−1p12-31;+1q31-42;−2q34-36;**+3q24-26**;−17p
OV401 (Serous; II)	+3q;**+3q24**;+**3q26**;−4q;+5p;−5q;−6;−7p;+**8q24**;+**11q23-24**;−18q21-22;+20;−Xq21-qters
OV402 (Serous; II)	+1q41-44;−2q24-32;+3q24-27;−5q21-23;−6q21-23;+11q13;−13q21-31;−18q12-22;+19;+20q;−Xq
OV176 (Clear Cell; III)	+**3q25-26**;+8q;+17q;+**20q12-13**
OV453 (Clear Cell; III)	+3q24-26.3;+17q24-25
OV522 (Clear Cell; III)	+**2p24-25**
OV623 (Clear Cell; III)	+8q
OV78 (Endo; III)	+1q21-41;−2p14-16;+3q13.1-13.3;−4q;+5p;+8q21.1-24;+9p21-23;+11q12-13;+17q23-25;−18q21-22;+20q;−X
OV93 (Endo; III)	+1q32-44;+2p24-25;+2q23-24;+3q13.3-qter;+3q24-26;−4q;−5;−6q22-qter;−8p;+8q;+9p22-23;+12p12-13;−13q31-34;−18q12-23;+20q13
OV110 (Endo; III)	+1p36;−4q13-32;−5q13-22;+8q24;+10;+11q24-25;+12p12-13;−14q23-24;+16q21-23;+19q;+20q
OV259 (Endo; III)	−1p13-22;+1q;+2q35-37;+**3q13**;+**3q25-27**;−4q23-qter;+5p;−5q14-qter;−6q;−7p;−11;+**12p12-13**;−13q;+19q;+**20q12-13**;+**22q12.2-12.3**;−Xq
OV4 (Serous III)	−2q;+**3q26.1-26.3**;−4q22-24;−5q13-23;−6q16.3-25;+**7q33-35**;+**8q23-24**;+**11q13-14**;+**12p11-13**;−18q12.3-22;+20q12-13.2
OV11 (Serous III)	−4q;−5q13.3-23.2;+**8q23-24**;+**11q23-24**;+12p12-13;−Xq21-qter
OV16 (Serous III)	+1q;−18q22;+22;−Xq23-25
OV29 (Serous III)	**+1q21-22**;−2q22-24;−5q;+8q;+**8q23-24**;+**11q12-13**;−18q12-qter;+**18p11.2-11.3**;+20q;−Xq
OV72 (Serous III)	+1p32-36.3;+1q31-32;−2q22-34;+5p13-15;−5q;+6p21.1-pter;+**7p21**;+8;+**8q23-24**;−9p;+**11q12-13**;+**12p12-13**;−18q12-qter;+19;+20;−Xq
OV150 (Serous III)	+**1q21-24**;+**2p24**;−3p;+**3q26-1-26.3**;−4p;+**5p14-15**;−6q13-21;+**8q24**;−9p24;+**11q13**;+**12p12-13**;−13q21-22;16p12-13.2;−18q;+20q12-13;−Xq
OV392 (Serous III)	+3q26;−4q221;−5q12-21;−6q24-27;+7q32-qter;+8q24.1-24.3;+12p13;−13q14-22;+20q;−X
OV448 (Serous III)	+**1p36**;−2q31-34;−5q14-23.3;+**17q24-25**;+19;+**20q12-13.2**;−X
OV319 (Endo; IV)	−1p31;+1q;+**2p24**;+2q24;+**3q21-26**;−4q32-qter;+**8q11.2-21.2**;+**8q23-24**;−9p23;−13q31;−15q21-qter;−18q;+**20q13**;−X

[a] Boldface denotes amplification.

CGH Analysis. To investigate whether any of the results from the gene expression analysis could be associated with genomic alterations, we performed CGH on a set of 29 ovarian tumors. The overall trend suggested similar changes in both early- and late-stage tumors (Table 5; Fig. 7). There were, however, conspicuous differences between the two tumor groups. For instance, the CGH results indicated that losses are more common than gains in the early-stage tumors. Consistent losses were observed at chromosomal regions of 2q (5 of 12), 4q (8 of 12), 5q (8 of 12), 6q (6 of 12), 13q (6 of 12), 18q (3 of 14), and Xq (5 of 12). Additional losses were also observed on chromosomes 7, 9, and 15 (2 cases); and 3, 12, 17, and 21 (1 case each). Gains involving chromosomes 20 (7 of 12); 1 (6 of 12); 3 and 8 (5 of 14); 19 (4 of 12); 11, 12, 16, 17, and 22 (3 of 14); 2, 5, 6, and 18 (1 of 12) were also observed. The chromosomal arms that showed frequent loss in the late-stage tumors were nearly identical to those observed in the early-stage tumors, and included 2q (5 of 17), 4q (9 of 17), 5q (9 of 17), 6q and 13q (5 of 17), 18q (6 of 13), and Xq (10 of 17). Less frequent losses were seen on chromosomes 9 (3 cases); 1p (2 cases); and 3, 7, 8, 11, 14, and 15 (1 case each). Gains on chromosomes 8 (76%), 20 (70%), 1 and 3 (58%), 12 (52%), 11 (41%), and 2 (35%) were evident in at least six of the late-stage tumors. Less frequent gains were observed on chromosomes 19 and 17 (4 of 17); 5 and 7 (3 of 17); 9, 16, and 22 (2 of 13); and 6 and 10 (1 case each).

Amplifications were mainly confined to the late-stage ovarian tumors. These involved regions at 8q23–24 (8 cases); 20q12–13 (6 cases); 3q24–26, 11q12–13, and 12p11–13 (5 cases each); 2p24–25 (3cases); 1p36, 9p21–23, 11q24–25, and 17q24–25 (2 cases each); and 1 case each on 1q32–44, 2q23–24, 3q13, 5p14–15, 7p21, 7q35, 8q11–21, and 22q12. In contrast, amplifications in the early-stage cancers were observed in just six instances: three times at chromosome 3q24–26 and one case each on 3q23, 8q23–24, and 11q24–25.

DISCUSSION

This report represents the first communication of an investigation involving the genome-wide examination of changes in gene expression and chromosomal regions for early- and late-stage ovarian cancer. In our cDNA microarray analysis, we identified several differentially expressed genes the potential role of which in carcinogenesis have been described previously. However, this analysis also identified several genes, both of known and unknown function, the role of which in tumor development has yet to be elucidated.

We validated the differential expression of several of the genes identified through microarray analysis by semiquantitative RT-PCR of RNA from both ovarian tumor cell lines and primary tumors. The down-regulation (at least 5-fold) of genes such as hydroxy-δ-5 steroid dehydrogenase, 3β (*HSD3B1*; Ref. 44), steroidogenic acute regulatory protein (*StAR*; Ref. 45, 46), *amphiregulin* (47), *glutathione S-transferase A3* (48), paternally expressed *gene 3* (49), and *integral membrane protein 2A* (50) have not previously been associated with ovarian cancer. Other genes such as *decorin* (41, 51), *platelet-derived growth factor receptor* α (52), *cadherin 11* (21), *cyclin D2* (53), *E2F*-related transcription factor *DP1* (33), *NOEY2* (6), and secreted frizzled related protein (*SFRP*; Ref. 54), have functions that can be or have been linked with carcinogenesis. Our expression analysis further revealed the consistent up-regulation of several genes, including *HE4* (40), *matrilysin* (*MMP 7*), *ceruloplasmin ferroxidase* (38), *claudin 4* (39), *cyclin D1* (55), *mucin 1* (56) *protease serine 8* (57), *keratins 7* (58) and *8* (28), as well as several ESTs. We have also validated the expression levels of *HE4*, *MMP7*, *ITM2A*, *HSD3B1*, and *PEG3* by real time RT-PCR in a smaller panel of primary tumors, and the results were similar to the semiquantitative RT-PCR analysis (data not shown).

To address the issue of whether the changes that we observed at the expression level were present at the genomic level, CGH analysis was performed on 12 early- and 17 late-stage tumors. Chromosomes 2, 4, 5, 6, 13, and 18 showed regions of loss in both stages at a similar frequency (59–61). Previous genomic sequence copy number screenings of ovarian tumors by conventional and molecular cytogenetics have reported similar findings (62). High copy number gains or amplifications were mainly observed in the late-stage tumors (62).

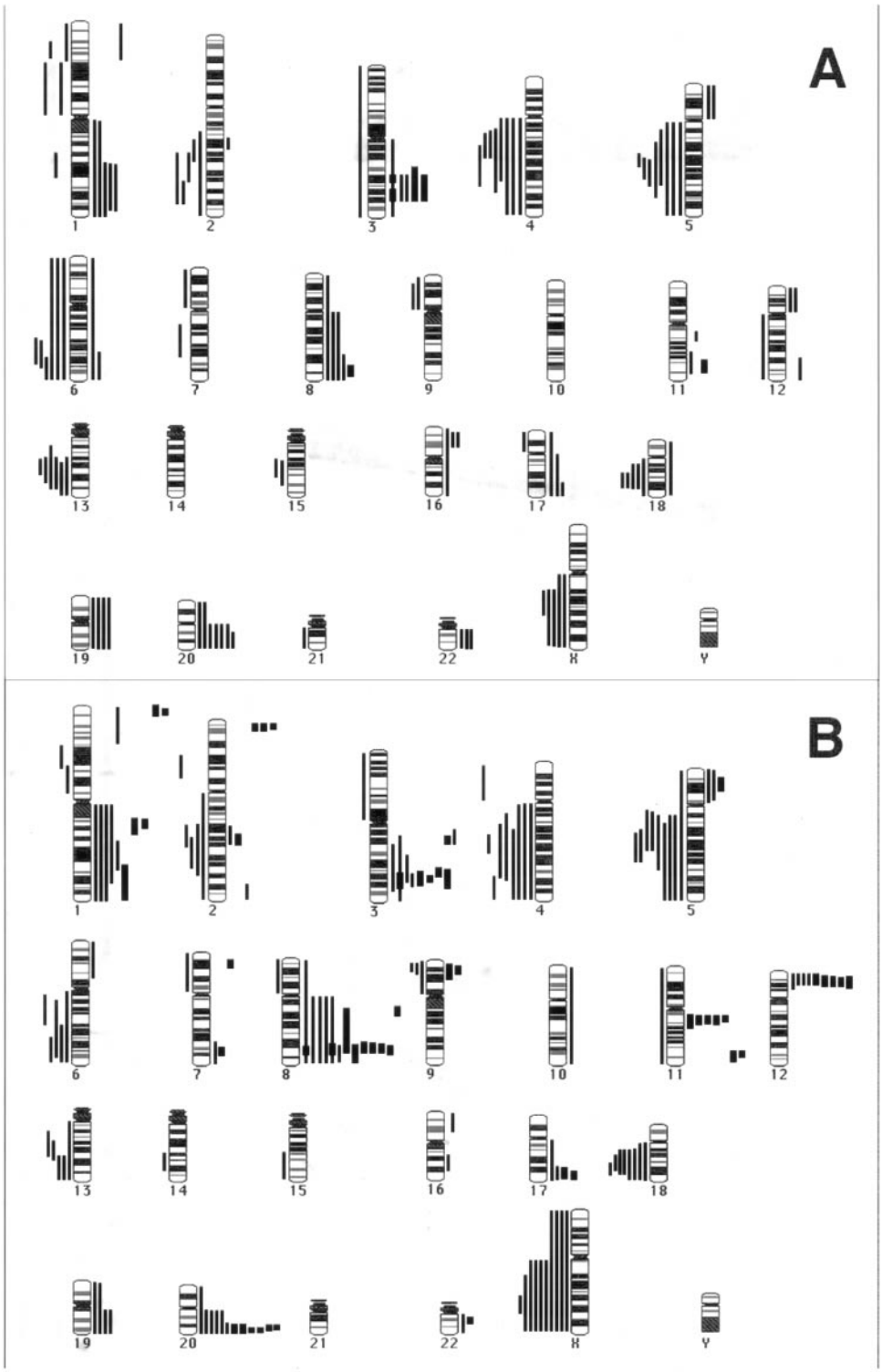

Fig. 7. Ideograms of the results of CGH analysis of 29 ovarian tumors. *Vertical lines to the right of schematic chromosomes*, gains; *vertical lines to the left*, losses. *Thicker line to the right*, the presence of high copy gain/amplification. *A*, CGH analysis of early-stage (I/II) tumors. Copy number changes of 12 tumors indicate a pattern of losses. In particular, loss of chromosomes 2, 4, 5, 6, 13, and 18 are frequent. Gains were less frequent, mainly involving chromosomes 1, 3, 6, 8 11, 19, and 20. *B*, CGH analysis of late-stage (III/IV) tumors. Copy number changes of 17 tumors indicate a pattern of losses and gene amplification; *thicker lines*, high level gains/amplifications (~>5 copy number). In contrast to the early-stage ovarian carcinomas, there are more chromosomal gains, and more tumors with localized gene amplification, predominantly involving the 3q24–26, 8q23–24, 11q12–13, 12p12–13, and 20q12–13 region. Other amplifications were detected on chromosomal arms 1p, 1q, 2p, 2q, 5p, 7q, 9p, 11q, 16p, 17q, and 18p. Losses were common on chromosomes 2, 4, 5,6, 13, and 18.

Chromosomal regions involved in amplification were 3q24–26 (63), 8q23–24 (64), 11q12–13 (55), 12p11–12 (65), 17q24–25 (66), and 20q12–13 (67).

Inspection of the data generated by CGH and expression profiling allows one to speculate on a mechanistic basis for the differential expression of at least some of the genes. For example, CGH results indicated high-level gains of 8q24 and 11q13 in multiple tumors (Fig. 8), and the microarray analysis of the same tumors indicated an increase in expression of genes/ESTs from these same regions: *i.e.*, *cyclin D1* (*PRAD1*), *uncoupling protein 2*, *folate receptor*, *and matrix metalloproteinase 7*, from 11q13; and *MYCC* (68), *PTK2 protein tyrosine kinase 2* (69), *RAD21* homologue (70), and several ESTs from 8q24 (data not shown), respectively. From the combined results, it seems reasonable to consider that the overexpression of these genes may be the result of gene amplification. Similarly, loss of genomic sequences from chromosomal region 4q could be responsible for the low expression of *LIM* (71), *Hevin* (72), *MAP kinase 10* and several ESTs detected by the expression profiling from the same region.

The data from the microarray analysis revealed that the majority of genes that were differentially expressed in ovarian cancer showed

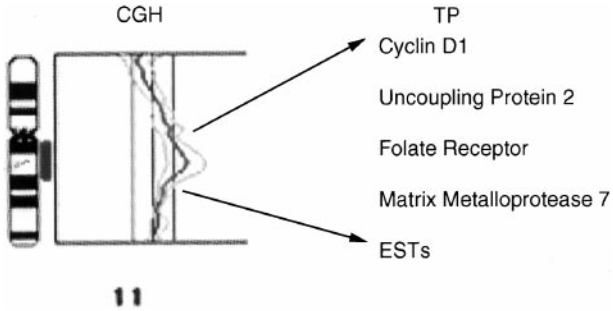

Fig. 8. CGH profile of an ovarian tumor illustrating a high copy gain/amplification in the 11q12–13 region of chromosome 11. The transcription profile of the same tumor by cDNA microarray analysis exhibited overexpression of cyclin D1, uncoupling protein 2, folate receptor, metalloproteinase 7, and some ESTs from this region.

aberrant transcript levels in both early- and late-stage tumors. At initial consideration, such results seem contrary to the genetic dogma associated with solid-tumor evolution that holds that gene alterations, and presumably, therefore, gene expression changes, become more prevalent as tumors become increasingly malignant. Interestingly, the CGH data indicate some differences between early- and late-stage tumors, especially the more common finding of regional gain and/or amplification in the late-stage tumors. Consequently, the CGH data provide some support for the genetic evolution of this solid tumor.

The inconsistency observed between the expression profiling and the CGH data could potentially be attributable to epigenetic changes such as methylation. Inactivation of genes attributable to CpG island methylation is very well documented (73). Evidence seems to indicate that these changes are probably very early events in carcinogenesis (74, 75). This may partially explain why the majority of genes are aberrantly regulated in early-stage tumors. It is also well documented that amplification events in carcinogenesis are late events (76–78) and is consistent with what is observed from the CGH data. Abnormal expression of genes is not attributable only to gene amplification or gains. Other genomic changes like rearrangements (translocations) are also responsible for alteration in gene expression. CGH can only aid in detecting net chromosomal losses or gains in tumor cells and does not account for rearrangements, thus overlooking changes that could be responsible for abnormal expression detected by microarray analysis.

An aspect of our finding that may be especially important to the clinical treatment of ovarian cancer is the determination of extensive, aberrant gene expression in early-stage tumors. One of the assumptions of most screening strategies is that a significant fraction of stage III/IV disease metastasizes from stage I lesions. These data are consistent with that possibility. In addition, poorly differentiated stage I cancers have been treated with cytotoxic chemotherapy. These data suggest that early-stage lesions have most of the genetic changes required for metastatic spread, consistent with a need for aggressive therapy. Further study of the genes that show consistent, high-level expression changes may result in the identification of markers useful for early cancer detection and may further point to potential therapeutic targets.

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