EXHIBIT 4

Gene Expression Profiles of BRCA1-Linked, BRCA2-Linked, and Sporadic Ovarian Cancers

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Background: Germline mutations in BRCA1 and BRCA2 are responsible for 5%–10% of epithelial ovarian cancers, but the molecular pathways affected by these mutations are unknown. We used complementary DNA (cDNA) microarrays to compare gene expression patterns in ovarian cancers associated with BRCA1 or BRCA2 mutations with gene expression patterns in sporadic epithelial ovarian cancers and to identify patterns common to both hereditary and sporadic tumors. *Methods:* Tumor samples from 61 patients with pathologically confirmed epithelial ovarian adenocarcinoma with matched clinicopathologic features were studied, including 18 with BRCA1 founder mutations, 16 with BRCA2 founder mutations, and 27 without either founder mutation (termed sporadic cancers). The cDNA microarrays contained 7651 sequence-verified features. Gene expression data were analyzed with a modified two-sided F test, with $P<.0001$ considered statistically significant. The expression level of six genes was also studied with reverse transcription–polymerase chain reaction. *Results:* The greatest contrast in gene expression was observed between tumors with BRCA1 mutations and those with BRCA2 mutations; 110 genes showed statistically significantly different expression levels ($P<.0001$). This group of genes could segregate sporadic tumors into two subgroups, "BRCA1-like" and "BRCA2-like," suggesting that BRCA1-related and BRCA2-related pathways are also involved in sporadic ovarian cancers. Fifty-three genes were differentially expressed between tumors with BRCA1 mutations and sporadic tumors; six of the 53 mapped to Xp11.23 and were expressed at higher levels in tumors with BRCA1 mutations than in sporadic tumors. Compared with the immortalized ovarian surface epithelial cells used as reference, several interferon-inducible genes were overexpressed in the majority of tumors with a BRCA mutation and in sporadic tumors. *Conclusions:* Mutations in BRCA1 and BRCA2 may lead to carcinogenesis through distinct molecular pathways that also appear to be involved in sporadic cancers. Sporadic carcinogenic pathways may result from epigenetic aberrations of BRCA1 and BRCA2 or their downstream effectors. [J Natl Cancer Inst 2002;94: 990–1000]

Germline mutations of BRCA1 and BRCA2 tumor suppressor genes are responsible for 5%–10% of all epithelial ovarian cancers *(1)*. However, little is known about the molecular mechanisms involved in BRCA1 and/or BRCA2 mutation-associated (termed BRCA-linked) ovarian carcinogenesis. For example, it is not known whether BRCA1 and BRCA2 mutations affect common or unique molecular pathways in ovarian

cancer or whether these pathways overlap with those involved in the formation of sporadic tumors. Both BRCA1 and BRCA2 proteins have been implicated in important cellular functions, including embryologic development, DNA damage repair, and transcriptional regulation *(2–5)*. The mechanisms by which BRCA1 and BRCA2 carry out these functions and potential differences between them remain largely unknown. Gene expression profiling with microarrays has become a powerful tool for molecular analysis and classification of a number of different tumors *(6–9)*. This investigation had two goals. First, we used complementary DNA (cDNA) microarrays to examine the role of BRCA mutations in ovarian carcinogenesis by comparing gene expression in cancers associated with germline BRCA1 and BRCA2 mutations and in sporadic ovarian cancers. Second, our experimental design also provided an opportunity to investigate gene expression patterns common among all tumor types, thus identifying genes that may be associated with the transformed state in both hereditary and sporadic ovarian cancers.

METHODS

Tumor Samples and Determination of BRCA Status

After Institutional Review Board approval, tumor samples from 61 patients with pathologically confirmed epithelial ovarian adenocarcinoma from the Memorial Sloan-Kettering Cancer Center were studied, including 18 linked to BRCA1 mutations, 16 linked to BRCA2 mutations, and 27 without either mutation and classified as sporadic. All patients were self-identified as Ashkenazi Jews and, after informed consent, underwent genotyping for germline founder mutations in BRCA1 (185delAG and 5382insC) and BRCA2 (6174delT), as previously described *(10)*. Those patients with a BRCA1 or BRCA2 mutation were categorized as having hereditary ovarian cancer, and those without such a mutation were categorized as having sporadic ovarian cancer. All tumor samples had been flash frozen, embedded in OCT medium, and stored at –80 °C. The rationale for and relative merits of this study design have been previously discussed in detail *(10)*.

RNA Extraction and cDNA Microarray Techniques

RNA was isolated with RNeasy columns (Qiagen, Valencia, CA), according to the manufacturer's instructions. The integrity of RNA was verified by denaturing gel electrophoresis. Total RNA was linearly amplified with a modification of the Eberwine method *(11)*. Briefly, total RNA was reverse transcribed by using a 63-nucleotide synthetic primer containing the T7 RNA polymerase binding site, 5′-GGCCAGTGAATTGTAATAC-GACTCACTATAGGGAGGCGG(T)$_{24}$–3′. Second-strand cDNA synthesis (producing double-stranded cDNA) was performed with RNase H, *Escherichia coli* DNA polymerase I, and *E. coli* DNA ligase (Invitrogen, Carlsbad, CA). After cDNA was made blunt-ended with T4 DNA polymerase (Invitrogen), it was purified by extraction with a mixture of phenol, chloroform, and isoamyl alcohol and by precipitation in the presence of ammonium acetate and ethanol. The double-stranded cDNA was then transcribed with T7 polymerase (T7 Megascript kit; Ambion, Austin, TX), yielding linearly amplified antisense RNA, which was purified with RNeasy mini-columns (Qiagen). Pooled total RNA from two simian virus 40-immortalized ovarian surface epithelial (IOSE) cell lines was amplified and used as the reference for cDNA microarray analysis. In addition, RNA from a

histologically normal postmenopausal ovary (a gift from Dr. Laurel Rice, University of Virginia, Charlottesville) was used in the reverse transcription–polymerase chain reaction (RT–PCR) experiments. The cDNA microarray chips contained 7651 total features and were manufactured at the National Cancer Institute microarray facility. Four micrograms of amplified RNA was reverse transcribed and directly labeled with cyanine 5-conjugated deoxyuridine 5′-triphosphate (dUTP) (tumor RNA) or cyanine 3-conjugated dUTP (IOSE RNA). Hybridization was performed in a solution of 5× saline sodium citrate and 25% formamide for 14–16 hours at 42 °C. Slides were washed, dried, and scanned with an Axon 4000a laser scanner (Axon Instruments, Union City, CA). A detailed protocol for RNA amplification, cDNA probe labeling, and hybridization is available on the Web at http://nciarray.nci.nih.gov/reference/index.shtml. Genepix software (Axon Instruments) was used to analyze the raw data, which were then uploaded to a relational database maintained by the Center for Information Technology at the National Institutes of Health. The cDNA clones used are identified by their Integrated Molecular Analysis of Genomes and their Expression Consortium (I.M.A.G.E.) clone number.

Data Analysis

The logarithmic expression ratios for the spots on each array were normalized by subtracting the median logarithmic ratio for the same array. Data were filtered to exclude spots with a size of less than 25 μm, spots with an intensity of less than two times background or less than 300 U in both red and green channels, and any poor-quality or missing spots. In addition, any features found to be missing in more than 10% of the arrays were not included in the analysis. Application of these filters resulted in the inclusion of 6445 of the total 7651 features in subsequent analyses. Statistical comparison between tumor groups was performed with BRB Array Tools software (http://linus.nci.nih.gov/ ~brb/tool.htm), consisting of a modified F test with $P<.0001$ considered statistically significant. This stringent P value was selected in lieu of the Bonferroni correction for multiple comparisons, which was deemed excessively restrictive.

In addition to statistical analysis, multidimensional scaling and hierarchical clustering techniques with a Pearson correlation metric and average linkage were used for evaluating overall gene expression *(12)*. When applicable, all statistical tests were two-sided.

Semiquantitative RT–PCR

Five samples from each tumor group were selected at random, and the expression level of six genes was studied. For each sample, 3.5 μg of total RNA was reverse transcribed by using oligo(dT) primers, 400 U of Superscript II reverse transcriptase (Invitrogen), all four deoxyribonucleoside 5′-triphosphates (each at 10 m*M;* Invitrogen), and 40 U of RNase inhibitor (Promega, Madison, WI). Reverse transcription was performed in a total reaction volume of 40 μL, of which 1 μL was subsequently used for each PCR. Preliminary experiments were performed to identify the optimal cycle number for each gene. Thirty cycles was found to be optimal for all amplified RNAs except for HLA-DRB1 and CD74, which were amplified for 26 cycles. PCR was performed with the GeneAmp PCR kit (PE Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. Gene-specific primer sequences were as follows: ACTB = 5′-ATGTGGATCAGCAAGCAGGA-3′ and 5′-GGTG-

GCTTTTAGGATGGCAA-3′; HE4 = 5′-TTCGGCTTCACC-CTAGTCTCA-3′ and 5′-AGAGGGAATACAGAGTCCCGAA-3′; ZFP36 = 5′-ACCCTGATGAATATGCCAGCA-3′ and 5′-GCTACTTGCTTTTGGAGGGTA-3′; RGS1 = 5′-GACTCTT-ATCCCAGGTTCCTCA-3′ and 5′-TGACTCCCTGGTTT-TAAGAGCA-3′; CD74 = 5′-CCAGTCCCCATGTGAGAGCA-3′ and 5′-AGCTGATAACAAGCTTGGCTGA-3′; TOP2A = 5′-TGTCCCTCCACGAGAAACAGA-3′ and 5′-CGTACAGATTTT-GCCCGAGGA-3′; and HLA-DRB1 = 5′-GCGAGTTGAGC-CTAAGGTGA-3′ and 5′-TTGAAGATGAGGCGCTGTCA-3′.

RESULTS

Clinicopathologic Characteristics of BRCA-Linked and Sporadic Ovarian Cancers

All tumors in this study were obtained from individuals screened for the founder mutations in BRCA1 or BRCA2. To minimize confounding variables, BRCA1-linked, BRCA2-linked, and sporadic tumors of similar stage, grade, and histology were selected (Table 1). The majority of tumors in all three groups were characterized by advanced stage, moderate to high grade (grade 2 or 3), and a predominance of serous histology. Hence, the clinicopathologic parameters of our samples were well-matched and in agreement with those reported previously for these tumor types *(10,13)*.

Global Assessment of Gene Expression Differences Among Tumor Groups

Before investigating specific intergroup differences, overall patterns of gene expression in the three tumor types were assessed. Multidimensional scaling, based on the expression levels
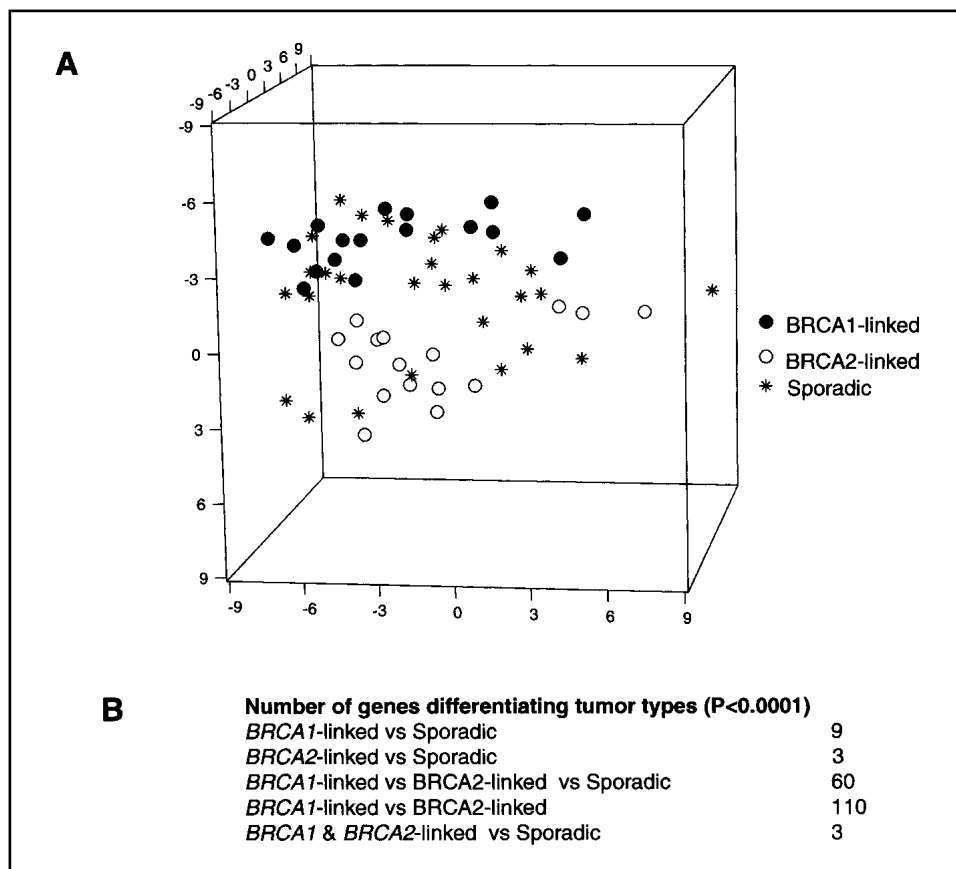
Table 1. Clinicopathologic features of tumor samples

	BRCA1-linked (n = 18)	BRCA2-linked (n = 16)	Sporadic (n = 27)
Median age*, y	50 ± 11	60 ± 9	69 ± 11
Stage, No. (%)			
I	2 (11.1)	0	0
II	0	2 (12.5)	3 (11.1)
III	11 (61.1)	12 (75)	24 (88.9)
IV	5 (27.8)	2 (12.5)	0
Grade, No. (%)			
1	0	0	0
2	4 (22.2)	6 (37.5)	8 (29.6)
3	14 (77.8)	7 (43.8)	16 (59.3)
No.	0	3 (18.7)	3 (11.1)
Histology†, No. (%)			
Serous	9 (50)	12 (75)	16 (59.3)
Endometrioid	3 (16.7)	0	2 (7.4)
Mucinous	0	0	0
Clear cell	2 (11.1)	0	0
Adenocarcinoma NOS	3 (16.7)	3 (18.8)	9 (33.3)
Mixed	1 (5.5)	1 (6.2)	0

*F test, P = .0002. Data are the median ± standard deviation.

†χ^2 test, P = .17 for differences in histology among tumor groups. NOS = not otherwise specified.

of all 6445 filtered genetic elements in the microarray, revealed that BRCA1-linked and BRCA2-linked tumors had distinct molecular profiles. In contrast, the sporadic samples showed a more heterogeneous distribution pattern, with many patterns clustering near the patterns of BRCA1-linked or BRCA2-linked samples (Fig. 1, A). The multidimensional scaling results indicated that gene expression patterns for BRCA1-associated and

Fig. 1. Overall expression differences between BRCA1-linked, BRCA2-linked, and sporadic ovarian epithelial cancers. **A**) Multidimensional scaling model based on the overall gene expression (all 6445 filtered spots) in BRCA1-linked, BRCA2-linked, and sporadic tumors (see also supplemental Fig. 1 for a rotating movie of the multidimensional scaling model and supplemental Table 1 for the raw data (http://jncicancerspectrum.oupjournals.org/jnci/content/vol94/issue13/). **B**) The magnitude of differences in gene expression between various tumor groups as determined by the number of genes differentially expressed among the tumor groups when the uniform statistical cutoff $P<.0001$ is applied.
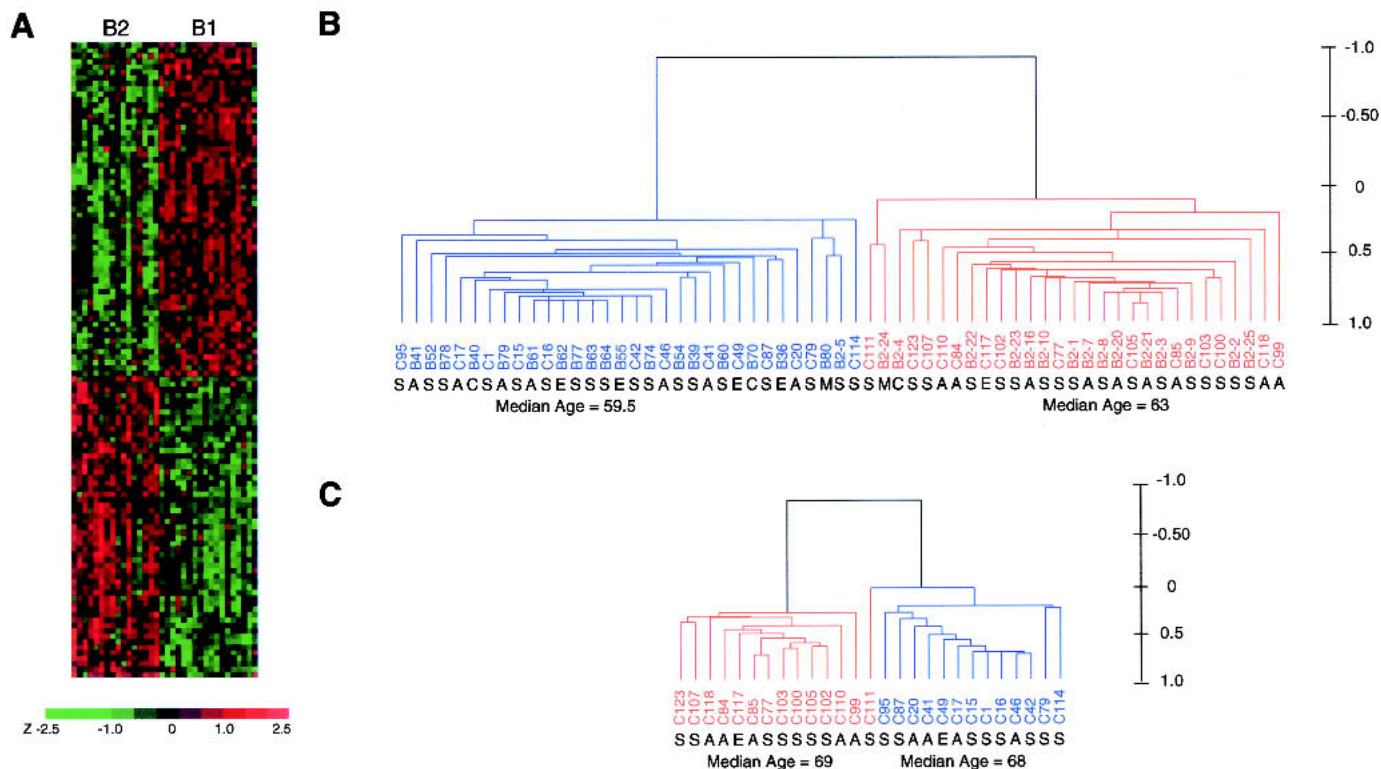

Number of genes differentiating tumor types (P<0.0001)

BRCA1-linked vs Sporadic	9
BRCA2-linked vs Sporadic	3
BRCA1-linked vs BRCA2-linked vs Sporadic	60
BRCA1-linked vs BRCA2-linked	110
BRCA1 & *BRCA2*-linked vs Sporadic	3

Fig. 2. BRCA1- and BRCA2-discriminating genes also segregate sporadic ovarian cancers into two groups. **A)** Hierarchical clustering of 110 nonredundant genes with statistically significant differential expression between BRCA1-linked (B1) and BRCA2-linked (B2) tumors (modified F test, *P*<.0001). The **red** and **green** intensities represent standard normal deviation (*Z* score) values from each gene's mean expression level (represented as **black**) across all 61 tumor samples. **B)** Hierarchical clustering of sporadic and BRCA-linked tumor samples based on the expression pattern of the 110 BRCA-discriminating genes. The two major clusters correspond to BRCA1-like (**blue**) and BRCA2-like (**orange**) patterns of gene expression. B = BRCA1-linked tumor; B2 = BRCA2-linked tumor; C = sporadic tumors. Tumor histology for each sample is indicated below the sample label as follows: S = serous; A = adenocarcinoma not otherwise specified; E = endometrioid; C = clear cell; M = mixed features. **C)** Hierarchical clustering of sporadic samples in the absence of BRCA-linked tumors. Note the maintenance of cluster integrity. Each sporadic sample is colored according to its cluster identity in **B.** The median age in years is shown for each major cluster.

BRCA2-associated groups would be the most different and that gene expression patterns for each of the BRCA groups and the sporadic tumors would have fewer differences. In support of this hypothesis, only a few genes showed statistically significant (*P*<.0001) differential expression between the sporadic tumors and the BRCA1-linked or BRCA2-linked tumors, whereas 110 genes were differentially expressed between BRCA1-linked and BRCA2-linked tumors (Fig. 1, B). Differential gene expression among all three groups was also performed, and it identified 60 genes whose expression segregated BRCA1-linked, BRCA2-linked, and sporadic tumors (modified F test, *P*<.0001). Fifty-one of these 60 genes were also among the statistically significant discriminators of BRCA1 and BRCA2 tumors, highlighting the distinct gene expression profiles of these two groups. In addition, the expression profile of the combined BRCA1-linked and BRCA2-linked group was remarkably similar to that of the sporadic tumors, as demonstrated by only three genes showing differential expression (*P*<.0001) between these two groups. These observations were in agreement with the multidimensional scaling analysis and demonstrated that the overall patterns for gene expression of the BRCA1-linked and BRCA2-linked tumors were distinct from one another. Furthermore, the gene expression profiles of the sporadic tumors appeared to share features of BRCA1-linked or BRCA2-linked cancers.
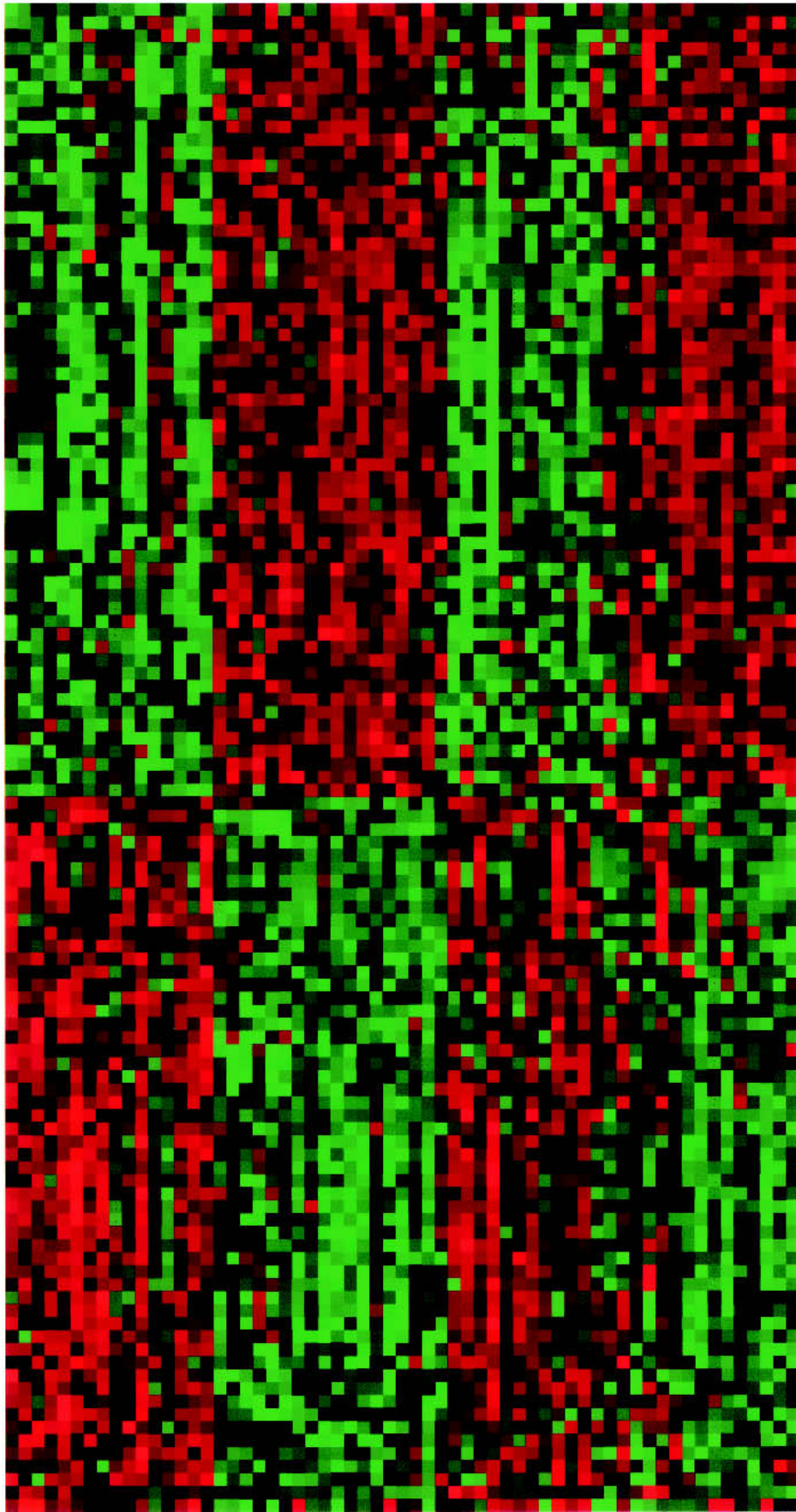
The group of 110 genes whose expression statistically significantly differentiated the BRCA1-linked and BRCA2-linked

tumors was further investigated by use of hierarchical clustering (Fig. 2). As expected, the BRCA-associated tumors showed distinct and contrasting expression profiles (Fig. 2, A). The sporadic samples also segregated into two groups based on the expression patterns of the same 110 genes; each sporadic sample had a molecular profile similar to that of the BRCA1-linked or the BRCA2-linked tumors. This observation was illustrated by the hierarchical clustering of all samples that revealed distinct BRCA1-like and BRCA2-like clusters (Fig. 2, B). To ensure that the BRCA-linked samples were not biasing the observed clustering patterns, the hierarchical architecture of gene expression in sporadic tumors was examined separately. Even in the absence of the BRCA-linked samples, two distinct cluster phenotypes were observed, each composed of those sporadic samples that previously grouped with BRCA1-linked and BRCA2-linked tumors (Fig. 2, C). Tumor histology and patient age were also evaluated for possible confounding effects on the observed BRCA1-like and BRCA2-like clusters. Neither variable was found to influence clustering patterns (Fig. 2, B and C).

Genes Differentially Expressed Between BRCA1-Linked and BRCA2-Linked Ovarian Carcinomas

The preceding analysis of overall gene expression patterns established that the same genes whose expression differentiated BRCA1-linked and BRCA2-linked tumors also identified two

Fig. 3. Molecular profiles of all 61 tumors as defined by the 110 nonredundant genes whose expression statistically significantly differentiates BRCA1 and BRCA2 tumors (*P*<.0001). The **red** and **green** color intensities represent expression levels shown as standard normal deviation (*Z* score) values from each gene's mean expression level (represented as **black**) across all 61 tumor samples. The symbol for each gene is followed by the I.M.A.G.E. (Integrated Molecular Analysis of Genomes and their Expression Consortium) clone number of the corresponding complementary DNA spotted on the array.

major subpopulations of sporadic cancers (Fig. 3). As such, these genes may represent important mediators of common genetic pathways in ovarian cancer and/or carcinogenesis. Many of these genes are involved in important cellular functions including signal transduction, RNA processing and translation, chemokine signaling and immune modification, and DNA repair. The BRCA1-associated tumors were characterized by the higher relative expression of AKT1 and lower relative expression of PTEN. In addition, the expression of UBL1 (also known as SUMO-1 and sentrin) was higher in BRCA1-associated tumors than in BRCA2-associated tumors. This molecule interacts with RAD51 and RAD52 and may have a regulatory role in homologous recombination *(14)*. The preferential expression of UBL1 in the BRCA1-linked samples may be relevant to possible differences in DNA repair actions of the BRCA tumor suppressor genes.

The BRCA2-linked tumors showed higher relative gene expression of WNT2 and SFRP4, which are members of the wnt/β-catenin/T-cell transcription factor (TCF) signaling pathway. WNT2 has recently been reported to be overexpressed in a number of solid tumors including breast, colorectal, and gastric cancers *(15)*. The secreted frizzled-related proteins (SFRPs) are modulators of the wnt signaling cascade, and, in some cellular contexts, their expression has been correlated with apoptosis *(16,17)*. The SFRP4 gene is overexpressed in endometrial hyperplastic tissue and carcinomas and in both infiltrating and *in situ* breast carcinomas and, thus, may play an early role in carcinogenesis *(18)*. Another notable observation is that both BRCA1-linked and BRCA2-linked tumors preferentially expressed proto-oncogenes commonly altered in hematologic malignancies. BRCA1 tumors expressed higher levels of RUNX1/AML1, whereas BRCA2-associated samples preferentially expressed TAL1/SCL. Both of these proto-oncogenes are transcription factors involved in proliferation, and their preferential expression in BRCA1-linked and BRCA2-linked tumors may indicate that the activation of such a proliferation driver is a necessary step in ovarian carcinogenesis.

Gene Expression Differences Between BRCA-Linked and Sporadic Tumors

Nine nonredundant genes showed statistically significant differential expression between BRCA1-linked and sporadic tumors (*P*<.0001; Fig. 4, A). A noteworthy observation was that three of these genes—WAS, PCTK1, and UBE1—have been mapped to Xp11.23, and all three genes were expressed more highly in the BRCA1-linked tumors than in BRCA2-linked and sporadic tumors. This observation seemed unlikely to be explained by chance alone because only 35 of the total 6445 filtered spots (0.5%) on our microarray represented genes mapped to Xp11. To further investigate this pattern, we considered a larger group of 53 genes differentially expressed between BRCA1-linked and sporadic tumors under the less stringent statistical significance level of *P*<.001. Among this group, three additional genes—SMC1L1, ARAF1, and EBP—were discov-

ered that also mapped to the Xp11.23 locus and showed higher mean expression in BRCA1-associated samples (Fig 4, D). Thus, six of 53 genes differentially expressed between BRCA1-linked and sporadic samples (*P*<.001) mapped to Xp11.23 and all showed higher mean expression in BRCA1-linked tumors. *In silico* analysis of the location of these genes revealed that they were all confined to a 5-megabase region of DNA in Xp11.2 (Ensemble database).

Only two genes were found to be differentially expressed between the BRCA2-linked and sporadic tumors at the statistical significance level of *P*<.0001 (Fig. 4, B). The gene designated LOC51760, also known as the B/K (brain/kidney) protein gene, is moderately homologous to the synaptotagmin family of vesicular transport molecules. The second differentially expressed gene is low-density lipoprotein-related protein-associated protein-1 (LRPAP1, also known as α$_2$-macroglobulin receptor-associated protein-1). To date, to our knowledge, there is no other report of an association between these genes and BRCA2 or ovarian cancer.

In the final comparison, we investigated differences in gene expression between the combined BRCA-linked group and the sporadic group and found only three nonredundant, differentially expressed genes (Fig. 4, C). All three genes were among the group of genes that differentiated BRCA1-linked and sporadic samples. This finding is consistent with our observation that the molecular profiles of sporadic ovarian cancers and BRCA1-linked or BRCA2-linked cancers share important features.

Gene Expression Features Distinguishing Ovarian Cancers From Ovarian Surface Epithelial Cells

The second aim of this study was to identify genes commonly overexpressed in ovarian tumors irrespective of their hereditary or sporadic nature, because such genes may be associated with the transformed state. We compared gene expression in all 61 primary tumor samples with that in the reference IOSE cells. Using the selection criterion of a twofold or greater expression ratio relative to the IOSE reference in at least two thirds of all tumors, we generated a list of 201 nonredundant genes and expressed sequence tags. The top 25 overexpressed and 25 underexpressed named genes (by order of magnitude) are presented in Fig. 5. This analysis revealed two potentially important functional groups of genes that are overexpressed in ovarian cancers. The first group contained several interferon-inducible genes (HLA-DRB1, HLA-DRB5, HLA-DRA, HLA-DPA, CD74, IFITM1, and IFITM2). The second group contained immediate-early response genes (BRF2, ZFP36, SGK, and FOS). In addition, several genes previously reported to be overexpressed in ovarian epithelial tumors were present in the list of genes overexpressed in tumors compared with IOSE cells (Fig. 5). One such gene, HE4, has been shown to be overexpressed in ovarian cancer by several investigators *(9,19–21)* and is currently being evaluated as a possible ovarian tumor marker. Elevated levels of CLU, CD24, and MUC1 gene expression were also observed, confirming the previously reported overexpression of these genes in ovarian cancer *(9,19,20)*. Fig. 5 contains additional

candidate genes for future investigations of potential biomarkers for ovarian cancer.

Semiquantitative RT–PCR and cDNA Microarray Data

To validate the array data, semiquantitative RT–PCR (sqRT–PCR) analysis of several messenger RNAs (mRNAs) was performed in a representative subset of tumors containing five BRCA1-linked RNA samples, five BRCA2-linked samples, and five sporadic samples. Expression of TOP2A, RGS1, CD74, HE4, HLA-DRB1, and ZFP36 genes was evaluated by sqRT–PCR with β-actin as a normalizing control. Because data obtained from cDNA microarrays are in the form of relative expression ratios between tumors and the reference sample, we
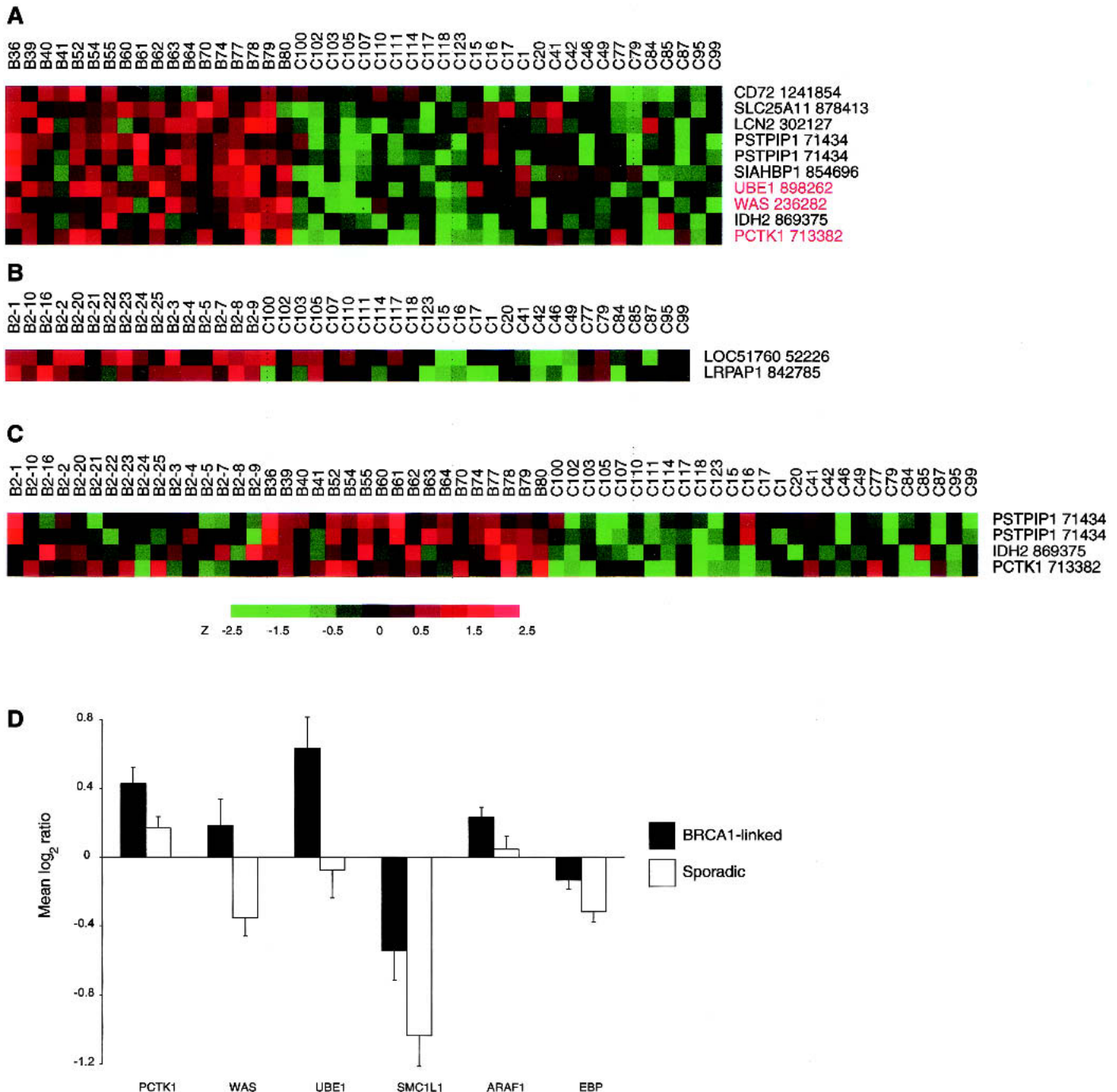

Fig. 4. Gene expression differences between BRCA-linked and sporadic tumors. A two-sided modified F test with a statistical significance level of *P*<.0001 was used to evaluate genes differentially expressed between tumor types. The **red** and **green** color intensities represent expression levels shown as standard normal deviation (*Z* score) values from each gene's mean expression level (represented as **black**) across all 61 tumor samples. The symbol for each gene is followed by the I.M.A.G.E. (Integrated Molecular Analysis of Genomes and their Expression Consortium) clone number of the corresponding complementary DNA spotted on the array. **A)** Genes differentially expressed between BRCA1-linked (B followed by a number) and sporadic (C followed by a number) samples. Genes located on Xp11 appear in **red. B)** Genes differentially expressed between BRCA2-linked (B2) and sporadic (C) samples. **C)** Genes differentially expressed between the combined BRCA1-linked and BRCA2-linked group (B and B2, respectively) and the sporadic (C) samples. **D)** BRCA1-linked tumors exhibit statistically significantly higher expression levels (*P*<.001) of all six genes mapped to Xp11.23 compared with the sporadic cancers. **Error bars** are the 95% confidence interval surrounding the mean.
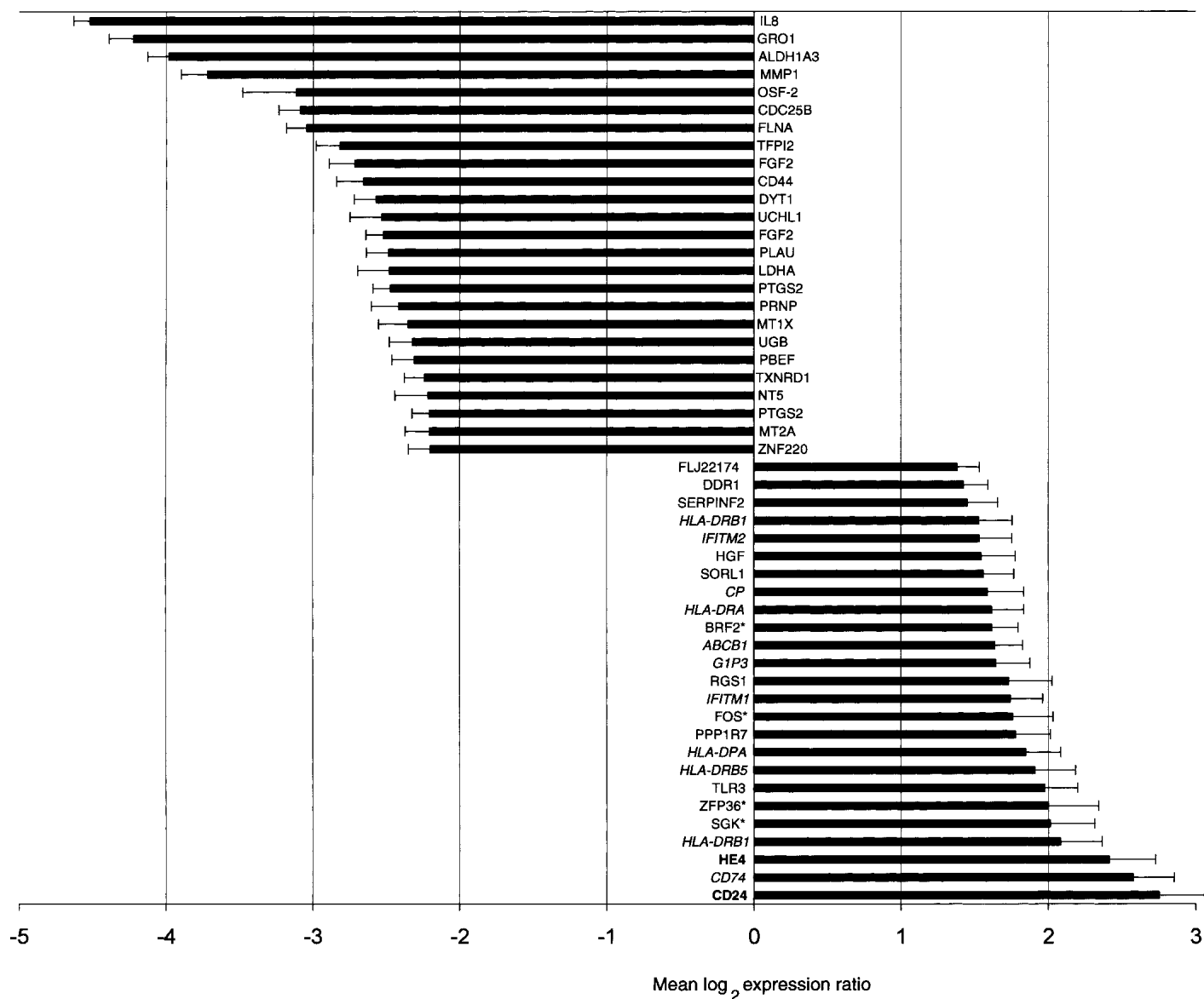
Fig. 5. Evaluation of gene expression patterns common to BRCA-linked and sporadic tumors. The 50 genes with the greatest (twofold or greater) overexpression and the 50 genes with the greatest underexpression in at least two thirds of all tumors compared with the IOSE reference cell line are shown. **Error bars** are 95% confidence intervals surrounding the mean. HE4 and CD24 genes (shown in boldface type) have been previously reported to be overexpressed in ovarian cancers. Several of the overexpressed genes have been demonstrated to be interferon-responsive and are shown in italic type. An **asterisk** denotes immediate-early response genes.

also included an IOSE cell RNA sample and a normal ovarian RNA sample in the sqRT–PCR experiments for comparison. The results of these sqRT–PCR experiments were consistent with the cDNA microarray relative expression data for all six genes evaluated (Fig. 6). As anticipated from previous reports *(9,19–21)* and our microarray results (Fig. 5), HE4 expression was consistently higher in all 15 tumor samples than in the IOSE reference cell line and a normal ovary (Fig. 6). Compared with normal ovary, invariant chain, also known as CD74, was found to be overexpressed in 11 of 15 tumors, and RGS1 was found to be overexpressed in 13 of 15 tumors tested by use of sqRT–PCR (Fig. 6). Both genes were among the top 25 overexpressed genes in tumors (Fig. 5).

In the microarray experiments, several members of the immediate-early response cascade showed elevated expression in

tumors compared with IOSE cells (see above); however, some of these genes have previously been shown to have lower expression in ovarian cancer than in normal ovary *(9,21)*. This discrepancy indicated that the elevated relative ratio observed in our experiments may be driven by low expression levels of these genes in IOSE cells grown in culture. To test this hypothesis, sqRT–PCR was used to compare ZFP36 (an immediate-early gene, also known as G0S24 and Tis11) expression in tumors with that in normal ovary and IOSE cells. As suspected, normal ovary had one of the highest expression levels of ZFP36, followed by that of the majority of tumors (Fig. 6), and the lowest expression level was observed in IOSE cells. In a similar instance, microarray experiments revealed that TOP2A gene expression was lower in all tumors than in the IOSE cell reference (data not shown). Previous investigations had reported variable
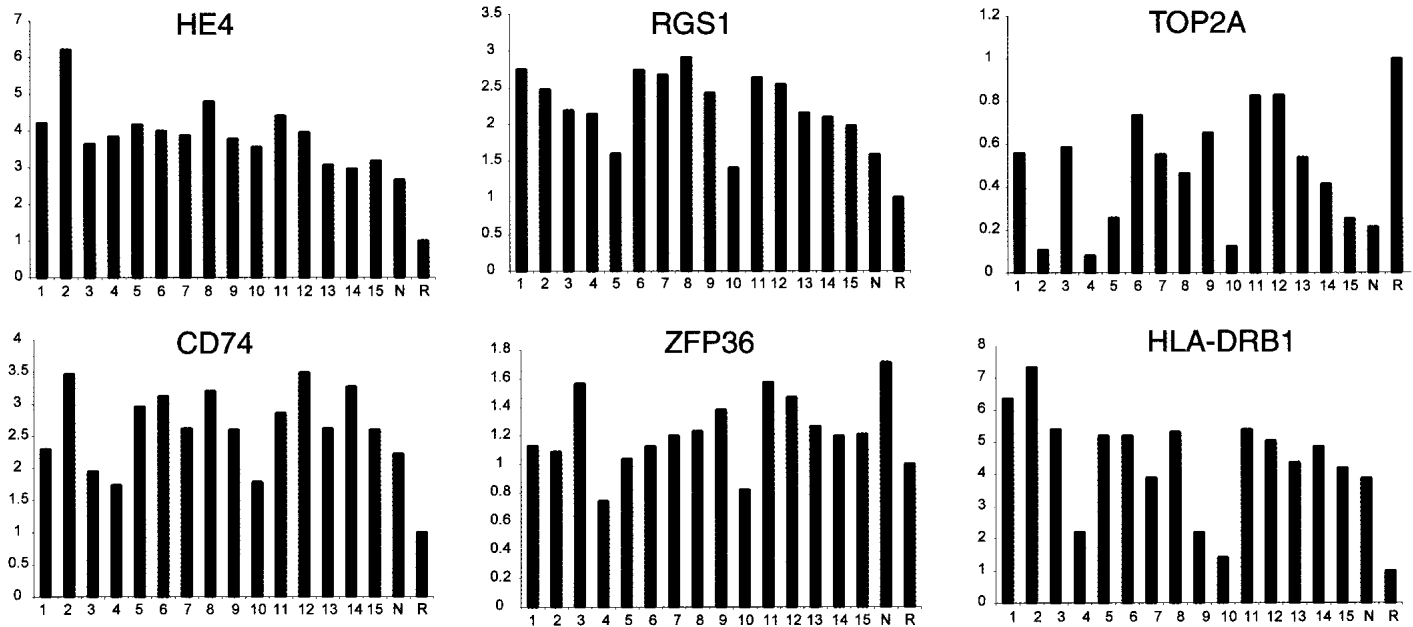
Fig. 6. Semiquantitative reverse transcription–polymerase chain reaction (sqRT–PCR) analysis of gene expression to confirm the complementary DNA microarray data. Expression patterns of select genes were examined with sqRT–PCR in representative BRCA1-linked (**bars 1–5**), BRCA2-linked (**bars 6–10**), and sporadic (**bars 11–15**) samples. The expression level of each gene in the tumor samples was compared with those of normal postmenopausal ovary (**bar N**) and the reference IOSE cells (**bar R**). All data were normalized to the amount of β-actin and are presented as the fold expression compared with that of the IOSE reference RNA.

but often higher expression of this gene in ovarian cancer *(22,23)*. We, therefore, further evaluated TOP2A expression by sqRT–PCR. The expression of TOP2A was found to be highest in the reference IOSE RNA, thus explaining the microarray observations. Furthermore, compared with the expression level in the normal postmenopausal ovary, 12 of the 15 tested tumor samples showed elevated (and variable) TOP2A gene expression, confirming previous observations *(22,23)*.

DISCUSSION

Our investigation reveals that BRCA1 and BRCA2 germline mutations lead to tumors with distinct molecular phenotypes. Furthermore, the same BRCA-discriminating molecular pathways also appear in non-BRCA-associated sporadic ovarian cancers, as demonstrated by the ability of the genes whose expression discriminates BRCA1-linked and BRCA2-linked tumors to define two major subgroups within the sporadic tumors. Thus, these BRCA-associated pathways are likely to play an important role in both hereditary and sporadic ovarian cancers and may therefore represent suitable therapeutic targets.

Hedenfalk et al. *(24)* recently published their findings on gene expression profiles of BRCA-linked and sporadic breast cancers. It is difficult to directly compare their results with our observations because of inherent differences between the tumor types studied (breast versus ovarian), the experimental design, and the composition of the cDNA microarrays used in the two studies. Nonetheless, in terms of overall gene expression patterns, these investigators also found the largest number of differentially expressed genes between BRCA1 and BRCA2 samples. However, this report did not address the possible presence of "BRCA-like" expression patterns within the sporadic group that may have been difficult to discern because of the small number of samples.

The observation that BRCA-associated gene expression pro-files are recapitulated in major subsets of sporadic tumors suggests that molecular mechanisms common to both hereditary and sporadic ovarian carcinogenesis exist. The most obvious possible etiology for the presence of such common molecular pathways would be the disruption of BRCA function in sporadic tumors. Somatic mutations in the BRCA genes are uncommon *(25,26)*, but methylation-induced transcriptional silencing of the BRCA1 gene has been demonstrated in approximately 15% of sporadic breast and ovarian cancers *(27–30)*. Although the clinical significance of this observation remains unclear *(31)*, the fact that BRCA1 promoter hypermethylation is observed selectively in breast and ovarian tumors and not in other tumors provides circumstantial evidence for its pathologic significance *(27,29)*. In addition, the recent report of a BRCA1-like expression profile in a sporadic breast tumor with BRCA1 promoter hypermethylation is also consistent with a role for epigenetic alterations of BRCA1 in some sporadic cancers *(24)*. Promoter methylation represents only one possible mechanism for BRCA functional inactivation. Furthermore, genetic or epigenetic alterations of BRCA-interacting proteins or other downstream mediators may produce carcinogenic sequelae similar to those resulting from BRCA dysfunction. Regardless of the nature of the initial alteration, the ensuing downstream molecular events may lead to the activation of dichotomous pathways resembling those associated with a BRCA1 or a BRCA2 mutation.

Our data derived from clinical BRCA-linked samples also raise interesting mechanistic questions. The exact functions of BRCA1 and BRCA2 remain poorly understood. Both tumor suppressors have been implicated in DNA repair, chromatin remodeling, and transcriptional regulation *(2–5)*, suggesting that BRCA1 and BRCA2 have the same, or very similar, functions in the carcinogenic process. In contrast, distinct BRCA1-associated and BRCA2-associated expression profiles in the resultant tumors point to functional differences between the BRCA proteins. Some of these differences may be in transcriptional con-

trol. Further investigation of the transcriptional regulatory functions of BRCA1 and BRCA2 may reveal potentially unique roles for these tumor suppressors and will provide additional insights into the biologic behavior of tumors associated with their dysfunction.

An unexpected finding in our investigation was the disproportionate number of genes located on Xp11 that showed higher mean expression in BRCA1-linked tumors relative to sporadic tumors. Because the differences in the mean expression for each gene between BRCA1-linked and sporadic tumors are modest, this observation is more likely to be the result of transcriptional dysregulation than of gene amplification. The short arm of chromosome X has been identified as a key region whose loss is associated with ovarian agenesis or premature ovarian failure, which are commonly observed in Turner's syndrome and related disorders *(32,33)*. However, during early development, all embryos with partial or complete loss of an X chromosome initially undergo ovarian and follicular development. The final phenotype is the result of accelerated atresia of ovarian tissue rather than agenesis *per se*. The short arm of the X chromosome is likely to contain genes involved in ovarian maintenance *(32)*. Thus, the overexpression of such potential ovarian survival factors on the X chromosome through a mechanism involving the loss of BRCA1, may be involved in ovarian carcinogenesis. In support of such an X chromosome-linked mechanism, BRCA1-linked ovarian cancers have been reported to have a higher frequency of aberrations on the X chromosome, such as nonrandom X chromosome inactivation *(34)* and loss of heterozygosity of regions in Xp and Xq *(35)*. Further investigation of the genes on the X chromosome, particularly in the Xp11.23 region, may reveal potential candidate genes for interaction with and/or regulation by BRCA1 that may be involved in ovarian carcinogenesis.

We also investigated the common molecular features of ovarian cancer by comparing gene expression in tumor samples and in the IOSE reference cells. IOSE cells served as a useful experimental control by allowing us to compare gene expression in ovarian tumors with that of a biologically relevant epithelium that was proliferating yet nontransformed and nontumorigenic. We do, however, recognize that the reference IOSE cell lines have a gene expression profile similar but not identical to that of normal ovarian surface epithelium *(20)*. Despite this limitation, we believe that valuable information is likely to be gained by comparing gene expression in tumors and IOSE cells. The biologic validity of this approach was confirmed by the identification of several genes, including HE4, CLU, and CD24, previously reported to be overexpressed in ovarian cancer compared with normal ovarian epithelium *(9,21,36)*. In addition, CD74 and CD24 have been shown to be overexpressed in other malignancies and may represent future common therapeutic targets *(37–39)*. Further investigation of other genes found in this study to be overexpressed in ovarian tumors may identify potential markers for ovarian cancer.

Many of the genes found to be overexpressed in tumors are characterized as interferon inducible, including several members of the major histocompatibility complex class II family. However, this induction may occur through an alternative pathway, because no increase in interferon-γ or the key downstream transcription factor class II transactivator (CIITA) was observed. In support of this hypothesis, other investigators have reported that ovarian cancers do not express interferon-γ *(40)*. Furthermore,

induction of major histocompatibility complex class II genes independent of interferon-γ and CIITA has been described *(41,42)*. The mechanism for this induction is not well-understood but may involve changes in histone acetylation *(43)*. Interferon-independent induction of other normally interferon-inducible genes has also been reported. One example is the induction of 9–27 (IFITM1) and 1-8d (IFITM2) in p53-deficient leukemia cells after ionizing radiation; this induction was shown to be independent of interferon-γ and interferon regulatory factor-1 (IRF1) *(44)*. This finding parallels our observation of IFITM1 and IFITM2 overexpression in ovarian tumors (Fig. 5) that have a high incidence of TP53 mutations *(13,45)*. Further investigation of this alternative pathway in ovarian cancer may lead to insights into the biology of these tumors and their interaction with the immune system.

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