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GENE-EXPRESSION PROFILES IN HEREDITARY BREAST CANCER

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ABSTRACT

Background   Many cases of hereditary breast cancer are due to mutations in either the *BRCA1* or the *BRCA2* gene. The histopathological changes in these cancers are often characteristic of the mutant gene. We hypothesized that the genes expressed by these two types of tumors are also distinctive, perhaps allowing us to identify cases of hereditary breast cancer on the basis of gene-expression profiles.

Methods   RNA from samples of primary tumors from seven carriers of the *BRCA1* mutation, seven carriers of the *BRCA2* mutation, and seven patients with sporadic cases of breast cancer was compared with a microarray of 6512 complementary DNA clones of 5361 genes. Statistical analyses were used to identify a set of genes that could distinguish the *BRCA1* genotype from the *BRCA2* genotype.

Results   Permutation analysis of multivariate classification functions established that the gene-expression profiles of tumors with *BRCA1* mutations, tumors with *BRCA2* mutations, and sporadic tumors differed significantly from each other. An analysis of variance between the levels of gene expression and the genotype of the samples identified 176 genes that were differentially expressed in tumors with *BRCA1* mutations and tumors with *BRCA2* mutations. Given the known properties of some of the genes in this panel, our findings indicate that there are functional differences between breast tumors with *BRCA1* mutations and those with *BRCA2* mutations.

Conclusions   Significantly different groups of genes are expressed by breast cancers with *BRCA1* mutations and breast cancers with *BRCA2* mutations. Our results suggest that a heritable mutation influences the gene-expression profile of the cancer. (N Engl J Med 2001;344:539-48.)

INHERITANCE of a mutant *BRCA1* or *BRCA2* gene (numbers 113705 and 600185, respectively, in Online Mendelian Inheritance in Man, a catalogue of inherited diseases) confers a lifetime risk of breast cancer of 50 to 85 percent and a lifetime risk of ovarian cancer of 15 to 45 percent.[1-6] These germ-line mutations account for a substantial proportion of inherited breast and ovarian cancers,[7] but it is likely that additional susceptibility genes will be discovered.[8,9]

Certain pathological features can help to distinguish breast tumors with *BRCA1* mutations from those with *BRCA2* mutations. Tumors with *BRCA1* mutations are high-grade cancers with a high mitotic index, "pushing" tumor margins (i.e., noninfiltrating, smooth edges), and a lymphocytic infiltrate, whereas tumors with *BRCA2* mutations are heterogeneous, are often relatively high grade, and display substantially less tu-

bule formation. The proportion of the perimeter with continuous pushing margins can distinguish both types of tumors from sporadic cases of breast cancer.[10] Tumors with *BRCA1* mutations are generally negative for both estrogen and progesterone receptors, whereas most tumors with *BRCA2* mutations are positive for these hormone receptors.[11-14] These differences imply that the mutant *BRCA1* and *BRCA2* genes induce the formation of breast tumors through separate pathways.

The BRCA1 and BRCA2 proteins participate in DNA repair and homologous recombination and probably other cellular processes.[15] A cell with a mutant *BRCA1* or *BRCA2* gene, which therefore lacks functional BRCA1 or BRCA2 protein, has a decreased ability to repair damaged DNA. In animal models, this defect causes genomic instability.[16] In humans, breast tumors in carriers of mutant *BRCA1* or *BRCA2* genes are characterized by a large number of chromosomal changes, some of which differ depending on the genotype.[17]

In this study, we examined breast-cancer tissues from patients with *BRCA1*-related cancer, patients with *BRCA2*-related cancer, and patients with sporadic cases of breast cancer to determine whether there are distinctive patterns of global gene expression in these three kinds of tumors.

METHODS

Patients and Biopsy Specimens

Patients with primary breast cancer and who had a family history of breast or ovarian cancer, or both, that was compatible with a dominant mode of inheritance were referred for genetic counseling to the Oncogenetic Clinic of Lund University Hospital. These patients were asked to provide a blood sample and to sign an informed-consent form authorizing an analysis for *BRCA1* and *BRCA2* mutations. Mutation analysis was performed as described previously.[18] Biopsy specimens of primary breast tumors from patients with germ-line mutations of *BRCA1* (seven patients) or *BRCA2* (eight tumors from seven patients) were selected for analysis. In addition, seven patients with sporadic cases of primary breast cancer whose family history was unknown were also identified. These patients had either estrogen-receptor–negative, aggressive tumors (characterized by aneuploidy and a high fraction of cells in S phase) or estrogen-receptor–positive, less aggressive tumors. Total RNA was extracted from flash-frozen tumor specimens, which had been stored at −80°C, with the use of the RNeasy Maxi Kit (Qiagen) and Trizol reagent (GIBCO BRL) according to the manufacturers' recommendations.[19]

The studies were approved by the institutional review boards of both Lund University and the National Human Genome Research Institute of the National Institutes of Health.

Microarrays of Complementary DNA

We obtained samples of complementary DNA (cDNA) with verified sequences[20] under a Cooperative Research and Development Agreement with Research Genetics. Gene names are listed according to build 110 of the UniGene human-sequence collection (available at the UniGene Web site: http://www.ncbi.nlm.nih.gov/UniGene/build.html). The 6512 cDNAs we used represent 5361 unique genes: 2905 are known and 2456 are unknown genes.

Microarrays were hybridized and scanned, and image analysis was performed as described previously (Fig. 1).[20-22] The reference cell line, MCF-10A (American Type Culture Collection, CRL-10317), a nontumorigenic breast-cell line, was an internal standard against which each tumor was compared (not a biologic control). RNA from normal breast epithelial cells was included for comparison (Fig. 2B).

Tissue Microarrays

A microarray of breast-cancer tissue (Fig. 1), constructed as previously described,[23] consisted of samples of 113 primary breast tumors, in duplicate, derived from a population-based series of patients from southern Sweden in whom the disease had been diagnosed before the age of 40 years. The patients consisted of 23 with *BRCA1* mutations, 17 with *BRCA2* mutations, 20 with familial breast cancer (defined as a history of breast or ovarian cancer in at least one first-degree relative) but no *BRCA1* or *BRCA2* mutations, 19 with possibly familial breast cancer (defined as a history of breast or ovarian cancer in at least one second-degree relative) but no *BRCA1* or *BRCA2* mutations, and 34 with sporadic breast cancer. The duplicate core-tissue–biopsy specimens (diameter, 0.6 mm) were obtained from the least differentiated regions of individual paraffin-embedded tumors.

Analysis of DNA Methylation

Patterns of DNA methylation in the CpG island of the *BRCA1* gene were determined by a methylation-specific polymerase chain reaction.[24]

Statistical Analysis

Tests for associations between each type of mutation (*BRCA1* or *BRCA2*) and clinical variables were performed with Fisher's exact test for categorical variables and the Wilcoxon–Mann–Whitney test for continuous and ordered variables. Reported P values are exact and have not been corrected for multiple comparisons (30 variables were tested). All P values are two-sided.

In the analyses involving cDNA microarrays, a total of 3226 genes with an average intensity (level of expression) of more than 2500 pixels among all samples, an average spot area of more than 40 pixels, and no more than one sample in which the size of the spot area was 0 pixels were included.[22] A conservative estimate of experimental variance (involving hybridization of pairs of cDNAs on different days) indicated that our observations fell within the 95 percent confidence interval of 0.61 to 1.65 for a mean value of 1.0.

We used a class-prediction method to determine whether the patterns of gene expression could be used to classify tumor samples into two classes according to the presence or absence of *BRCA1* and *BRCA2* mutations (positive or negative for *BRCA1* mutations and positive or negative for *BRCA2* mutations), with use of a compound covariate predictor.[25] We estimated the misclassification rate using leave-one-out cross-validation and used random permutations of the class-membership indicators to determine the significance of the results.

We used three methods to generate lists of genes with different levels of expression among the groups of patients with breast cancer: modified F tests and t-tests, a weighted gene analysis, and mutual-information scoring (InfoScore). InfoScore uses a ranking-based scoring system and combinatorial permutation of sample labels to produce a rigorous statistical benchmarking of the over-abundance of genes whose differential expression pattern correlates with sample type (information available at http://www.labs.agilent.com/resources/techreports.html). An agglomerative hierarchical clustering algorithm was used to investigate any relation among the statistically significant discriminator genes.[19,20] We also used multidimensional scaling to show the correlation of expression of given subgroups of genes among various tumor samples.[20] In this three-dimensional rendering of the data, samples with similar expression profiles lie closer to each other than those with dissimilar profiles.

Supplemental Information

Additional information on the methods, clones, genes, samples, fluorescence-intensity ratios, and statistical methods is available at
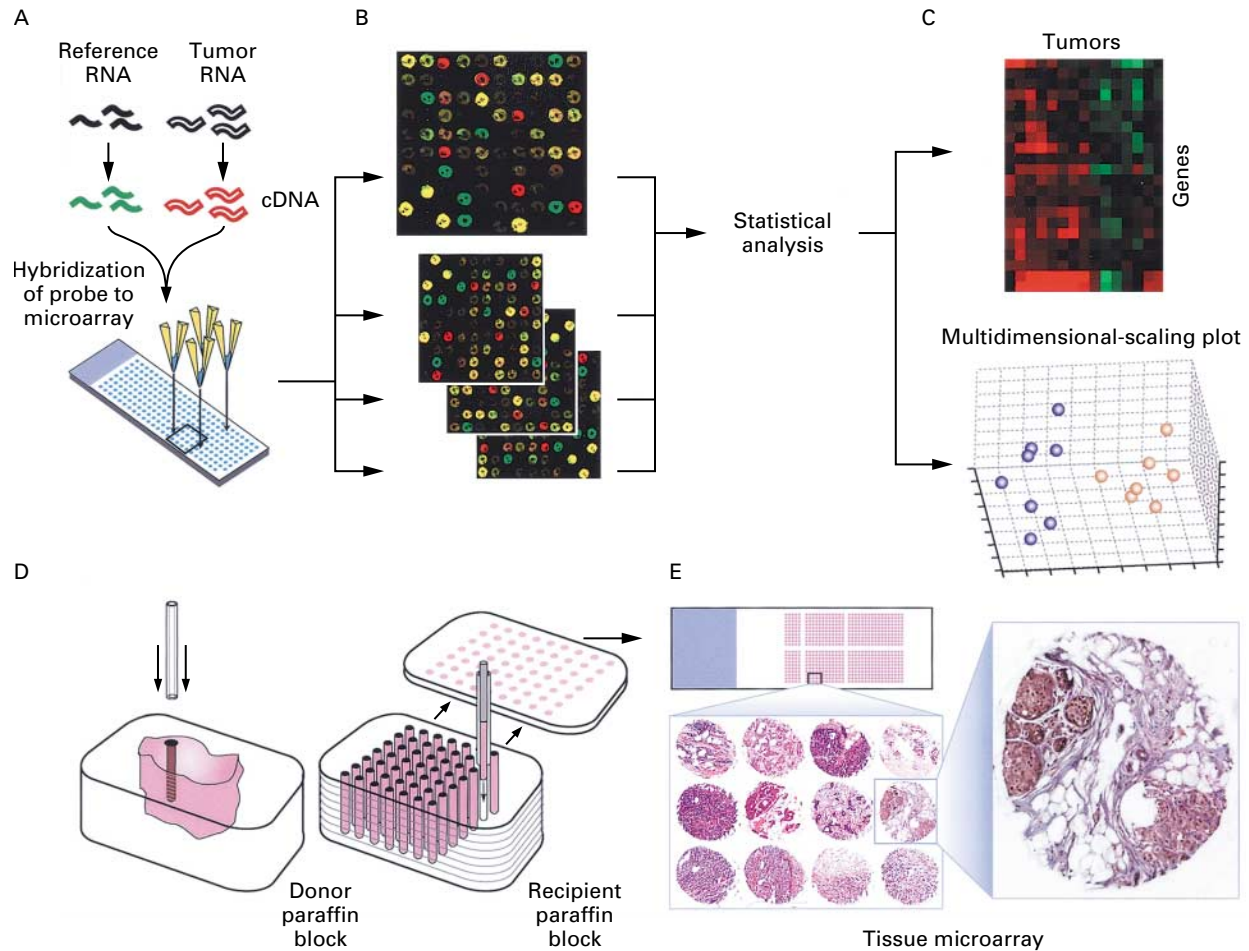
Figure 1. Overview of Procedures for Preparing and Analyzing Microarrays of Complementary DNA (cDNA) and Breast-Tumor Tissue.
As shown in Panel A, reference RNA and tumor RNA are labeled by reverse transcription with different fluorescent dyes (green for the reference cells and red for the tumor cells) and hybridized to a cDNA microarray containing robotically printed cDNA clones. As shown in Panel B, the slides are scanned with a confocal laser scanning microscope, and color images are generated for each hybridization with RNA from the tumor and reference cells. Genes up-regulated in the tumors appear red, whereas those with de-creased expression appear green. Genes with similar levels of expression in the two samples appear yellow. Genes of interest are selected on the basis of the differences in the level of expression by known tumor classes (e.g., *BRCA1*-mutation–positive and *BRCA2*-mutation–positive). Statistical analysis determines whether these differences in the gene-expression profiles are greater than would be expected by chance. As shown in Panel C, the differences in the patterns of gene expression between tumor classes can be portrayed in the form of a color-coded plot, and the relations between tumors can be portrayed in the form of a multidi-mensional-scaling plot. Tumors with similar gene-expression profiles cluster close to one another in the multidimensional-scaling plot. As shown in Panel D, particular genes of interest can be further studied through the use of a large number of arrayed, paraffin-embedded tumor specimens, referred to as tissue microarrays. As shown in Panel E, immunohistochemical analyses of hundreds or thousands of these arrayed biopsy specimens can be performed in order to extend the microarray findings.

http://www.nejm.org and at http://www.nhgri.nih.gov/DIR/Microarray.

RESULTS

Characteristics of the Tumors

Mutations in seven carriers of *BRCA1* mutations and seven carriers of *BRCA2* mutations were con-firmed by direct sequencing (Table 1). Specimens were also obtained from seven patients with sporadic pri-mary breast cancer. Tumors were classified patholog-ically according to criteria of the Breast Cancer Link-age Consortium[10,26,27]; all slides were read by a single pathologist. Grading was performed according to a previously described method.[28] The pathological re-sults for our cohort were similar to those of earlier studies.[10,12,26,29-31] All tumors with *BRCA1* mutations were grade 3, most had lymphocytic infiltration and extensive pushing margins, most tended to grow in sheets, and several had confluent necrosis; there was one atypical medullary carcinoma. These features as a whole were not as common among patients with *BRCA2* mutations.[30,31] As expected, estrogen and pro-
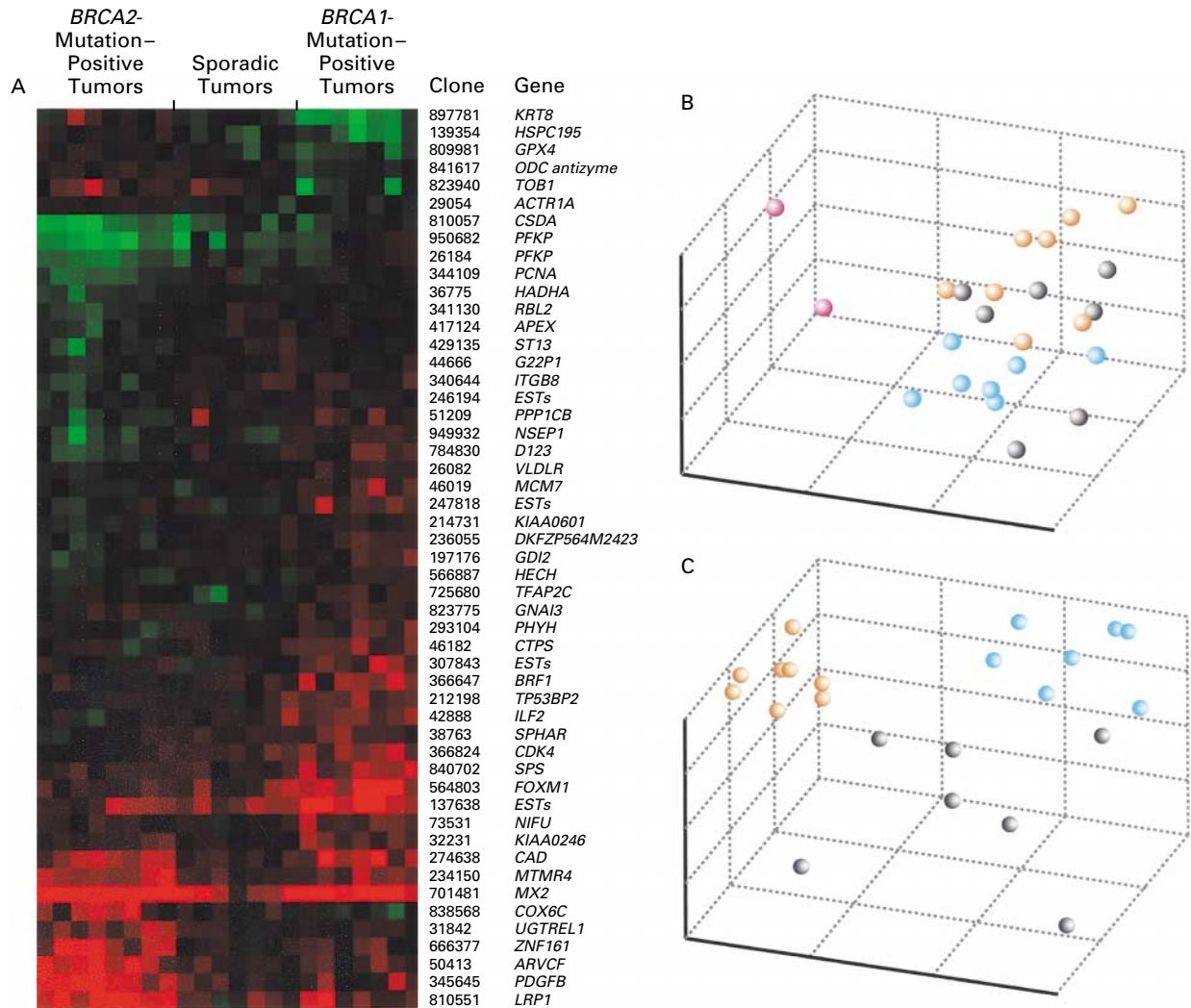
Figure 2. Identification of Genes That Can Be Used to Differentiate *BRCA1*-Mutation–Positive, *BRCA2*-Mutation–Positive, and Sporadic Cases of Primary Breast Cancer.

Panel A shows the 51 genes that best differentiated among the three types of tumors, as determined by a modified F test ($\alpha = 0.001$). Panel B shows the multidimensional-scaling plot of the seven samples from patients with *BRCA1*-mutation–positive breast tumors (blue circles), eight samples from patients with *BRCA2*-mutation–positive tumors (tan circles), seven samples from patients with sporadic tumors (gray circles), and two samples of normal mammary epithelial cells (pink circles) that included all 3226 genes that met the criteria for inclusion in the analysis. Panel C shows the multidimensional-scaling plot of the 22 primary-tumor samples that included the 51 genes that best differentiated the three types of tumors, as evidenced by the clustering of the *BRCA2*-mutation–positive samples and the *BRCA1*-mutation–positive samples.

gesterone receptors were absent in tumors from all the patients with *BRCA1* mutations and also from one patient with a *BRCA2* mutation.[11,12]

Use of Gene-Expression Profiles to Identify Hereditary Breast Cancers

Fluorescence-intensity ratios were calculated and gene-expression profiles were generated for each sample. The gene-expression profiles were used to determine which of the genes expressed by the tumors correlated with the *BRCA1*-mutation–positive tumors,

the *BRCA2*-mutation–positive tumors, and the sporadic tumors. Figure 2A shows the results of a modified F test, which yielded 51 genes ($\alpha = 0.001$) whose variation in expression among all experiments best differentiated among these types of cancers. The multidimensional-scaling plot of the 22 samples from patients with primary breast cancer and 2 samples of normal mammary epithelial cells that included all 3226 genes that met the criteria for inclusion is shown in Figure 2B. The multidimensional-scaling plot of the 22 samples from patients with primary breast cancer that in-

TABLE 1. CHARACTERISTICS OF BREAST-CANCER TISSUE FROM PATIENTS WITH *BRCA1*-MUTATION–POSITIVE, *BRCA2*-MUTATION–POSITIVE, OR SPORADIC CASES OF PRIMARY BREAST CANCER.*

PATIENT NO. AND TYPE OF CANCER	MUTATION	TYPE OF INVASIVE CARCINOMA	GRADE (SCORE)†	GROWTH AS SOLID SHEET	PUSHING MARGINS	ESTROGEN-RECEPTOR STATUS‡	PROGESTERONE-RECEPTOR STATUS‡	PLOIDY	CELLS IN S PHASE
				% of tumor					%
BRCA1-mutation–positive									
1	C1806T	Ductal, NST	3 (8)	>75	25–75	−	−	HD	20
2	2594delC	Ductal, NST	3 (8)	25–75	<25	−	−	MP	15
3	5382insC	Ductal, NST	3 (9)	25–75	25–75	−	−	AP	25
4	T300G	Ductal, NST	3 (9)	>75	ND	−	−	AP	ND
5	1201del11	Atypical medullary	3 (9)	>75	>75	−	−	AP	22
6	C1806T	ND	ND	ND	ND	−	−	AP	15
7	1201del11	Ductal, NST	3 (9)	>75	>75	−	−	AP	26
BRCA2-mutation–positive									
8	5445del5	Ductal, NST	3 (9)	>75	<25	+++	+	D	13
9	A3058T	Ductal, NST	2 (6)	25–75	None	+++	−	AP	10
10§	2024del5	Ductal, NST	3 (9)	25–75	25–75	+	++	AP	15
10§	2024del5	ND	ND	ND	ND	ND	ND	ND	15
11	4486delG	Ductal, NST	3 (9)	>75	>75	−	−	AP	23
12	C6293G	Pleomorphic lobular	2 (6)	25–75	None	++	+++	ND	ND
13	A3058T	Ductal, NST	2 (7)	25–75	25–75	++	−	D	6.8
14	4486delG	ND	ND	ND	ND	+++	+++	TP	6.2
Sporadic									
15	ND	Ductal	ND	ND	ND	++	++	D	4.7
16	ND	Ductal	ND	ND	ND	+	+	D	9.2
17	ND	Ductal	ND	ND	ND	+++	−	AP	12
18	ND	Tubular	ND	ND	ND	+	+++	MP	14
19	ND	Ductal, lobular	ND	ND	ND	−	−	AP	15
20	None	Ductal	ND	ND	ND	−	−	AP	18
21	ND	Ductal	ND	ND	ND	−	−	AP	17

*All patients but Patient 14 were women. NST denotes no specific type, HD hypodiploid, MP multiploid, AP aneuploid, ND not determined, D diploid, and TP tetraploid.

†The histologic grade was based on the aggregate score for three variables (mitotic frequency, nuclear pleomorphism, and tubular differentiation) as follows: grade 1 indicated a well-differentiated tumor (1 to 5 points), grade 2 a moderately differentiated tumor (6 or 7 points), and grade 3 a poorly differentiated tumor (8 or 9 points).

‡The receptor status was considered to be negative (−) if receptor levels were less than 10 fmol per milligram of protein, positive (+) if levels were 10 to 25 fmol per milligram of protein, strongly positive (++) if levels were 26 to 200 fmol per milligram of protein, and very strongly positive (+++) if levels were more than 200 fmol per milligram of protein.

§Patient 10 had unilateral tumors.

cluded the 51 genes that best differentiated among the three types of tumors is shown in Figure 2C.

We used a class-prediction method to determine whether the gene-expression profiles of the 22 breast-tumor samples accurately identified them as positive or negative for *BRCA1* mutations or as positive or negative for *BRCA2* mutations. For the analysis of all 22 tumor samples, 9 genes were differentially expressed between *BRCA1*-mutation–positive tumors and *BRCA1*-mutation–negative tumors, and 11 genes were differentially expressed between *BRCA2*-mutation–positive tumors and *BRCA2*-mutation–negative tumors ($\alpha=0.0001$) (Table 2). All 7 tumors with *BRCA1* mutations and 14 of 15 tumors without *BRCA1* mutations were correctly identified in the *BRCA1* classification. Five of 8 tumors with *BRCA2* mutations and 13 of 14 tumors without *BRCA2* mutations were correctly identified in the *BRCA2* classification. The accuracy of these classifications was sig-

nificant as compared with randomized data. Only 0.3 percent of data sets in which *BRCA1* classifications were permuted resulted in the misclassification of one or fewer samples, and only 4.0 percent of data sets in which *BRCA2* classifications were permuted resulted in the misclassification of four or fewer samples. Similar results were obtained when we applied naive Bayesian classifiers.[32]

Taken together, these results suggest that the gene-expression profiles of *BRCA1*-mutation–positive and *BRCA2*-mutation–positive tumors are generally distinctive and differ from each other as well as from those of sporadic tumors. However, identification of the *BRCA2*-mutation–positive and *BRCA2*-mutation–negative tumors was less accurate than the identification of *BRCA1*-mutation–positive and *BRCA1*-mutation–negative tumors. Of the three samples that were misclassified in the *BRCA2* classification, two had the earliest truncating mutation among the eight *BRCA2*

TABLE 2. CLASSIFICATION OF HEREDITARY BREAST CANCERS ACCORDING TO THE GENE-EXPRESSION PROFILE.

CLASSIFICATION	NO. OF SAMPLES ANALYZED	NO. OF DIFFERENTIALLY EXPRESSED GENES*	NO. OF MISCLASSIFIED SAMPLES	PERCENTAGE OF RANDOM PERMUTATIONS WITH MISCLASSIFICATIONS†
BRCA1-mutation–positive vs. *BRCA1*-mutation–negative	22	9‡	1 (*BRCA1*-mutation–positive, 0; *BRCA1*-mutation–negative, 1)	With ≤1 misclassification, 0.3
BRCA2-mutation–positive vs. *BRCA2*-mutation–negative	22	11§	4 (*BRCA2*-mutation–positive, 3; *BRCA2*-mutation–negative, 1)	With ≤4 misclassifications, 4.0

*The results were determined with use of the standard two-sample t-test ($\alpha = 0.0001$).

†There was a total of 1000 permutations for each classification.

‡Nine clones (212198, 897781, 840702, 566887, 307843, 247818, 26082, 46019, and 366647) were differentially expressed between *BRCA1*-mutation–positive tumors and *BRCA1*-mutation–negative tumors.

§Eleven clones (31842, 666377, 50413, 784830, 29054, 36775, 51209, 340644, 344109, 366824, and 345645) were differentially expressed between *BRCA2*-mutation–positive tumors and *BRCA2*-mutation–negative tumors.

mutations identified in the study (Table 1), and the other came from a man with breast cancer. The gene-expression profile of his *BRCA2*-mutation–positive tumor was very similar to the profiles of the other such tumors, but the expression of a small subgroup of genes could have caused the misclassification.

Figure 3 shows the way in which we identified the genes that are most important in distinguishing a *BRCA1*-mutation–positive breast cancer from a *BRCA2*-mutation–positive breast cancer. A total of 176 such genes were identified by all three statistical methods (modified t-test, weighted gene analysis, and mutual-information scoring). This list shows that *BRCA1* and *BRCA2* tumors differ significantly in their gene-expression profiles. Within this list is a large block of genes (shown in red in Fig. 3A) that are up-regulated in *BRCA1*-mutation–positive samples but not in *BRCA2*-mutation–positive samples. Examination of individual genes in this block suggests the coordinated transcriptional activation of two major cellular processes in *BRCA1*-mutation–positive samples: DNA repair and apoptosis. DNA-repair pathways are reflected by genes (e.g., *MSH2*)[33] that participate in the activation of cellular responses to stress. In addition,
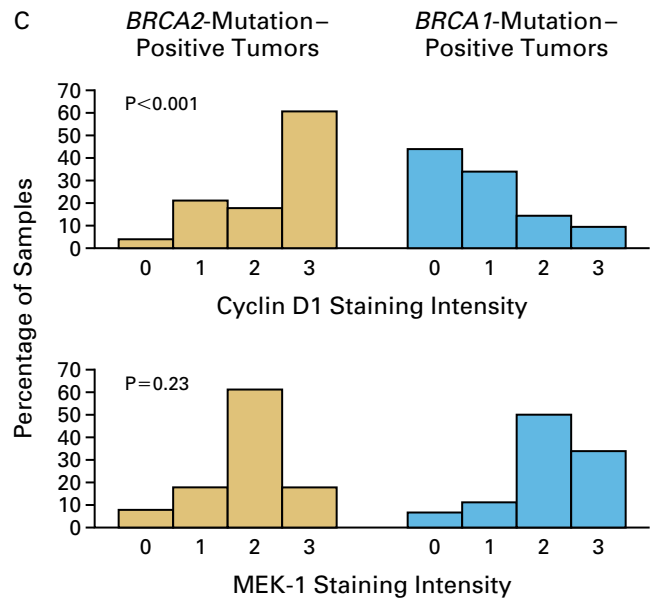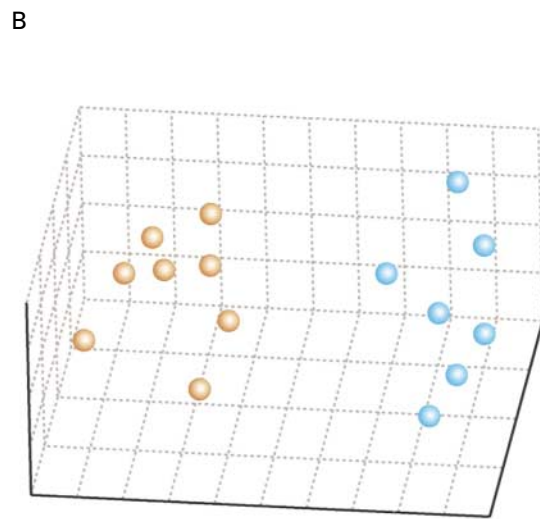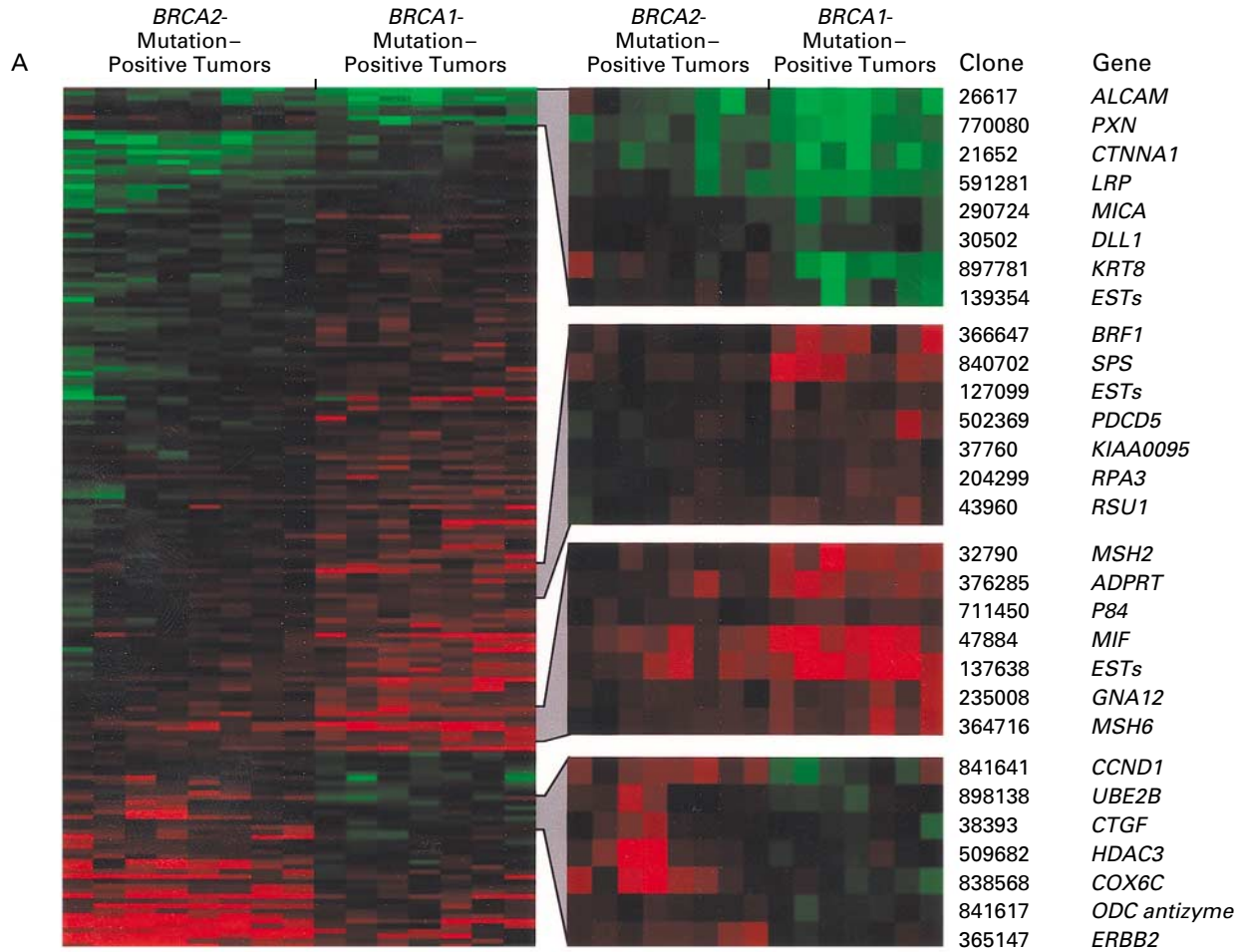
BRCA1-mutation–positive tumors display increased expression of genes associated with inducing apoptosis (e.g., *PDCD5*)[34] and decreased expression of genes involved in suppressing apoptosis (e.g., *CTGF*).[35]

This finding suggests that the mutation of *BRCA1* leads to a constitutive stress-type state. The cellular response to damaged DNA is complex and includes the activation of "checkpoints" in the cell cycle, DNA repair, and changes in gene transcription — all these functions involve the proteins encoded by *BRCA1* and *BRCA2*.[15] The finding that *BRCA1*-mutation–positive tumors have increased expression of genes involved in a response to stress should provide further insight into the different functions of the two genes.

High-Density Tissue Microarrays

A high-density microarray of breast-cancer tissue (Fig. 1D and 1E)[23] was used to determine whether levels of proteins encoded by selected genes (as measured by immunohistochemical analysis) correlate with the cDNA microarray results. Figure 3C illustrates the results for two genes (encoding cyclin D1 and mitogen-activated protein kinase kinase 1 [MEK-1]) against a microarray containing 113 breast-cancer spec-

Figure 3 (facing page). Analysis of Genes Discriminating Breast Cancers with *BRCA1* Mutations from Those with *BRCA2* Mutations. Three statistical methods were used to generate lists of genes that discriminate between the *BRCA1*-mutation–positive and *BRCA2*-mutation–positive breast tumors; the three lists were then combined into a consensus list consisting of 176 genes. Panel A shows the *BRCA1*-mutation–positive and *BRCA2*-mutation–positive samples of breast-cancer tissue with regard to the level of expression of the 176 genes on the consensus list. Panel B shows the resulting multidimensional-scaling plot; it illustrates the ability of these 176 genes to separate *BRCA1*-mutation–positive tumors (blue circles) from *BRCA2*-mutation–positive tumors (tan circles). Panel C shows the results of staining of tissue microarrays with antibodies against cyclin D1 and MEK-1. The average nuclear intensity is considered to be 0 in the absence of staining, 1 in the presence of weak staining, 2 in the presence of moderate staining, and 3 in the presence of strong staining. Each analysis included 23 *BRCA1*-mutation–positive samples and 17 *BRCA2*-mutation–positive samples. Each tumor was represented on the array by two cores; the agreement in scores between each pair was high as measured by a weighted kappa statistic. The Wilcoxon–Mann–Whitney test was used to test for differences between *BRCA1*-mutation–positive and *BRCA2*-mutation–positive tissues (with use of the mean score for both cores). P values are two-sided and exact. The specimens used in the analysis of cDNA microarrays and the tumor-microarray analyses differed but were from the same institution (Lund University Hospital).

A

Clone	Gene
26617	*ALCAM*
770080	*PXN*
21652	*CTNNA1*
591281	*LRP*
290724	*MICA*
30502	*DLL1*
897781	*KRT8*
139354	*ESTs*
366647	*BRF1*
840702	*SPS*
127099	*ESTs*
502369	*PDCD5*
37760	*KIAA0095*
204299	*RPA3*
43960	*RSU1*
32790	*MSH2*
376285	*ADPRT*
711450	*P84*
47884	*MIF*
137638	*ESTs*
235008	*GNA12*
364716	*MSH6*
841641	*CCND1*
898138	*UBE2B*
38393	*CTGF*
509682	*HDAC3*
838568	*COX6C*
841617	*ODC antizyme*
365147	*ERBB2*

imens obtained from the same referring hospital that provided all the samples used in cDNA-microarray analyses.

The intensity of staining for cyclin D1 differed significantly (P<0.001): *BRCA2*-mutation–positive tumors displayed more intense staining than *BRCA1*-mutation–positive tumors, a finding that is consistent with the expression of cyclin D1 in cDNA-microarray experiments (P<0.001 by the t-test) (Fig. 3C). As expected, the negative control MEK-1, the gene for which was not on the consensus gene list, had similar levels of expression in the two types of tumors (P=0.23) (Fig. 3C and http://www.nhgri.nih.gov/DIR/Microarray).

Effect of DNA Methylation on Gene Expression

In our analysis, only one tumor (from Patient 20, who had sporadic breast cancer) was misclassified as positive for a *BRCA1* mutation (Table 2 and Fig. 2C). As compared with the specimens from the other six patients with sporadic breast cancer, this specimen had a markedly reduced level of expression of *BRCA1,* perhaps because of an unrecognized mutation of *BRCA1* in this patient. On further investigation, the tumor was found to have phenotypic characteristics (e.g., negativity for estrogen receptors, a high grade, and a ductal location) that were consistent with the common clinical and pathological profiles of a *BRCA1*-mutation–positive breast cancer.

On approval by the institutional review board, the patient was contacted and agreed to be tested for a germ-line mutation in *BRCA1*. Using sequence-based mutation analysis and a chip-based system of mutation detection,[36] we found no mutation in the *BRCA1* gene. We then analyzed the *BRCA1* promoter region for aberrant methylation, which is known to silence *BRCA1* in sporadic cancers with no mutations in the gene.[24,37] Testing (in a blinded fashion) of all specimens of sporadic tumors from our study indicated that the misclassified tumor (from Patient 20) was the only one with hypermethylation of the *BRCA1* promoter region, indicative of the inactivation of *BRCA1* (Fig. 4). This result was corroborated by the finding that this tumor exhibited by far the lowest level of *BRCA1* messenger RNA of all the samples in the study.

DISCUSSION

Studies of the pathological features of breast cancer suggest that cancers with underlying germ-line mutations in *BRCA1* and *BRCA2* differ from each other and from cancers that do not carry these mutations.[10,26] However, methods to classify these cancers on the basis of such features have been prone to error and subjective interpretation. Our study, although limited in terms of the number of specimens, indicates that gene-expression technology can increase the specificity of the molecular classification of breast cancer.

Early reports suggested that there is a loss of es-

trogen and progesterone receptors in tumors with *BRCA1* mutations,[11-14] whereas tumors with *BRCA2* mutations are more variable in this respect but often have such receptors.[11] For this reason, some of the differences we found in the levels of expression of various genes between *BRCA1*-mutation–positive and *BRCA2*-mutation–positive breast cancers are probably due to differences in the genes whose expression is associated with these receptors. Nevertheless, these differences cannot explain all the findings. For example, one breast-cancer sample with a *BRCA2* mutation lacked estrogen and progesterone receptors, yet its gene-expression profile was very similar to those of the receptor-positive cancer specimens with *BRCA2* mutations. Also, many of the genes that were differentially expressed in receptor-positive and receptor-negative sporadic tumors did not distinguish between *BRCA1*-mutation–positive or *BRCA2*-mutation–positive tumors. Conversely, many of the genes that identified hereditary breast cancers were unable to separate receptor-positive from receptor-negative sporadic breast cancers. These results, together with those of a recently published study by Perou et al.,[38] indicate that cDNA microarrays can readily distinguish estrogen-receptor–positive from estrogen-receptor–negative sporadic breast tumors.

We used several statistical approaches to evaluate the patterns of gene expression in the breast-cancer specimens. Of the 22 specimens that we studied, the class-prediction method misclassified one sporadic tumor as positive for a *BRCA1* mutation, three *BRCA2*-mutation–positive samples as negative for a *BRCA2* mutation, and one tumor sample as positive for a *BRCA2* mutation. The different patterns of gene expression among the three types of breast cancer on microarray analyses therefore represent useful ways of distinguishing these types, but the method is clearly imprecise in determining the presence or absence of *BRCA2* mutations. The use of microarrays covering a larger proportion of the genome and the analysis of larger numbers of tumors may make possible a more precise molecular classification of breast cancer.

Our finding that a case of sporadic breast cancer appeared to arise from a *BRCA1* mutation prompted us to investigate the mechanism of the inactivation of this gene in this specimen. We found that the down-regulation of the expression of *BRCA1* in this tumor was associated with hypermethylation of the promoter region. This suggests that cDNA microarrays may be of use in identifying sporadic breast tumors with a phenotype resembling that of a *BRCA1*-mutation–positive breast cancer.[37] This unexpected finding prompted consideration of whether to contact the patient to request that she undergo testing for *BRCA1* mutations.

The institutional review boards of the participating centers initially waived the requirement to obtain the patients' consent to use these specimens, with the stip-
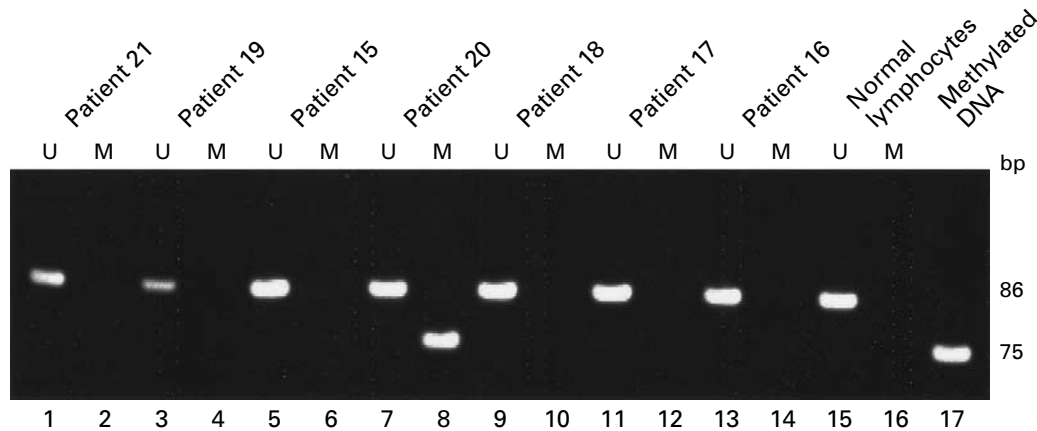
Figure 4. Methylation Analysis of the *BRCA1* Promoter Region in Tumor Samples from Seven Patients with Sporadic Breast Cancer.

A methylation-specific polymerase-chain-reaction assay was used to distinguish unmethylated alleles (U) from methylated alleles (M) of *BRCA1* on the basis of sequence changes produced by treating DNA with bisulfite, which converts unmethylated (but not methylated) cytosines to uracil, followed by a polymerase-chain-reaction assay involving primers designed for either methylated or unmethylated DNA.[24] The methylated product is 75 bp long, and the unmethylated product is 86 bp. DNA from normal lymphocytes was used as a negative control, and in vitro methylated DNA was used as a positive control.

ulation that the investigators would not contact subjects with the results. This approach was implemented to avoid providing results to subjects without their prior consent to receive results. The investigators and the institutional review boards evaluated this unanticipated finding, noting that patients with breast cancer who have a *BRCA1* mutation are at greater risk for ovarian cancer and breast cancer in the contralateral breast than patients with breast cancer who do not have a *BRCA1* mutation[39] and that preventive surgery (oophorectomy and mastectomy) might increase the life expectancy of such patients.[40] In addition, further research to determine why this sporadic breast tumor had a gene-expression profile similar to that of the *BRCA1*-mutation–positive samples might improve our understanding of breast cancer.

The institutional review boards agreed that the patient could be contacted to disclose the finding and request that she undergo further evaluation but asked that her primary physician make the final decision and be the initial conveyer of the information. The primary physician's established relationship with the patient placed him in the best position to weigh the clinical benefits and the harm of conveying this information. To avoid similar problems in future studies in which personal identifiers are retained, obtaining subjects' consent to be contacted in the event of a relevant finding should be strongly considered. One approach would be to incorporate such explicit consent in the surgical consent process. Whenever there appears to be a compelling need to contact a subject for clinical reasons, this decision should involve both the institutional review boards and the subject's physician.

Perhaps the most striking finding of our study is that tumor samples from patients with germ-line mutations in *BRCA1* and those from patients with such mutations in *BRCA2* differ significantly in their global patterns of gene expression, even though both mutant genes lead to breast and ovarian cancer. Study of the molecular differences between these cancers may improve our understanding of the way in which pathologically different breast cancers arise in carriers of *BRCA1* or *BRCA2* mutations. Our results indicate that a heritable mutation influences the gene-expression profile of a tumor.

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