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Altered Gene Expression in Morphologically Normal Epithelial Cells from Heterozygous Carriers of *BRCA1* or *BRCA2* Mutations

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Abstract

We hypothesized that cells bearing a single inherited “hit” in a tumor suppressor gene express an altered mRNA repertoire that may identify targets for measures that could delay or even prevent progression to carcinoma. We report here on the transcriptomes of primary breast and ovarian epithelial cells cultured from *BRCA1* and *BRCA2* mutation-carriers and controls. Our comparison analyses identified multiple changes in gene expression, in both tissues for both mutations, that were validated independently validated by real-time RT-PCR analysis. Several of the differentially expressed genes had been previously proposed as cancer markers, including mammaglobin in breast cancer and serum amyloid in ovarian cancer. These findings demonstrate that heterozygosity for a mutant tumor suppressor gene can alter the expression profiles of phenotypically normal epithelial cells in a gene-specific manner; these detectable effects of “one-hit” represent early molecular changes in tumorigenesis that may serve as novel biomarkers of cancer risk and as targets for chemoprevention.

Keywords

Breast cancer; Gynecological cancers: ovarian; BRCA1 and BRCA2 mutation; Tumor suppressor gene; Single-hit mutation; Transcriptome; Biomarkers

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Introduction

The notion of multistep carcinogenesis posits that rate-limiting mutations accumulate in a single cell and its progeny, marking recognizable histopathological transitions in the target tissues (1,2). The time required for this accumulation affords the opportunity to test whether pharmacological and/or dietary interventions can delay or prevent the transition to malignancy (3). Early targeted intervention would be optimally performed on persons with a very high risk of developing a specific cancer, as with those individuals who carry a germline mutation in a gene known to impose such a risk. Rationale for such an intervention is provided by early studies that demonstrated that cells heterozygous for a cancer-predisposing mutation could show abnormalities in tissue cultures; “one-hit” effects in heterozygous cells were seen in morphologically normal cultured fibroblasts and in epithelial cells derived from Familial Adenomatous Polyposis (FAP) patients (4-6). Furthermore, we have recently reported specific changes in protein expression in colonic epithelial cells from FAP patients (7). Support for the significance of these early changes comes from observations of similar aberrations in corresponding cancer cells, (7,8,9). Such changes may play a role in progression to malignancy and therefore constitute targets for strategies to delay or prevent such progression in FAP and in other genetic predispositions to cancer.

With this rationale in mind we have undertaken an investigation of two of the most common predisposing genes, *BRCA1* (10,11) and *BRCA2* (12,13, and references therein) in two important target tissues, breast and ovary. We note that previous reports on benign cells associated with breast cancer already suggest the possibility of such heterozygous effects.

We have compared the transcriptomes of primary breast and ovarian epithelial cultures from patients predisposed to cancer, bearing monoallelic *BRCA1* or *BRCA2* mutations, with corresponding cultures from control individuals. We demonstrate that the morphologically normal epithelial cells from mutation carriers exhibit abnormalities in a gene-specific and tissue-specific manner, consistent with detectable single-hit effects. These alterations constitute possible molecular targets for intervention on the path to cancer.

Materials and Methods

Subject accrual and biopsy specimens

All subjects were recruited with the approval of the FCCC Institutional Review Board, irrespective of gender, race and age. Individuals with a personal history of cancer and subjects treated previously with either chemotherapy or radiation were ineligible. Eligible cases included unaffected at-risk women in the Fox Chase Family Risk Assessment Program who were shown to be carriers of *BRCA1* or *BRCA2* mutations. In particular, six *BRCA1*, six *BRCA2* mutation carriers, and six healthy controls were accrued for breast specimens and an equal number for ovary specimens. Normal breast and ovary specimens were obtained by prophylactic oophorectomy or mastectomy or breast reduction surgery.

Cell culture establishment

Surgical breast specimens were placed in transport medium (serum-free Ham's F-12), containing 100 U/ml penicillin, 100 μg/ml of streptomycin, 10 μg/ml ciprofloxacin, 10 μg/ml gentamicin, 2.5 μg/ml of Amphotericin B and 100 U/ml of Nystatin. The tissue was finely minced using sterile disposable scalpels and transferred to a tube containing 25 ml of 200 U/ml solution of collagenase (Sigma) prepared in DMEM with 2 g/l of NaHCO3, supplemented with 160 U/ml of Hyaluronidase, 0.5 μg/ml hydrocortisone, 10 μg/ml insulin, 10 ml of Antibiotic/Antimycotic (Gibco) and 10% horse serum. The tissue was digested overnight at 37°C in a rotating water bath and then centrifuged at 2200 rpm for 10 minutes. The supernatant

was carefully decanted to a sterile tube. The tissue was rinsed four times with transport medium, resuspended in culture medium, and centrifuged one last time. The tissue was then plated in a swine skin gelatin (Sigma)-coated T-25 flask. Cells were cultured for 24 hours in High Calcium Medium and then refed with Low Calcium Medium 24 hours later. High Calcium Medium consists of DMEM/F12 1:1 without calcium (Gibco), supplemented with 5% chelated horse serum, 20 ng/ml EGF, 100 ng/ml cholera toxin, 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, 1.05 mM calcium chloride, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml ciprofloxacin, and 0.25 μg/ml Amphotericin B. Low Calcium Medium was the same recipe supplemented with 0.04 mM calcium chloride (14). Cells were cultured four to six weeks until the flask was confluent.

Oophorectomy specimens were collected under aseptic conditions and placed in transport medium (M199:MCDB105, 1:1) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. The ovaries were processed to establish epithelial cell cultures by gently scraping the ovarian surface with a rubber policeman. Cells were centrifuged and resuspended in fresh medium (M199:MCDB105, 1:1), supplemented with 5% FBS, penicillin, streptomycin, glutamine and 0.3 U/ml insulin, and transferred to tissue culture flasks coated with skin gelatin; they were refed every four days and passaged once they reached confluency.

All the breast and ovarian samples were treated with the same tissue-specific culture conditions, including timing for passaging and harvesting. Importantly, all the samples were de-identified, including notation on carrier or control status, and no significant difference in growth or apoptosis among them was noted. At harvest, all cultures were in log phase.

RNA extraction and amplification

Total RNA was prepared from cultured cells by extraction in guanidinium isothiocyanate-based buffer containing β-mercaptoethanol and acid phenol. RNA integrity was evaluated on the Agilent 2100 Bioanalyzer. All samples showed distinct peaks corresponding to intact 28S and 18S ribosomal RNAs and therefore were included in the analysis. Amplification of total RNAs was achieved using the one-cycle Ovation™ biotin system (NuGEN Technologies, Inc., San Carlos, CA) as previously described (15).

Hybridization and microarray analysis

For each sample a total of 2.2 μg of ssDNA, labeled and fragmented with the NuGEN kit, was hybridized to Affymetrix arrays (Human U133 plus 2.0), following the manufacturer's instructions as previously described (15). After washing and staining with biotinylated antibody and streptavidin phycoerythrin, the arrays were scanned with the Affymetrix GeneChip Scanner 3000 for data acquisition.

Real-time reverse transcriptase-PCR (RT-PCR) validation of microarray data

Validation of microarray findings was conducted by real-time RT-PCR, using TaqMan Low Density Arrays (LDA, microfluidic cards from Applied Biosystems). A 48-gene custom made array (44 candidate biomarkers + 4 housekeeping genes) was designed and prepared by Applied Biosystems. The entire panel of 48 genes was tested across breast and ovarian samples. All samples were tested in quadruplicate to ensure accuracy and reproducibility.

Data were obtained in the form of threshold-cycle number (C_t) for each candidate biomarker identified and the housekeeping gene *HPRT1* for each genotype (*BRCA1, BRCA2, WT*). For each gene, the C_t values were normalized to the housekeeping gene and the corresponding ΔC_t values obtained for each genotype. Relative quantitation was computed using the Comparative C_t method (Applied Biosystems Reference Manual, User Bulletin #2) between

BRCA1 mutants and *WT* primary cell RNAs. The relative quantitation is the ratio of the normalized amounts of target for *BRCA1* mutant for *WT* RNAs, and is computed as $2^{(-\Delta C_t)}$ where ΔC_t is the difference between the mean ΔC_t values for *BRCA1* mutant and the mean ΔC_t values for *WT* RNAs. We repeated the relative quantitation analyses for *BRCA2* mutant and *WT* RNA samples.

Statistical analysis

We considered breast and ovarian samples for each of the three genotypes: *BRCA1*, *BRCA2* and mutation negative or wild type (*WT*). There were 6 biological replicates in each experimental condition resulting in a total of 18 samples for each target organ. For each sample, we obtained probe-level data in the form of raw signal intensities for 54675 probe sets from Affymetrix.CEL files. Raw data for each target organ were preprocessed separately using the Robust Multi-chip Average (RMA) method proposed by Irizarry et al. (16,17).

We applied the variance-stabilizing and normalizing logarithmic transformation to the data before analysis, and used the Local Pooled Error (LPE) method (18) for class comparisons. LPE is based on pooling errors within genes, and between replicate arrays for genes whose expression values are similar. All comparisons were two-sided. The Benjamini-Hochberg step-up method (19) was applied to control the False Discovery Rate (FDR). Genes were defined as differentially expressed, based on statistical significance as well as biological significance. Genes showing an FDR of less than the desired cut-off were considered statistically significant. We accepted an FDR cutoff of 0.20 for breast and 0.10 for ovary. These cut-offs were selected in order to obtain similar numbers of genes. Biological significance was measured as fold change; i.e., the ratio of the mean expression profiles between two conditions. Genes showing more than 2-fold change in either direction (up or down regulated) were considered biologically significant. Differentially expressed genes from each of the above filters were combined, and a list of common genes showing statistical and biological significance was identified. These genes were subsequently validated using RT-PCR. The analytical tools available in the R/BioConductor package (http://www.r-project.org, http://www.bioconductor.org), bioNMF (20) and TMev (http://www.tm4.org) were utilized in these computations.

Data mining analysis

Pathway and association analyses were conducted to obtain additional insight into the functional relevance of the changes observed. Up and down regulated genes for these exploratory analyses were selected as described above, but using a more relaxed p-value cutoff of 0.001. Gene ontology (GO) functional categories enriched in differentially expressed genes were identified using conditional hyper-geometric tests in the GOstats package (R/BioConductor). A p-value cut off of 0.01 was used in selecting GO terms. Furthermore, gene networks were generated using Ingenuity Pathways Analysis version 6.5 (Ingenuity® Systems, www.ingenuity.com). Gene Set Enrichment Analysis (GSEA) (21) was performed against the lists of differentially expressed genes for *BRCA1*-*WT* and *BRCA2*-*WT* comparisons. Gene sets from MSigDB (21), including positional, curated, motif and computational sets, were tested. Default parameters were chosen, except that the maximum intensity of probes was only selected while collapsing probe sets for a single gene.

Next we compiled and analyzed publicly available microarray gene expression data from the following: i) a study of mammary gland side population cells (22); ii) a study of two different human breast epithelial cell types, BPEC (breast primary epithelial cells) and HMEC (human mammary epithelial cells), compared to their transformed counterparts (23); iii) a molecular characterization of cancer stem cells in MMTV-Wnt1 murine breast tumors (24); iv) profiles of hereditary breast cancer (25) and v) a set of annotated genes involved in DNA damage repair. The data from these studies were analyzed (a description of the methods is given in

supplementary materials). The differentially expressed genes between *BRCA1*, *BRCA2* and *WT* from our study were compared against the above mentioned gene sets using GSEA.

Results

Genome-wide transcriptome analysis of single-hit BRCA1 and BRCA2 and mutation-negative breast and ovarian epithelial cell cultures

We are interested in the growth behavior of cells that are precursors of cancer; therefore, we chose to study primary cells multiplying in culture. Morphologically normal primary breast and ovarian epithelial cells were established from BRCA1 and BRCA2 mutation carriers and mutation-negative individuals (Fig. 1). Demographic and mutation data of mutation carriers and control individuals are shown in Table 1. The Table shows that our population is mostly Caucasian, and that carriers and controls are well matched for age, race, parity, menopausal status and body mass index, with the only exception being the group of BRCA1 carriers donating ovarian epithelial cells in which there is a predominance of pre-menopausal women. This is due to the fact that carriers of highly penetrant BRCA1 mutations were advised to undergo prophylactic oophorectomy. We conducted microarray studies of these primary epithelial cultures in order to identify differentially expressed genes between BRCA1 mutant and BRCA2 mutant single-hit cells and WT cells, for each target organ.

Class comparison analyses (*BRCA1* vs. wild type, and *BRCA2* vs. wild type) revealed notable changes in gene expression, indicating that heterozygous mutations in *BRCA1* and *BRCA2* do indeed affect the expression profiles of cultured primary epithelial cells from the relevant target organs, breast and ovary.

Breast Epithelial Cells

Table 2 summarizes selected gene expression fold-changes on a linear scale in breast cells versus controls. The secretoglobin family of genes (*SCGB2A1*, *SCGB2A2* and *SCGB1D2*), of unknown function, is highly up-regulated in BRCA1 mutant breast cells. The genes have been recently described as novel serum markers of breast cancer with significant prognostic value (26,27); approximately 80% of all breast cancers overexpress this complex (26). In *BRCA1* cells, we observed 3-fold up-regulation of mammaglobin (*SCGB2A2*, FDR:0.06, P value: 4×10^{-10}) and 12-fold increase of lipophilin B and C (*SCGB1D2* and *SCGB2A*, FDR:0.06, P value:2×10^{-8} and FDR:0.16, P value:0.0002, respectively). We also detected in *BRCA1* cells a 3-fold up-regulation of the chitinase 3-like 1 gene (FDR:0.06, P value:1.2×10^{-7}), that has proliferative effects on stromal fibroblasts and chemotactic effects on endothelial cells. It can promote angiogenesis, and high serum levels of this protein have been found in patients with glioblastoma (28). IGFBP5 was 10-fold up-regulated in BRCA2 breast cells (FDR: 0.04, P value:1.9×10^{-7}). It is involved in the stimulation of growth and binding to extracellular matrix, independently of IGF, and is highly overexpressed in breast cancer tissues (29).

Several cell-to-cell interactions and cell-to-matrix adhesion genes were found to be downregulated in breast cells, including those that code for tensin 4 (*TNS4*, fold change: 0.26, FDR:0.06, P value:2.2×10^{-7} in BRCA1 and fold change: 0.5, FDR:0.03, P value:3.7×10^{-6}), mucin 16 (*MUC16*, fold change: 027, FDR:0.08, P value:3×10^{-5} in BRCA1 and fold change: 024, FDR:0.03, P value:2×10^{-6} in BRCA1) (in both *BRCA1* and *BRCA2*) and for keratin 14 (*KRT14*, fold change: 0.5, FDR:0.12, P value:0.0001 in BRCA1 and fold change: 0.5, FDR: 0.03, P value:2.2×10^{-10} in BRCA2) (in *BRCA2*). Lack of tensin 4 expression has been reported in prostate and breast cancers (30), suggesting that the down-regulation of tensin expression is a functional marker of cell transformation. Also, loss of keratins, which are necessary for proper structure and function of desmosomes, can cause an increase in cell flexibility and deformability, and may enable a tumor cell to detach from its epithelial layer, and metastasize.

Finally, mucin 1 (*MUC1* or *CA15-3*), known to be over-expressed in breast cancer (31), is down-regulated in 'single-hit' BRCA1 cells (fold change: 0.5, FDR:0.99, P value:0.02). Overrepresentation of even one glycoprotein may affect cell surface protein distribution, with effects on other membrane proteins, such as the downregulation of genes encoding mucin 16 and mucin 1 that we have detected in mutant breast cells. A box plot representation of the top differentially expressed genes for breast epithelial cells is shown in Fig. 2A.

Ovarian epithelial cells

Table 3 summarizes expression changes for selected genes in ovarian epithelial cultures. For example, 5-fold down-regulation of the cyclin B1/cdc2 complex (*CDC2*, FDR:0.04, P value: 7×10^{-5}), a key regulator controlling the G_2M checkpoint, was observed in BRCA1 mutant ovarian cells. Multiple genes implicated in the mitotic spindle checkpoint, such as nucleolar and spindle-associated protein 1 (*NUSAP-1*, fold change: 0.08, FDR:0.02, P value:9×10^{-9} in BRCA1) were down-regulated. *NUSAP-1* plays a crucial role in spindle microtubule organization, whereas *CENP-A*, which is down-regulated in BRCA2 mutants of breast cells (fold change: 0.5, FDR:0.03, P value:9×10^{-6}), is essential for centromere structure, function and kinetochore assembly. Since BRCA1 and BRCA2, in addition to their role in DNA repair, are also involved in checkpoint pathways, we suggest that inappropriate expression of these proteins could induce abnormal kinetochore function and chromosome mis-segregation, a potential cause of aneuploidy and a critical contributor to oncogenesis. Among up-regulated genes in *BRCA1* heterozygous ovarian epithelial cells is *SAA2*, an acute phase component of the innate immune system (fold change: 6.4, FDR:0.02, P value:2×10^{-7}), a candidate marker of epithelial ovarian cancer (32,33 and references therein). Among the differentially expressed genes in *BRCA2* mutant ovarian epithelial cells, matrix metalloproteinase 3 (*MMP3*) was upregulated 9- to 12-fold (FDR:0.1, P value:2×10^{-7}); the same tendency has been reported for MMP1 and MMP2 in ovarian cancers (34,35). Our data also show upregulation of *COX1* (cyclooxygenase 1, *PTGS1*, fold change: 6.6, FDR:0.1, P value:8×10^{-9}) in *BRCA2* ovarian epithelial cells, a finding that is consistent with the reported up-regulation of COX1, but not COX2, in ovarian cancer (36,37). A box plot representation of the top differentially expressed genes for ovarian epithelial cells is shown in Fig. 2B.

Real-time reverse transcriptase-PCR (RT-PCR) validation of microarray data

A validation study on select genes for breast and ovary was performed with total RNA using quantitative, real-time RT-PCR on low-density arrays (LDA). We selected 44 candidate biomarker genes and 4 housekeeping genes, and the entire panel of 48 genes was tested by the Comparative C_1 method across all breast and ovarian samples in quadruplicate using a custom-made array, to ensure accuracy and reproducibility. The real-time RT-PCR validation results and the comparisons with the original Affymetrix data are shown in Tables 2 and 3, for breast and ovary, respectively. There was a good correlation (Spearman's ρ) between microarray and LDA data for fold changes of candidate biomarkers in breast and ovarian cultures heterozygous for *BRCA1* (0.94 in each case). For candidate biomarkers originally identified in breast and ovarian cultures for one genotype (*BRCA1* or *BRCA2*), there was also a moderate to good correlation between microarray and LDA data for the other genotype (*BRCA2* or *BRCA1*). This is described in more detail in the Supplemental Section and the results are presented in Supplemental Table S1.

Functional Mining of Microarray Data

In order to define the biological underpinnings of the observed gene expression differences, we conducted additional mining of the microarray data using gene ontology, pathway and association analyses. Gene ontology analysis revealed overrepresentation of several biological processes in *BRCA1* and *BRCA2* mutant cells (Fig. 3) (see supplementary information). Next,

using differentially expressed genes as input to Ingenuity pathway analysis software, we generated networks and overlaid pathways onto genes to understand their interactions and functional importance. In the case of breast *BRCA1* cells, two gene networks had many down-regulated genes involved in G2/M DNA damage checkpoint regulation, DNA damage response, p38 MAPK signaling and tight junction signaling. Genes such as *ATR, PMS1* and *MCM6* that are involved in *BRCA1*-related DNA damage response were up-regulated in *BRCA1* heterozygous cells (Fig. 4A). Similarly, in breast *BRCA2* cells, one significant network was identified that contained genes involved in G1/S checkpoint regulation and ephrin receptor signaling (Fig. 4B). In *BRCA1* ovarian cells, two significant networks, involved in cell cycle control (G2/M DNA damage checkpoint regulation) and pyrimidine metabolism, and in glucocorticoid receptor signaling, contained down-regulated genes (Fig. 4C). We did not find any significant networks for genes differentially expressed in ovarian *BRCA2* heterozygous cells.

In order to identify unifying biological themes central to mutant breast and ovarian cells in comparison to *WT* cells, we used GSEA for the detection of complex relationships among co-regulated genes. GSEA is an analytical methodology that allows the evaluation of lists of differentially expressed genes of interest against known biological modules, such as gene sets specific to pathways, processes and profiles, of previous profiling experiments (21). To determine whether any specific pathway or profile is enriched in the four different gene lists, we tested 1,892 curated gene sets obtained through MsigDB which is a constituent database of gene sets available through GSEA. Table S3 shows the gene sets from MsigDb that are enriched in breast and ovarian samples. Fig. 5A is a heatmap of differentially expressed genes in both breast and ovary showing various gene sets that were identified to be enriched. We did not find any significant enrichment for canonical pathways. However, we found a variety of gene sets that are listed in Table S3. Among these, we found two stem cell-related gene sets.

Since the gene expression profiles in *BRCA1* and *BRCA2* mutant cells have similarities to those of stem and progenitor cells, we tested the four gene lists from this study against a cohort of gene sets obtained from various studies including breast stem cells from both human and mouse and DNA repair genes (22-24). Breast *BRCA1* and *BRCA2* cells show significant enrichment with the differentially expressed gene sets of transformed human mammary epithelial cells (HMEC) vs. control HMEC cells and breast primary epithelial cells (BPEC) vs. control BPEC population (Fig. 5B). We observed that down-regulated genes from breast BRCA1 heterozygous cells show significant association with transformed HMEC and BPEC cells suggesting that BRCA1 ‘single-hit’ cells and transformed breast primary cells share a common fingerprint.

Finally, we compared our breast *BRCA1* dataset to three datasets of Hedenfalk *et al*. on sporadic breast cancers as well as breast cancers in families with *BRCA1* and *BRCA2* mutations (25). The GSEA comparison revealed that our breast epithelial BRCA1 dataset is most similar to the *BRCA1* tumors with matching hits corresponding to the following genes: *KRT8, TGFB1, S100A2, S100P, EPHA2, TRIM29, OSBP2, FLNB and MUC1*. Fig. 5B shows genes from *BRCA1* and *BRCA2* mutant and sporadic tumors associated with breast *BRCA1* heterozygous cells. These genes have already been implicated in breast cancer. For instance, decreased levels of KRT8 in cytoplasm are detected in breast cancer cells (38). *TGFB1* is highly expressed in sporadic breast tumors or tumors from BRCA1 and BRCA2 kindreds, whereas in heterozygous BRCA1 breast cells it is downregulated. Calcium binding proteins A2 and P are downregulated across all tissues. Aberrant expression of S100A2 has been implicated in breast cancer. In all genes that are common to three tumor types (*BRCA1*, *BRCA2* and sporadic) and heterozygous *BRCA1* cells, expression of *TGFB1* is observed to be different. This finding confirms our hypothesis of the earliest significant molecular changes in “one-hit” cells and their relationship with transformed breast cells.

Discussion

Our study demonstrates that mRNA expression profiles are altered in morphologically normal breast and ovarian epithelial cells heterozygous for mutation in *BRCA1* or *BRCA2*, and include functionally critical genes. Remarkably, these single-hit cells bear significant transcriptomic changes that share features of the profiles of the corresponding cancer cells. It is well known that *BRCA1*- and *BRCA2*-mutant breast cancers exhibit distinct expression profiles (25), and the same is true for ovarian cancer (39). In the case of our single-hit breast and ovarian epithelial cell cultures, gene expression differences related to a given genotype clearly emerge when supervised methods are used (Tables 2 and 3), and they are reflected in separate clusters (Fig. 3). On the other hand, genome-wide unsupervised analyses using hierarchical clustering and non-negative matrix factorization revealed clusters that differentiate tissue of origin but not genotype (see supplemental material, Fig. S2).

Although these specific molecular changes are yet to be placed in the context of cancer initiation and progression, it should be noted that both BRCA proteins have clear functional links to transcription. Indeed, both are mediators of the cellular response to DNA damage that includes a transcriptional component (40,41). Of course, damage does occur in normal cells as a consequence of physiological DNA replication processes, although it is repaired with high efficiency (42), and BRCA1 and BRCA2 are part of a protein complex with RNA polymerase II and the CBP and p300 histone acetyltransferases that is involved in chromatin remodeling and transcription (43). Because of these links to transcription (44), alterations in the levels of BRCA1 and BRCA2 proteins in single-hit cells might be expected to lead to multiple gene expression differences.

Intriguingly, some of the gene expression profiles enriched in breast *BRCA1* one-hit cells are similar to those detected in stem and progenitor cells (Fig. 5A). This does not appear to be true for one-hit *BRCA2* breast epithelial cells. Indeed, recent findings from the Wicha laboratory indicate that the *BRCA1* gene is involved in regulating stemness and differentiation of breast progenitor cells (45). Also, more recently, human mammary epithelial cells from *BRCA1*-mutation carriers were found to form progenitor cell colonies on semisolid medium with higher plating efficiency as compared to mammary epithelial cells from reduction mammoplasty controls (46).

In general, we found more expression changes in *BRCA1 vs. WT* cells than with *BRCA2 vs. WT* cells (Table 2), which may reflect the fact that BRCA2 is primarily involved in double-strand break repair, whereas BRCA1 may also bridge double-strand break repair and signal transduction pathways. Thus, BRCA1 may act both as a sensor of DNA damage and as a repair factor, whereas BRCA2 is thought to be involved primarily in actual repair. Even small alterations in levels of the sensor (BRCA1) may have phenotypic consequences in terms of differentially expressed genes. This is reminiscent of animal models with hypomorphic alleles of the mismatch repair protein MSH2 (part of the damage sensor) that are proficient for mismatch repair *per se* but defective for activation of the cellular DNA damage response (47).

An additional important finding from this study is that some of the molecular changes detected correspond to candidate biomarkers described previously for breast and ovarian cancer, such as mammaglobin and serum amyloid protein for breast and ovarian cancer, respectively (Tables 2 and 3). Furthermore, GSEA analysis reveals that the dataset of breast epithelial *BRCA1* one-hit cells shows similarities to that of hereditary breast carcinomas associated with *BRCA1* mutations (Fig. 5B).

In conclusion, the findings from this study are largely consistent with what is known about the pathophysiology of *BRCA1* and *BRCA2* and sporadic cancers. However, there are genes with

abnormal patterns of expression that seem unrelated. Both their number and their unrelatedness are unexplainable based on what is known about *BRCA1* and *BRCA2* cancers, but they may be early changes associated indirectly with cancer initiation (7,8). For example, heterozygosity may trigger a phenomenon such as induction of expression of siRNAs, each of which might affect the expression of multiple genes

In principle, therefore, the genetic approach used in this study may serve as a model for the identification of biomarkers for epithelial malignancies in general, and for the use of such markers as targets for chemoprevention measures that would decrease the probability of a second “hit” or greatly reduce its occurrence.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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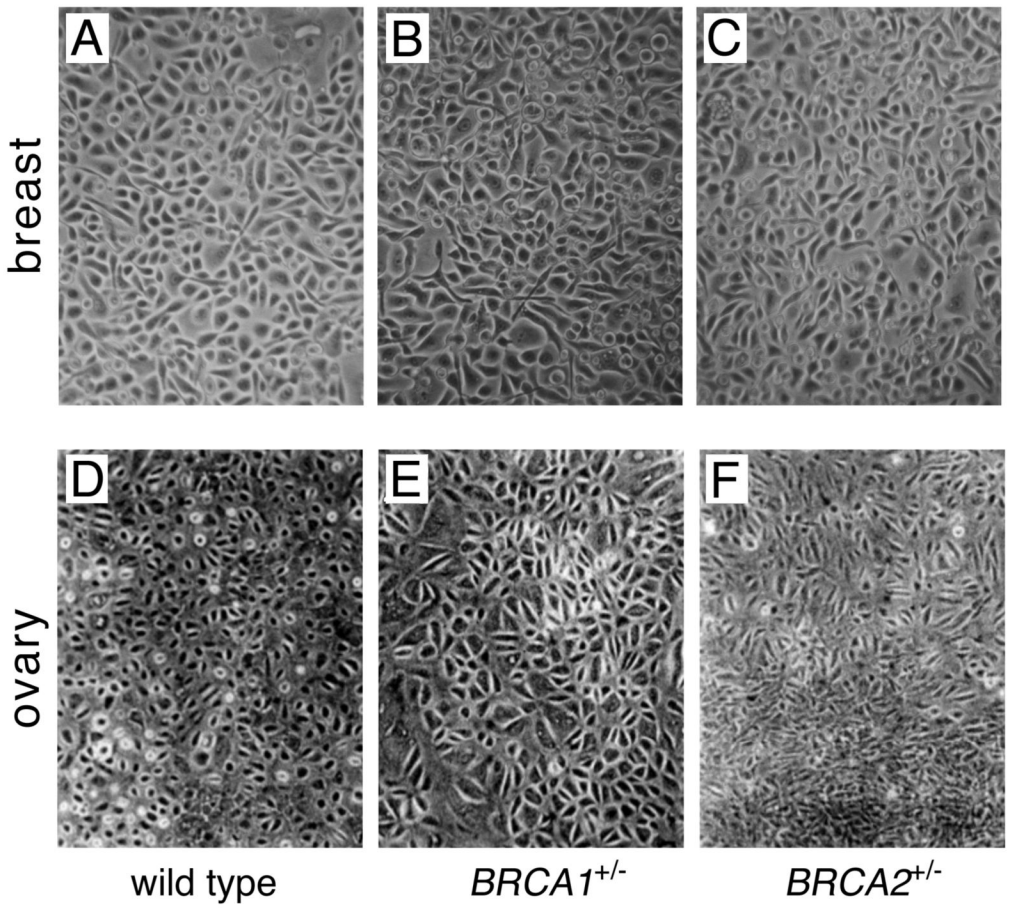


Figure 1.
Primary cultures of normal breast (A-C) and ovarian (D-F) epithelial cells from control individuals (A, D), *BRCA1* mutation carriers (B, E) and *BRCA2* mutation carriers (C, F). For each tissue of origin, cellular morphology is similar, irrespective of genotype.

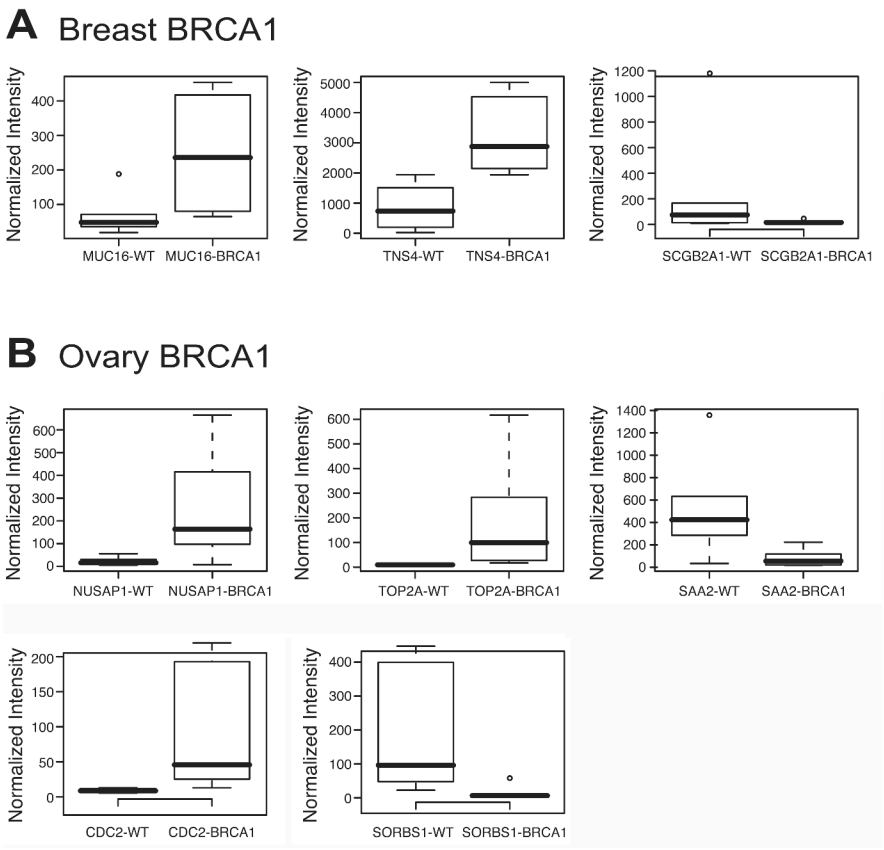


Figure 2. Gene expression differences in BRCA1 and BRCA2 heterozygous epithelial cells

Box plots are shown for the top differentially expressed genes for breast BRCA1 and ovary. Values are normalized expression values (intensity produced by RMA analysis).

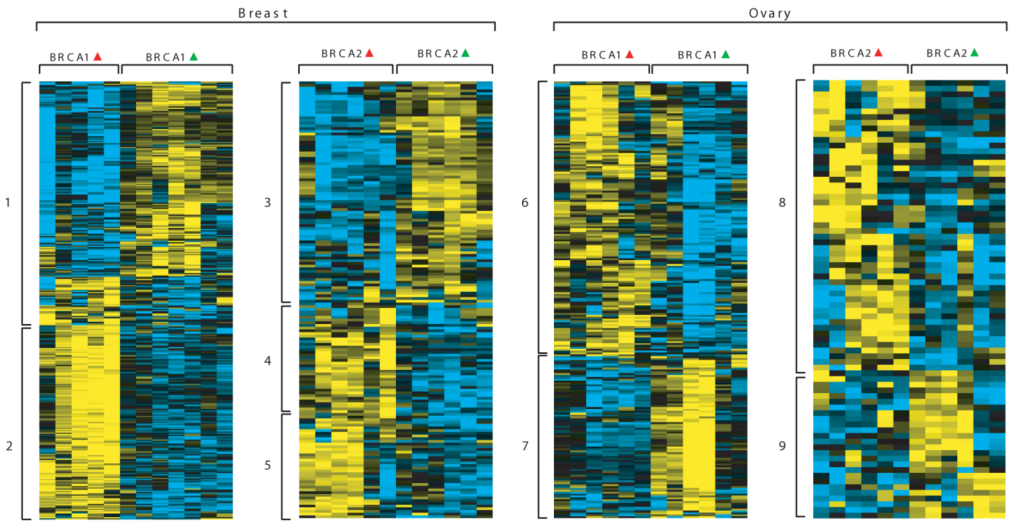
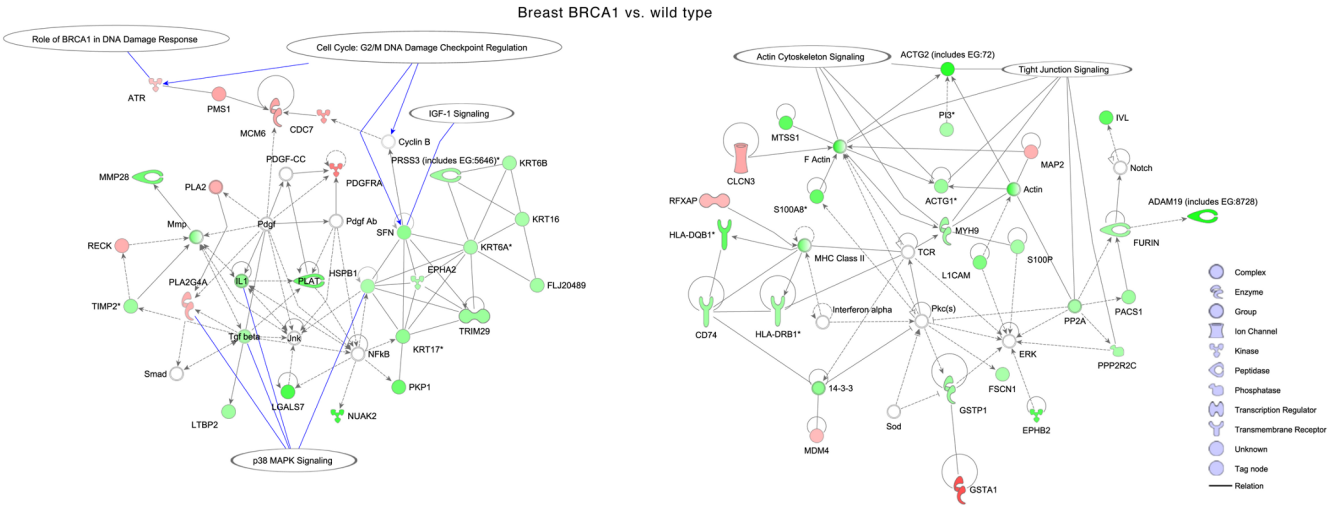
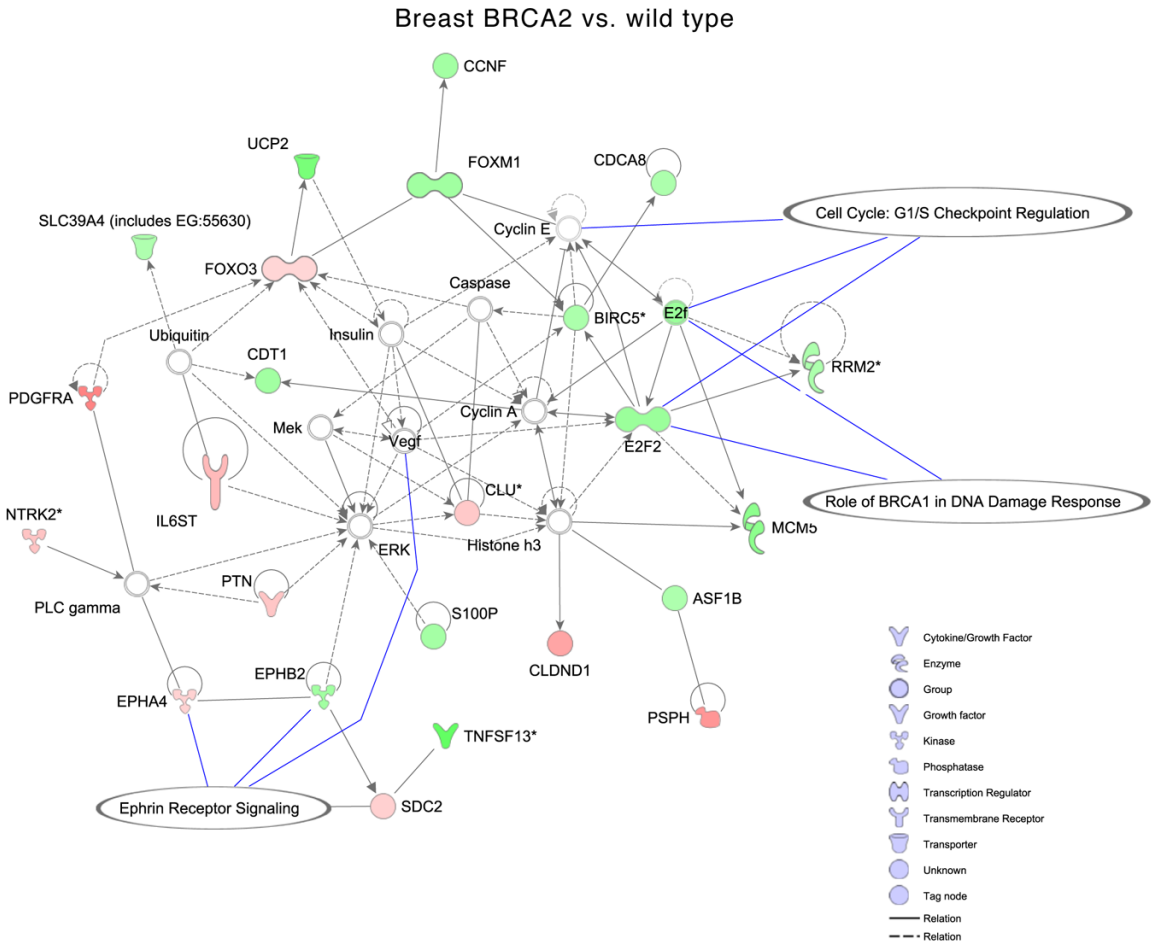


Figure 3. Gene expression patterns between *BRCA1*, *BRCA2* heterozygous and *WT* cells for breast and ovary

Each panel shows gene expression patterns represented as a heat map. Red and green deltas represent mutated and WT phenotype respectively. Rows are genes with their expression represented in yellow-blue color scale. Yellow and blue represent high to low respectively. The blocks marked with numbers to left side of each panel represent the enriched biological processes. The Gene Ontology (GO) biological processes enriched for ‘up’ (Yellow') and ‘down’ (‘Blue’) genes (numbers 1-9) are listed in Table S2.





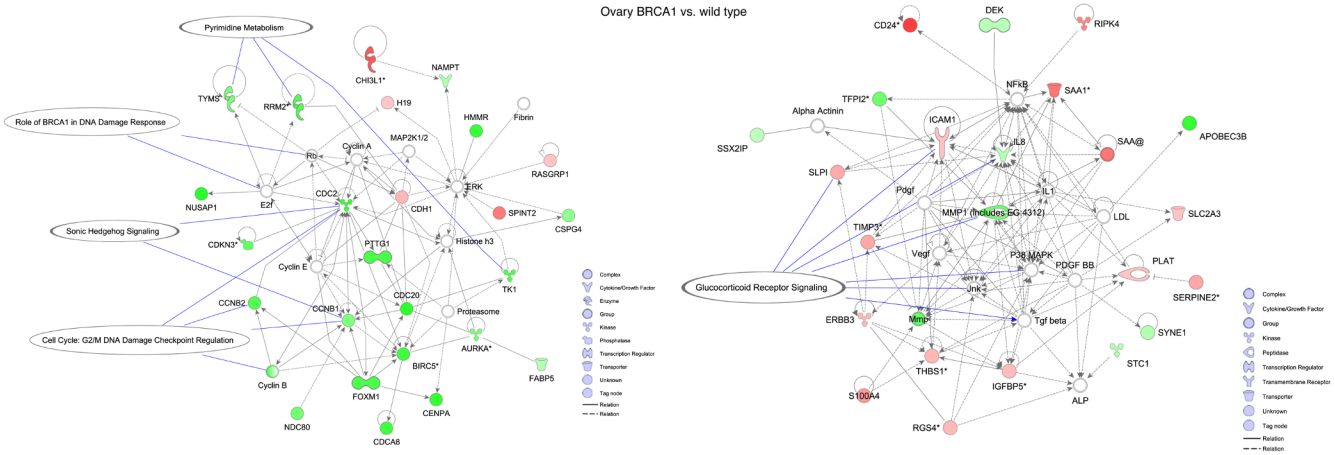
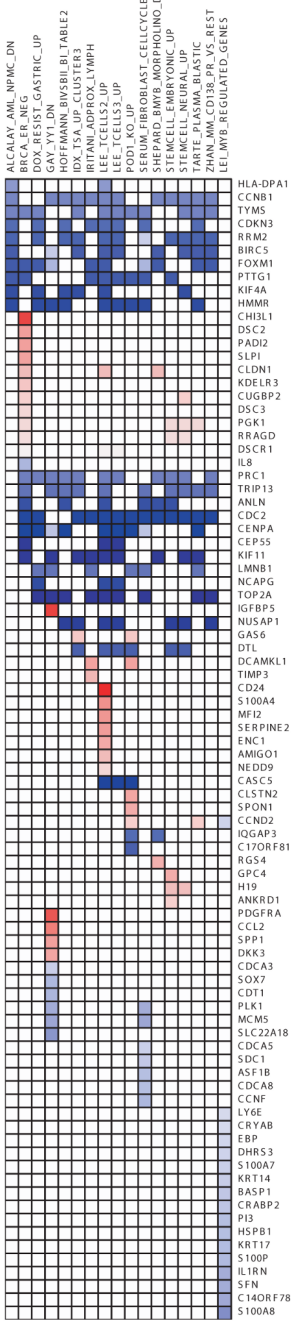


Figure 4. Ingenuity Pathway Analysis showing functional networks in one-hit cells

Selected significant canonical pathways are shown in relation to genes that are differentially expressed for: *A*, breast *BRCA1*; *B*, breast *BRCA2*; and *C*, ovarian *BRCA1* heterozygous cells.



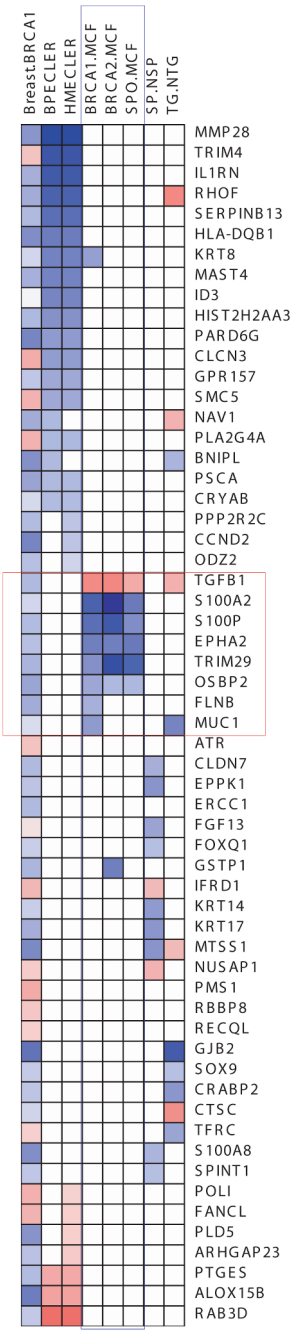


Figure 5. Association heat maps showing union of gene sets enriched for both *BRCA1* and *BRCA2* in breast and ovary heterozygous cells

Each row represents a gene whereas columns are gene sets enriched. Blue color indicates that the genes are down-regulated and red color indicates up-regulation. *A*, Association heatmap of genes in common between the indicated datasets (listed in Table S3) and primary breast *BRCA1* and *BRCA2* mutant cells. *B*, Association heatmap of genes in common between transformed human mammary epithelial cells (HMECs) and primary breast *BRCA1* and *BRCA2* heterozygous cells. In this figure HMEC refers to genes differentially expressed between HMEC-transformed (HMLER) vs. parental HMEC cells. Blue color indicates the down-regulation and red color indicates up-regulation. Breast.BRCA1 column indicates genes

differentially expressed in BRCA1 heterozygous cells from breast. SP.NSP indicates differentially expressed genes between mouse mammary side population and non-side population cells (22). TG.NTG column indicate genes differentially expressed between mouse cancer stem cells and non cancerous stem cells (24). Columns in blue box (BRCA1.MCF, BRCA2.MCF and SPO.MCF) are differentially expressed gene sets from Hedenfalk *et.al*. Genes in red box indicate the genes common to Hedenfalk and Breast.BRCA1.

Table 1

Demographic and mutation data of the patients enrolled in this study

Sample ID	Epithelial culture	BRCA	Mutation	Age	Race	Parity	Menopause status	Weight (pounds)	Height (inches)	Body mass index
218	Breast	B1	2800delAA; BRCA1 c.2681_2682del	45	C	2	Post	165	70	23.7
256	Breast	B1	R1835X; BRCA1 c5503C>T (p.R1835X)	50	C	2	Post	137	62	25.1
288	Breast	B1	185delAG; BRCA1 c.68_69delAG	70	C	2	Post	116	NA	NA
349	Breast	B1	1997insTAGT; BRCA1 c.1878_1879insTAGT	28	C	0	Pre	127	64	22.5
425	Breast	B1	T1194I, BRCA1 c.3581C>T (pT119I)*	45	C	5	Pre	126	64	22.3
479	Breast	B1	R1751X; BRCA1 c.5251C>T (p.R1751X)	48	C	3	Post	211	64	37.4
182	Breast	B2	6174delT; BRCA2 c.5945delT	44	C	0	Pre	115	66	19.1
197	Breast	B2	2912delC; BRCA2 c.2683delC	47	C	4	Pre	138	62	25.2
285	Breast	B2	R2394X; BRCA2 c.7181A>T	37	AA	3	Pre	234	63	41.4
300	Breast	B2	UN	45	C	2	Pre	131	64	23.2
304	Breast	B2	K745E; BRCA2 c.2233A>G (p.K745E)*	61	AA	2	Post	204	64	36.1
308	Breast	B2	UN	51	C	0	Post	NA	NA	NA
158	Breast	WT	—	59	C	2	Post	170	NA	NA
162	Breast	WT	—	74	C	NA	Post	130	NA	NA
161	Breast	WT	—	69	C	3	Post	125	NA	NA
169	Breast	WT	—	49	C	3	Per	NA	NA	NA
187	Breast	WT	—	31	C	0	Pre	161	65	27.6
181	Breast	WT	—	39	C	0	Post	102	NA	NA
166	Ovary	B1	IVS16-581del1014; BRCA1-c.4986-581del1014	28	C	2	Pre	NA	NA	18.3
183	Ovary	B1	IVS12-1632del3835; BRCA1-c.4185-1632del3855	43	C	2	Pre	NA	NA	NA
193	Ovary	B1	IVS16-581del1014; BRCA1-c.4986-581del1014	37	C	0	Pre	NA	NA	20.8
194	Ovary	B1	1632del5-ter503; BRCA1 c.1513del5 (p.503X)	43	C	2	Pre	NA	NA	NA
201	Ovary	B1	IVS12-1632del3835; BRCA1-c.4185-1632del3855	44	C	3	Pre	NA	NA	29.4
217	Ovary	B1	UN	46	C	NA	NA	NA	NA	NA
302	Ovary	B2	9663delGT; BRCA1 c9434_9435del	44	C	3	Pre	184	66	28.2
206	Ovary	B2	9132delC-ter2975; BRCA2 c.8903delC (p.2975X)	39	C	2	Pre	110	64	18.9
328	Ovary	B2	UN	38	C	3	Pre	189	NA	NA
431	Ovary	B2	6174delT; BRCA2 c.5945delT	54	C	0	Post	137	64	24.3

Sample ID	Epithelial culture	BRCA	Mutation	Age	Race	Parity	Menopause status	Weight (pounds)	Height (inches)	Body mass index
470	Ovary	B2	UN	57	C	1	Post	123	60	24
612	Ovary	B2	UN	42	C	3	Pre	145	66	23.4
178	Ovary	WT	—	60	C	0	Post	286	65	47.6
211	Ovary	WT	—	40	C	3	Pre	121	NA	NA
276	Ovary	WT	—	38	C	4	Pre	188	65	31.3
207	Ovary	WT	—	45	C	0	Post	130	61	24.6
121	Ovary	WT	—	33	C	0	Post	200	65	33.3
600	Ovary	WT	—	45	C	2	Pre	155	64	26.6

Abbreviations: WT, individual tested negative for a BRCA1 and BRCA2 alteration; UN, patient self reported prior to surgery but declined to share the specific mutation

*Missense mutation, unique to Breast Cancer Information Core (http://research.nhgri.nih.gov/bic/index.shtml), segregates with disease, suspected deleterious C, Caucasian; AA, African American

NA, information was not available

Table 2

Comparison between microarray and low density array (LDA) data for the candidate breast biomarkers; fold changes are shown for *BRCA1 vs. WT* and for *BRCA2 vs. WT* comparisons

A. *BRCA1 vs. WT* comparison

Gene Symbol	Gene Name	Affymetrix	LDA	Taq Man Assay
SCGB1D2	Secretoglobin, family 1D, member 2	12	5.2	Hs00255208_m1
SCGB2A1	Secretoglobin, family 2A, member 1	12	2.68	Hs00267180_m1
SCGB2A2	Secretoglobin, family 2A, member 2	3	3.8	Hs00267190_m1
CHI3L1	Chitinase 3-like 1	3	2.24	Hs00609691_m1
MCM6	minichromosome maintenance complex component 6	2.8	1.37	Hs00195504_m1
MUC1	Mucin 1, cell surface associated	0.5	0.25	Hs00159357_m1
LGALS1	Lectin, galactoside-binding, soluble, 1	0.5	0.33	Hs00169327_m1
KLK10	Kallikrein-related peptidase 10	0.5	0.15	Hs00173611_m1
ANXA8	Annexin A8	0.4	0.14	Hs00179940_m1
TNS4	Tensin 4	0.3	0.08	Hs00262662_m1
MUC16	Mucin 16, cell surface associated	0.3	0.17	Hs00226715_m1
GJB2	Gap junction protein, beta 2, 26kDa	0.2	0.12	Hs00269615_s1

B. *BRCA2 vs. WT* comparison

Gene Symbol	Gene Name	Affymetrix	LDA	Taq Man Assay
IGFBP5	Insulin-like growth factor binding protein 5	10	10.99	Hs00181213_m1
SPP1	Secreted phosphoprotein 1(Osteopontin)	3	2.3	Hs00167093_m1
RRM2	Ribonucleotide reductase M2 polypeptide	0.58	0.39	Hs00357247_g1
TNS4	Tensin 4	0.5	0.45	Hs00262662_m1
TNFSF13	Tumor necrosis factor superfamily, member 13	0.5	0.56	Hs00601664_g1
SFN	Stratifin	0.5	0.34	Hs00356613_m1
KRT14	Keratin 14	0.5	0.07	Hs00559328_m1
CENPA	Centromere protein A	0.5	0.57	Hs00156455_m1
BIRC5	Baculoviral IAP repeat-containing 5	0.5	0.43	Hs00153353_m1
MUC16	Mucin 16, cell surface associated	0.25	0.2	Hs00226715_m1
PAX8	Paired box gene 8	0.2	0.61	Hs00247586_m1

Table 3

Comparison between microarray and low density array (LDA) data for the candidate ovarian biomarkers; fold changes are shown for the BRCA1 vs. WT comparison and BRCA2 vs. WT comparisons

A. *BRCA1 vs. WT* comparison

Gene Symbol	Gene Name	Affymetrix	LDA	Taq Man Assay
SORBS1	Sorbin and SH3 domain containing 1	7	1.78	Hs00908953_m1
SAA2	Serum amyloid A2	5	9.18	Hs00763479_s1
SAA1;SAA2	Serum amyloid A1 and A2	5	17.47	Hs00761940_s1
CD24	CD24	5	49.89	Hs00273561_s1
MFI2	Antigen p97	3.5	7.47	Hs00195551_m1
SPON1	Spondin 1	2.5	8.22	Hs00391824_m1
THBS1	Thrombospondin 1	2	3.96	Hs00170236_m1
GAS6	Growth arrest-specific 6	2	5.91	Hs00181323_m1
CSPG4	Chondroitin sulfate proteoglycan 4	0.3	0.6	Hs00426981_m1
CCNB1	Cyclin B1	0.2	0.55	Hs00259126_m1
TOP2A	Topoisomerase (DNA) II alpha	0.1	0.07	Hs00172214_m1
NUSAP1	Nucleolar and spindle associated protein 1	0.1	0.22	Hs00251213_m1
CDC2	Cell division cycle 2, G1 to S and G2 to M	0.1	0.09	Hs00364293_m1

B. *BRCA2 vs. WT* comparison

Gene Symbol	Gene Name	Affymetrix	LDA	Taq Man Assay
MMP3	Matrix metallopeptidase 3	12.14	6.37	Hs00233962_m1
PTGS1	Prostaglandin-endoperoxide synthase 1	6.60	6.56	Hs00326564_s1
MMP1	Matrix metallopeptidase 1	2.05	24.16	Hs00233958_m1
KRT18	Keratin 18	0.35	0.18	Hs01920599_gH