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Research Article

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Determination of Molecular Markers for *BRCA1* and *BRCA2* Heterozygosity Using Gene Expression Profiling

Asher Y. Salmon[4], Mali Salmon-Divon[5], Tamar Zahavi[1], Yulia Barash[1], Rachel S. Levy-Drummer[3], Jasmine Jacob-Hirsch[2], and Tamar Peretz[1]

Abstract

Approximately 5% of all breast cancers can be attributed to an inherited mutation in one of two cancer susceptibility genes, *BRCA1* and *BRCA2*. We searched for genes that have the potential to distinguish healthy *BRCA1* and *BRCA2* mutation carriers from noncarriers based on differences in expression profiling. Using expression microarrays, we compared gene expression of irradiated lymphocytes from *BRCA1* and *BRCA2* mutation carriers versus control noncarriers. We identified 137 probe sets in *BRCA1* carriers and 1,345 in *BRCA2* carriers with differential gene expression. Gene Ontology analysis revealed that most of these genes relate to regulation pathways of DNA repair processes, cell-cycle regulation, and apoptosis. Real-time PCR was conducted on the 36 genes, which were most prominently differentially expressed in the microarray assay; 21 genes were shown to be significantly differentially expressed in *BRCA1* and/or *BRCA2* mutation carriers as compared with controls ($P < 0.05$). On the basis of a validation study with 40 mutation carriers and 17 noncarriers, a multiplex model that included six or more coincidental genes of 18 selected genes was constructed to predict the risk of carrying a mutation. The results using this model showed sensitivity 95% and specificity 88%. In summary, our study provides insight into the biologic effect of heterozygous mutations in *BRCA1* and *BRCA2* genes in response to ionizing irradiation-induced DNA damage. We also suggest a set of 18 genes that can serve as a prediction and screening tool for *BRCA1* or *BRCA2* mutational carriers by using easily obtained lymphocytes. *Cancer Prev Res; 6(2); 82–90. ©2013 AACR.*

Introduction

It is well established that hereditary cancer syndromes result from germline mutations in cancer susceptibility genes that lead to organ-specific cancers with distinct histologic phenotypes (1, 2). For example, women with a germline mutation in *BRCA1* or *BRCA2* have markedly increased risk of early-onset breast and ovarian cancer. Specifically, hereditary *BRCA1* and *BRCA2* mutations account for about 60% of inherited breast cancer (3). Knudson's 2 hit hypothesis implies that hereditary breast tumors result from inactivation of 2 alleles of tumor suppressor genes, *BRCA1* or *BRCA2*, which typically occurs in 2 "hits" (4). However, recent studies show that *BRCA* heterozygosis itself can contribute to breast cancer initiation (4, 5). In addition, no differences were found in the expression of BRCA1 protein between noncancer predisposed ovarian surface epithelial (NPOSE) cultures and cancer predisposed ovarian surface epithelial (POSE) cultures, which were derived from women harboring *BRCA1* germline mutations. This suggests that the wild-type (WT) *BRCA1* allele was not lost (6).

From a clinical point of view, identification of the *BRCA1* and *BRCA2* mutation carriers offers an opportunity to early identify or prevent the development of malignancy; therefore, the ability to determine which patients are more likely to carry *BRCA1* or *BRCA2* mutations is of great importance.

The structurally distinct proteins encoded by *BRCA1* and *BRCA2* genes regulate numerous cellular functions, including DNA repair, chromosomal segregation, gene transcription, cell-cycle arrest, and apoptosis (7). *BRCA1* and *BRCA2* are considered to be "gatekeeper" genes, which when mutated or abnormally expressed, cause disruption of normal cell biology, interrupt cell division or death control, and promote the outgrowth of cancer cells

Microarray studies show that *BRCA1* and *BRCA2* mutation status influences the gene expression profiles of distinct types of cells. For example, a recent study (8) shows that mRNA expression profiles are altered in morphologically normal breast and ovarian epithelial cells heterozygous for mutation in *BRCA1* or *BRCA2* and includes functionally critical genes. Another study shows that heterozygous *BRCA1* mutation carrier fibroblasts have a distinctive gene expression phenotype after radiation-induced DNA damage

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(9). Together, these data suggest that the accumulation of somatic genetic changes during tumor progression may follow a unique pathway in individuals genetically predisposed to cancer.

As DNA sequencing is expensive, time consuming, and does not allow the identification of all types of mutations (10), efforts have been devoted to develop more indirect methods for *BRCA* screening that can improve the selection of patients for sequence-based *BRCA* testing. Our aim in the present study is to investigate whether gene expression profiling could be used to distinguish between heterozygous *BRCA1* and *BRCA2* mutation carriers and control samples from lymphocytes after induced DNA damage. We used lymphocytes because they can be easily obtained from peripheral blood and can be used for diagnostic purposes. We show that induced DNA damage by exposure to irradiation resulted in significant differences in gene expression profiling in heterozygous *BRCA1* or *BRCA2* mutation carriers as compared with noncarriers.

Materials and Methods

Sample information

Fresh blood samples were obtained from proven *BRCA1* or *BRCA2* mutation carriers and mutation-negative women. All subjects were healthy women between ages 25 and 50 years, with no personal history of cancer. Individuals heterozygous for *BRCA1* or *BRCA2* germline mutations were identified in the *BRCA1* and *BRCA2* predictive testing program in the Oncogenetic Clinic of Hadassah University Medical Center (Jerusalem, Israel). Mutation-negative subjects were healthy women with a family history of breast cancer that were found to be noncarriers of the *BRCA1* or *BRCA2* mutation known to be present in their families. Written informed consent was obtained from all participating individuals before inclusion in the study, and the study protocol was approved by the local Ethics Committee of Hadassah Medical Center.

Lymphocytes preparation and RNA extraction

Lymphocytes were collected from fresh blood samples using LymphoPrep kit (Sigma) and short-term cultured for 6 days in RPMI-1640 with L-glutamine (Biological Industries), supplemented with 1% HEPES, 16% fetal calf serum, penicillin+streptomycin, and interleukin (IL)-2. Cells were γ-irradiated with 8 Gray (Gy) at a high-dose rate (0.86 Gy/min, Ortovoltage X-ray machine) as described (11). One hour later, RNA was extracted using EZ RNA kit (Biological Industries) according to manufacturer's instruction for further analysis. The integrity of all RNA samples was verified by 2% agarose gel electrophoresis before use in microarray experiments.

Microarray assay

The preparation and processing of labeled, fragmented cDNA for oligonucleotide microarray hybridization were conducted according to the Affymetrix protocols described in the technical manual (Affymetrix). We used the Affymetrix GeneChip Human Genome U133A 2.0 Array. Expression values for each probe were calculated using the Affymetrix Microarray Suite (MAS 5.0) software. GEO accession number: GSE39976.

Data analysis

Data analysis was done using GeneSpring GX software (Agilent Technologies).

Background adjustment, normalization, and summarization were done using Robust Multi-array Average methodology. The relative expression data for each probe set were then generated by normalizing each gene to the median of its own expression intensities across the entire experiment set (per gene normalization). Control probes and genes whose expressions were not changed across the experiment were removed from the list before statistical analysis was conducted. Differentially expressed genes were analyzed by one-way Welch ANOVA, with P value cutoff of 0.05 after Benjamini and Hochberg false discovery rate (FDR) multiple testing correction. Average linkage hierarchical clustering of the different experimental samples was obtained for selected genes using Pearson correlation as a similarity measure. Bootstrapping analysis was conducted for the assessment of the robustness of the cluster dendogram topology. Cluster members were categorized according to their biologic functions using The Database for Annotation, Visualization and Integrated Discovery (DAVID) tools (12). The Pathway Express tool (13) was used to characterize the responsive genes on molecular interaction networks in regulatory pathways.

Real-time reverse transcriptase-PCR and statistical analysis

The RNA was reverse transcribed by High Capacity cDNA Reverse Transcriptase and probed to the 48 well TaqMan Low Density Array (Applied Biosystems) according to the manufacturer's protocols. Differential expression was calculated using Data Assist Software (Applied Biosystems). All samples were tested in triplicate to ensure accuracy and reproducibility. One sample of control mutation-negative individual was loaded in each of the 96-well plates of the experiment, and it was verified that the verifiable known housekeeping control genes of that sample are expressed approximately in the same level for all plates. In addition, samples of *BRCA* mutation carriers group and of control mutation-negative individuals were distributed randomly across the 96-well plates, to reduce interassay variability. Subsequent validation of the results by 24-well TaqMan Low Density Array (Applied Biosystems) allowed us to use robust rank-based methods (Mann–Whitney test) to assess differential expression (17 normal vs. 40 *BRCA1* or/and *BRCA2* carriers). The significant differences were considered at $P \leq 0.05$. The construction of the receiver operating characteristic (ROC) curves was conducted through usage of the GraphRoc Program for Windows and the area under the curve (AUC) was calculated to evaluate the diagnostic performance of genes that were identified in the

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Table 1. Mutation data of the patients enrolled in the study

Mutation	Number of individuals
BRCA1-185del AG	34
BRCA1-5382 inc C	10
BRCA1-3875 4	2
BRCA1-A>T 1182	2
BRCA1-44184 del	2
BRCA1-3450delCAAG	2
BRCA2-6174delT	17
BRCA2-delTT6503	2
BRCA2-C>T9610	2
BRCA2-delCA995	2
BRCA2-6503delTT	2
BRCA2-4075delGT	2
BRCA2-delCA995	2
BRCA2-5950delCT	2
BRCA1-185delAG + BRCA2-6174del T	1

real-time PCR analysis as being differentially expressed in *BRCA1* and *BRCA2* carriers versus control individuals. Youden index (*J*) was used to define cutoff points on the ROC curves with the maximal sum of sensitivity and specificity (14).

Results

Total gene expression profile in *BRCA1* or *BRCA2* mutation carriers versus noncarriers

This study was aimed at defining features unique to irradiation stress effects on human lymphocytes. First, we wished to identify and characterize the gene expression profile in lymphocytes in response to ionizing irradiation. To accomplish this aim, lymphocyte cells were obtained from *BRCA1* or *BRCA2* mutation carriers and control mutation-negative individuals. The mutations identified in the *BRCA1* or *BRCA2* carriers are listed in Table 1.

To examine potential relationships between the expression profiles of control and *BRCA1* or *BRCA2* mutation carrier samples, microarray analyses were conducted. The Affymetrix GeneChip Human Genome U133A 2.0 Array was probed using cDNA obtained from lymphocytes from 9 proven unaffected carriers of *BRCA1*, 8 proven unaffected *BRCA2* carriers, and from 10 noncarrier healthy women. For each sample an individual chip was used. Hybridization experiments were carried out on RNA extracted from lymphocytes before irradiation and 1 hour following exposure to 8 Gy of ionizing irradiation. This irradiation dose was chosen according to the results of previous studies done by the first author of this article and others, determining the optimal irradiation dose–response for creating chromosomal damage in lymphocytes from *BRCA1* mutation carriers (15) and also by an expression study, which shows that heterozygous *BRCA1* mutation carrier fibroblasts have a distinctive gene

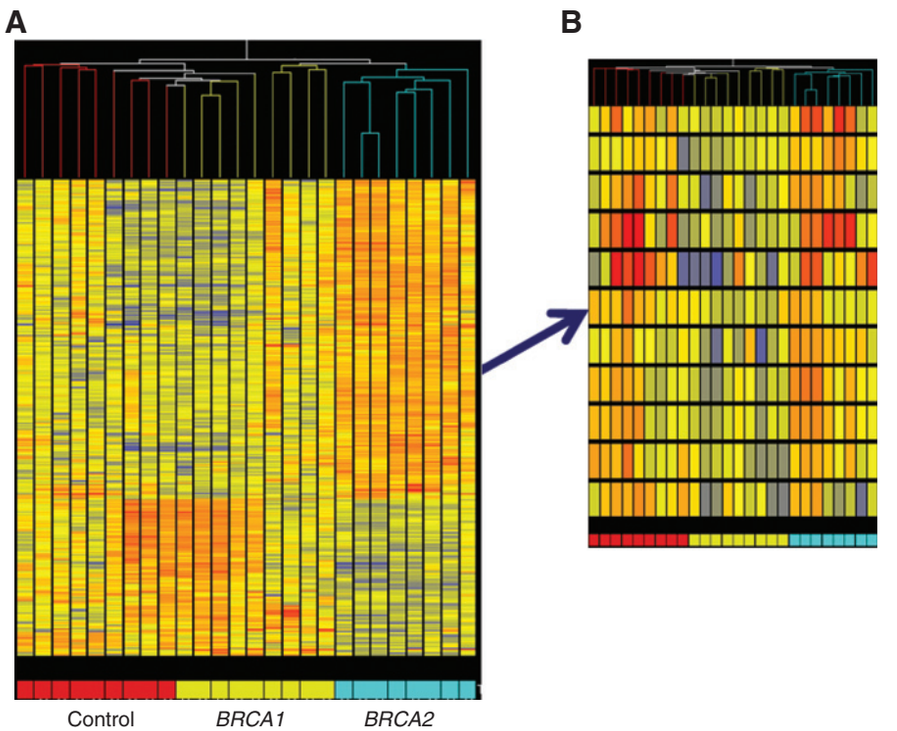

Figure 1. Gene expression patterns between *BRCA1*, *BRCA2* heterozygous and *WT* lymphocytes cells following ionizing irradiation. ANOVA analysis of *BRCA1* (yellow), *BRCA2* (blue), and control (red) gene expression. A, hierarchical clustering of the 3 conditions. The median value of the normalized probe intensities served as a reference to compare the expression of each gene across the 3 conditions. Probe intensities above and below the global median are denoted by shades of red and blue respectively, those at the global median level are colored yellow. Note the homogenous clustering of *BRCA2* as compared with the somewhat more heterogeneous clustering of *BRCA1*. B, cluster of 11 genes that were significantly underexpressed in *BRCA1* in comparison with *BRCA2* and control.

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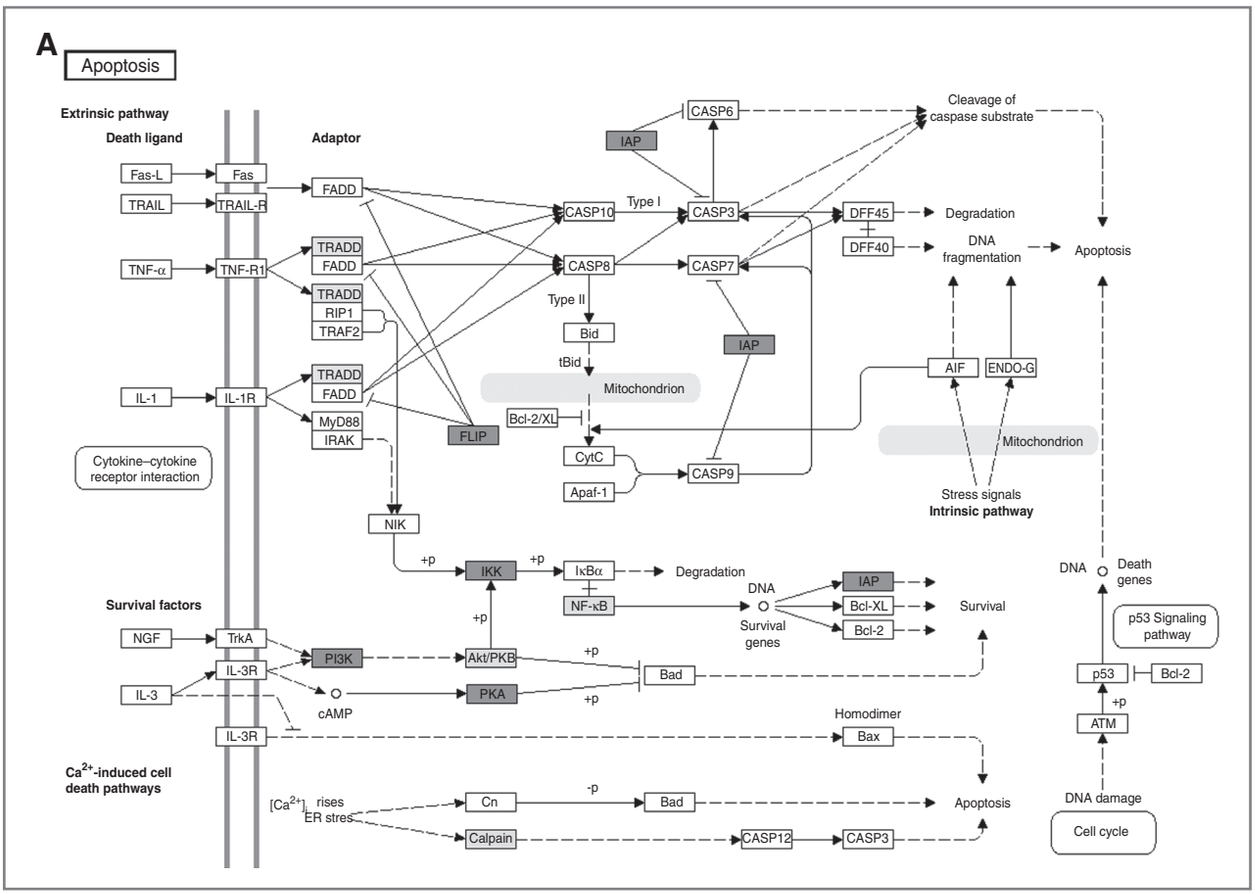

Figure 2. Pathway analysis showing functional networks in *BRCA2* heterozygous lymphocytes cells. Pathway express tool (13) was used to characterize the responsive genes on molecular interactions networks in regulatory pathways. Scheme of the apoptosis pathway (A) and

expression phenotype after radiation-induced DNA damage (9). No significant differences in gene expression profiles were detected in a preliminary study using RNA from nonirradiated lymphocytes from the 3 groups (data not shown). This result is consistent with findings in previous studies (4, 5). Following irradiation, differences in gene expression profiles between the 3 groups were observed. An ANOVA analysis and hierarchical clustering were conducted on those genes that showed a significantly different expression pattern between either the *BRCA1* or the *BRCA2* group as compared with the control group (FDR ≤ 0.05). The *BRCA2* mutation carrier group showed a homogenous, unique expression profile, unlike the *BRCA1* mutation carrier group, which showed a more heterogeneous expression pattern.

As shown in Fig. 1, there was a clear-cut distinction between upregulated genes in the *BRCA2* mutation carrier group and the control group. Moreover, expression patterns within the *BRCA2* mutation group were highly conserved among all samples. In contrast, the gene expression profile in *BRCA1* mutation carrier samples was less homogeneous; nevertheless, unique gene expression patterns could be identified. This is exemplified in Fig. 1B in which a set of genes are displayed, which were downregulated in *BRCA1* in comparison with both *BRCA2* and control cells. In total, 137 probe sets in *BRCA1* and 1,345 probe sets in *BRCA2* mutation carriers were differentially expressed ($P \leq 0.05$) when compared with the control samples. Using a 5% FDR (16), the number of *BRCA2*-differentiated genes was reduced to 596. This method was not applicable for the *BRCA1* group due to the lower homogeneity of the samples. To find the genes that differentiate the most between the groups, further selections were carried out: first, the genes with a minimum of a 2-fold expression between the *BRCA1* and *BRCA2* groups and the control groups were selected; second, from these genes only those with the most consistent pattern of expression in all samples within the same group were chosen. This selection resulted in a list of 36 genes. Interestingly, the function of most of these genes is related to transcription regulation processes and DNA binding.

Gene Ontology analysis of differentially expressed genes between *BRCA1* and *BRCA2* mutation carriers versus noncarriers

Gene Ontology and pathway analysis was conducted on a list of genes, which had different expression patterns for either the *BRCA1* or the *BRCA2* groups as compared with

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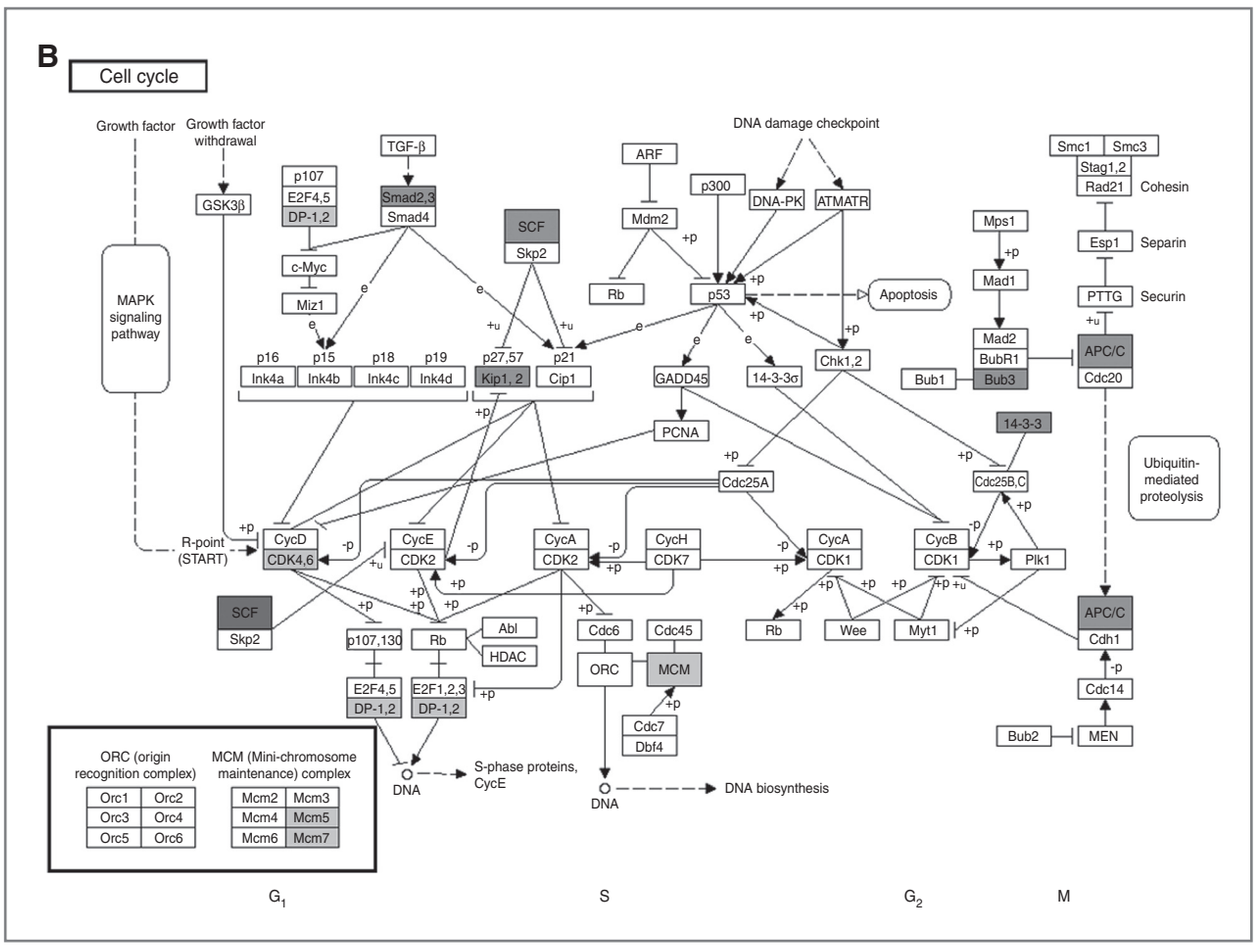

Figure 2. (*Continued*) cell cycle (B) were taken from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (28, 29). In dark gray are the genes that are overexpressed and in light gray are genes that are underexpressed in the *BRCA2* mutation carriers group compared with control. Among these are oncogenes, cell-cycle regulatory genes, and genes that function in transcription regulation and DNA damage repair. Rb, retinoblastoma; MAPK, mitogen-activated protein kinase; PCNA, proliferating cell nuclear antigen; APC, antigen-presenting cell; PI3K, phosphoinositide 3-kinase; NGF, nerve growth factor; IKK, inhibitor of IκB kinase; SCF, stem cell factor; FLIP, FLICE-like inhibitory protein; IAP, inhibitor of apoptosis; PKA, protein kinase.

the control group. The analysis of the genes revealed that most of them are predictably related to the gene expression regulation pathways, DNA repair processes (i.e., *DNAJ*, *RAD51*), cell-cycle regulation (i.e., cyclin H, *Kip1*), cancer associated (i.e., *RPS6KB1*, *RBL2*), and apoptosis (Fig. 2). Furthermore, a number of these genes were shown to function together (for example *SMURF2* and *RNF11*). Mutations in *BRCA1* have been shown to impair the homologous repair of double-stranded breaks in the DNA, and the BRCA1 protein has been shown to function in cell-cycle regulation (17). Therefore, these results might be relevant to the function of BRCA1 and BRCA2. The next largest group of genes is related to the hematologic and the immune systems (i.e., *HLA-DQB1*, granulysin), as is to be expected when tested in lymphocytes.

Real-time PCR validation of selected microarray transcripts

We conducted a real-time PCR analysis of those transcripts, which were identified as being differentially expressed in lymphocytes in response to ionizing irradiation between *BRCA1* and *BRCA2* mutation carriers and control mutation-negative individuals. In this study, we tested 17 samples from the *BRCA1* mutation carriers group, 10 samples from the *BRCA2* mutation carriers group (Table 1), and 12 samples of control mutation-negative individuals. As described earlier, we selected 36 candidate genes and 5 housekeeping genes, and the complete set of 41 genes was tested by TaqMan gene cards reverse transcriptase PCR (RT-PCR). Of these, 21 genes were shown to be significantly differentially expressed between control mutation-negative individuals versus *BRCA1* or *BRCA2* mutation carriers, with the $P < 0.05$ threshold defined as significant (Table 2). These genes are related to apoptosis, cell signaling, transcriptional activation, cell cycle and proliferation, nucleotide binding, DNA replication, and repair processes and may be of importance to disease pathophysiology. A number of these genes were found to have roles in ubiquitination. We also checked which of our 36 selected genes are included in the Sanger COSMIC v55 database as genes

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Table 2. Twenty-one genes that were significantly differently expressed in *BRCA1* or *BRCA2* mutation carriers as compared with control mutation-negative individuals.

Gene	*BRCA1*/control	*BRCA2*/control	*P* value
RAB-23GAP1	2.267857	2.70063	0.0009
NFAT5	1.820479	2.303268	0.0036
MRPS6	2.089909	2.321377	0.0041
AUH	2.16	2.2	0.0046
MID1IP1	1.945218	2.187696	0.0075
YTHDF3	1.509434	1.957825	0.0145
MARCH7	1.460844	1.927102	0.0147
ELF1	1.75	2	0.015
STAT5A	1.48776	1.933504	0.016
C6orf111	1.55	1.9	0.025
NR3C1	1.356322	1.79716	0.0257
NR4A2	1.494553	1.869281	0.0275
IFI44L	1.77931	1.842596	0.0297
RPL32	1.54199	1.860155	0.0333
SARS	1.759104	1.795918	0.0348
RP6K-1B1	1.494767	1.825334	0.0377
CDK-1N1B	1.68	1.81	0.041
RGS16	1.364034	1.736471	0.0463
DNAJC12	1.68	1.71	0.0491
EIF3S7	1.57	1.93	0.0491
SMURF2	1.487574	1.782943	0.0491

NOTE: Fold changes are shown for the control versus *BRCA1* comparison and control versus *BRCA2* comparison. The *P* value was calculated by Wilcoxon rank sum test. The *P* values between *BRCA2* and control are presented in the table (*P* values between *BRCA1* and control are not shown but are all less than 0.05).

having somatic mutations in cancer in benign neoplasms and other benign proliferations, *in situ* and invasive tumors, recurrences, metastases, and cancer cell lines (18). Interestingly, 17 of 36 genes were found to be mutated in ovary and breast cancer (Supplementary Table S1), implying that these genes may have a role in oncogenesis. Furthermore, 18 of 36 genes were found, by searching the medical literature (Pubmed database), to be associated with breast or ovary cancer (Supplementary Table S1).

Subsequently, to verify the accuracy of the results, we expanded our study and tested an additional 26 samples from the *BRCA1* mutation carriers group, 13 samples from the *BRCA2* mutation carriers group, 1 sample from *BRCA1* and *BRCA2* mutation carrier, and 17 samples from control mutation-negative individuals (Table 1). In addition to the 21 genes that were found to be differently expressed between control individuals versus *BRCA1* and *BRCA2* mutation carriers (Table 2), 4 verifiable known housekeeping genes were tested as a control. Results similar to those of the first experiment were obtained (Supplementary Table S2).

Development of assay to predict *BRCA1* and *BRCA2* mutation carriers

RNA expression analysis of the 21 selected genes by RT-PCR revealed statistically significant differences between *BRCA1* and *BRCA2* mutation carriers and the control mutation-negative individuals. At first, the selected genes were tested by ROC curve analysis as individual predictive genes, which showed that *CDKN1B* has the highest value for discriminating *BRCA1* and *BRCA2* mutation carriers from mutation-negative individuals in our sample set (AUC = 0.901; $P < 0.0001$; Table 3; Fig. 3). Next, we calculated combined sensitivity and specificity for the minimal numbers of genes to define an optimal minimum number of genes that could improve the diagnostic performance over a single gene. It was concluded that 3 of 21 genes reduced the specificity and sensitivity levels, so they were excluded from the final model. This resulted in the multiplex model that included 6 or more coincidental genes of 18 selected genes (Table 3). The model has sensitivity 95% and specificity 88% in the process of distinguishing *BRCA1* and *BRCA2* carriers from noncarriers. Supplementary Table S3 details the sensitivity and specificity for various minimal numbers of genes. At the point on the multiplex model ROC with the maximum sum of sensitivity and specificity, the positive predictive values for *BRCA1* and *BRCA2* mutation carriers groups were 92% and 100%, respectively. Because the mutation prevalence rate of the general tested population is much lower than one of the study sample (10% and 50%, respectively), the positive predictive value of the general population is expected to be lower. The negative predictive value was 88%.

Discussion

Our microarray analyses show that mRNA expression profiles in irradiated lymphocytes are altered in *BRCA1* or *BRCA2* mutation carriers compared with controls and include multiple functionally critical genes (Fig. 2). Because *BRCA1* and *BRCA2* have a transcription-activating function (19–21), alterations in the levels of *BRCA1* and *BRCA2* proteins in cells heterozygous for mutation in *BRCA1* or *BRCA2* might be expected to lead to multiple gene expression differences, and indeed Gene Ontology and pathway analysis revealed that these genes are predictably related to different biologic processes, such as apoptosis, cell signaling, transcriptional activation, cell cycle and proliferation, nucleotide binding, and DNA replication and repair processes and may be of importance to breast cancer pathophysiology (Fig. 2).

In general, the expression patterns in the tested *BRCA2* mutation group were highly conserved among all samples, whereas *BRCA1* mutation carriers showed greater heterogeneity in gene expression than did *BRCA2* carriers. This could be explained by the fact that *BRCA1* protein is an E3 ubiquitin ligase that functions as a mediator protein in homologous DNA double-strand break repair, cell-cycle checkpoint, transcription regulation, and apoptosis (22). The different functions are dependent on many *BRCA1* interacting proteins (23) and the downstream activation

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Table 3. The best 18 selected genes and their ROC curve AUC data

	AUC			Asymptotic 95% confidence interval	
Test result variable(s)	Area	SE	Asymptotic sig.	Lower bound	Upper bound
AUH	0.765	0.072	0.002	0.624	0.906
CDKN1B	0.901	0.041	0	0.821	0.982
EIF3D	0.719	0.07	0.009	0.583	0.856
ELF1	0.857	0.05	0	0.76	0.955
IFI44L	0.785	0.076	0.001	0.636	0.934
MID1IP1	0.826	0.055	0	0.718	0.935
MRPS6	0.862	0.059	0	0.746	0.978
NFAT5	0.732	0.066	0.006	0.604	0.861
NR3C1	0.76	0.065	0.002	0.632	0.888
NR4A2	0.795	0.068	0	0.663	0.927
RAB3GAP1	0.822	0.059	0	0.707	0.937
RGS16	0.827	0.063	0	0.703	0.951
RPS6KB1	0.599	0.082	0.243	0.437	0.76
SARS	0.679	0.073	0.033	0.536	0.822
SFRS18	0.768	0.061	0.001	0.649	0.888
SMURF2	0.731	0.066	0.006	0.601	0.86
STAT5A	0.843	0.057	0	0.731	0.954
YTHDF	0.826	0.054	0	0.72	0.933

of various genes (17). In contradistinction, only one biologic function is known for the *BRCA2* protein, which is its role in facilitating homologous recombination DNA repair.

Despite a large number of studies on *BRCA1* and *BRCA2* genes, the exact roles of *BRCA1* and *BRCA2* in *BRCA1*- and *BRCA2*-related cancers remain speculative. The common theory is that hereditary breast tumors are caused by complete inactivation of tumor suppressor genes, *BRCA1* and *BRCA2*, in 2 "hits" (24). However, a recent study shows that *BRCA1* heterozygous female mice had a higher incidence of ovarian tumors after irradiation without losing the second *BRCA1* allele (15). Moreover, reduction in BRCA1 protein impairs homologous recombination processes (25), indicating that haploinsufficency alone can compromise genome stability and lead to additional cancer-causing mutations. Finally, alterations in the levels of *BRCA1* and *BRCA2* proteins in heterozygous cells might be expected to lead to multiple gene expression differences in breast and ovarian epithelial cells (5) and in fibroblasts (4), suggesting again that *BRCA* heterozygosis itself can contribute to breast cancer initiation. Our results that mRNA expression profiles are altered following irradiation in *BRCA1* or *BRCA2* heterozygous cells compared with controls are also consistent with detectable heterozygous effects.

On the basis of the results obtained from microarray analyses and the real-time PCR analysis, an assay to predict *BRCA1* or *BRCA2* mutation carriers was designed including 18 genes that were expressed as downregulated in cells heterozygous for mutation in BRCA1 or BRCA2 in response to irradiation. Intriguingly, about half of these 18 genes have been found in the literature as implicated in breast cancer. For instance, basal activation of STAT5 in healthy human breast epithelia is frequently lost in invasive and metastatic human breast cancer. Indeed, loss of active STAT5 in breast cancer is correlated with poorly differentiated histology and poor prognosis (26). In addition, most human mammary tumors displayed decreased expression of the transcription-related gene *ELF1*, which was also found downregulated in our study (27). This finding supports our hypothesis of the significant molecular changes after radiation-induced DNA damage in heterozygous cells and their relationship with transformed breast cells.

Notably, our study was done in lymphocyte cells due to the fact that these cells can be easily obtained from peripheral blood for a simple assay to screen for *BRCA1* and *BRCA2* mutations. For this reason, the specific alterations in mRNA expression profiles that were found in our microarray analysis have yet to be placed in the context of breast cancer initiation and progression. However, our study can point to mechanistically important genes involved in the sensitivity to DNA damage caused by ionization irradiation.

Finally, the available diagnostic tests for mutation analysis of *BRCA1* and *BRCA2* are time and labor intensive, expensive, and do not allow for the identification of all types of mutation (e.g., mutations in the regulatory regions, which are found up and downstream to the structure gene). We developed a functional diagnostic tool for mutation analysis of *BRCA1* and *BRCA2* (*BRCA* test), which offers the following benefits: identification of high-risk individuals who carry one of the known or rare mutations in the structural gene and potentially also in its regulatory regions. However, additional studies would be needed to verify its clinical efficacy.

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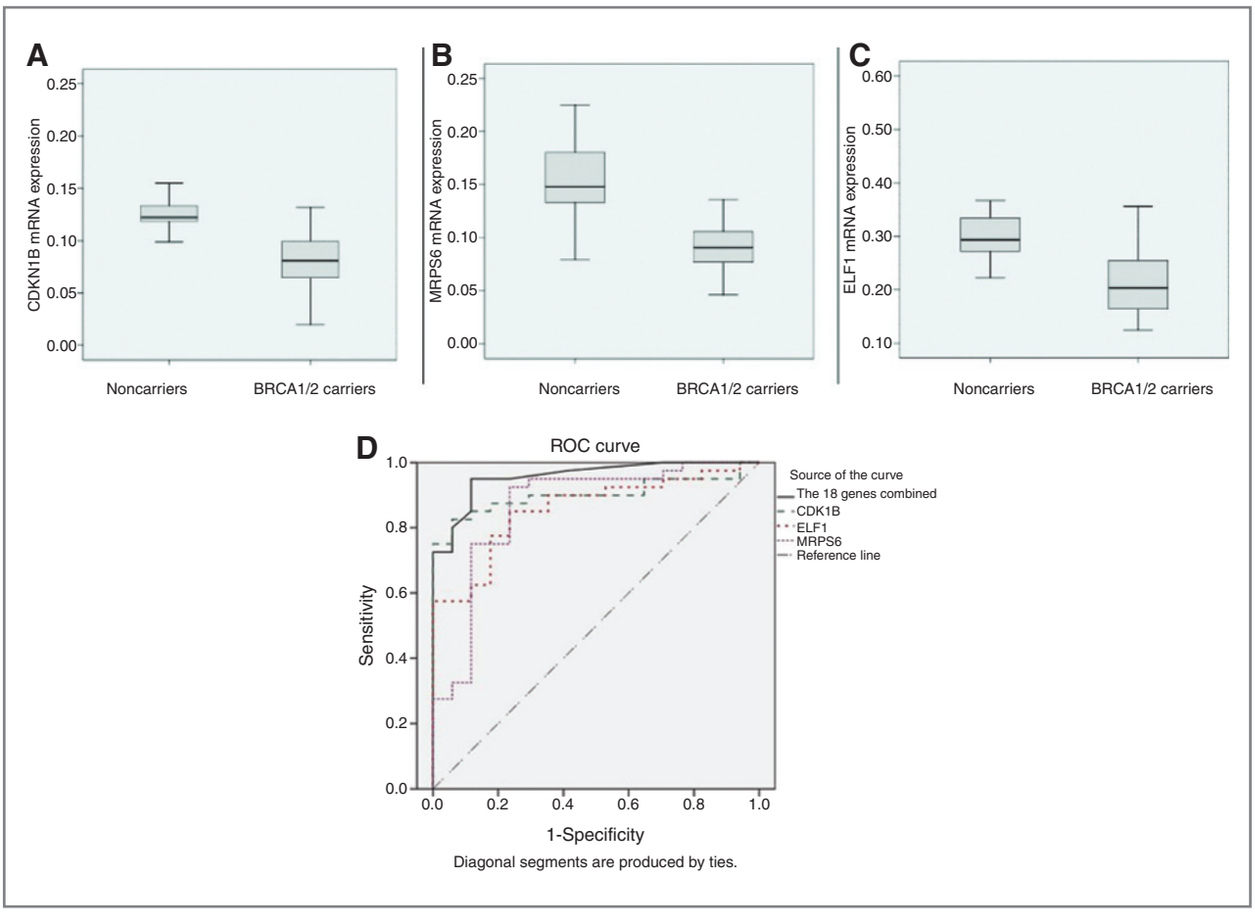

Figure 3. Gene expression differences in *BRCA1* and *BRCA2* heterozygous lymphocytes cells following ionizing irradiation. Comparison of mRNA expression levels of CDKN1B (A) MRPS6 (B), and ELF1 (C) in *BRCA1* and *BRCA2* carriers and noncarriers. The *P* values shown were obtained with the Mann–Whitney test. D, ROC curves of the 18 genes combined, CDKN1B, MRPS6, and ELF1. An AUC value close to 1 indicates great sensitivity and specificity of the method at carrier/noncarriers discrimination. The 18 genes combined has the highest value for discriminating *BRCA1* and *BRCA2* mutation carriers from mutation-negative individuals in our sample set (AUC = 0.957; $P < 0.0001$).

In conclusion, the findings from this study may provide additional insight into the role of the biologic effect of heterozygous mutations in *BRCA1* or *BRCA2* genes in cellular response to irradiation DNA damage, as well serving as a molecular functional tool that can be used to determine which patients are more likely to carry *BRCA1* or *BRCA2* mutations. This received functional assay shows a sensitivity of 95% and specificity of 88% in predicting mutation carrier status and will help to identify the presence of mutations in *BRCA1* and *BRCA2* genes in an individual in a sensitive, simple, inexpensive, and easily obtained fashion. In regions where sequencing is possible economically, it could be used on women with positive result in our test. In other regions, our test with adequate validation may serve as a substitute. We are currently further expanding our study to validate these assumptions.

Disclosure of Potential Conflicts of Interest

A.Y. Salmon has commercial research support from Micromedic, Israel and has honoraria from Speakers Bureau of Novaris, Pfizer, and Johnson & Johnson. T. Peretz has commercial research support from Micromedic, Israel and has ownership interest (including patents) in a patent owned by Hadassah Medical Organization. No potential conflicts of interest were disclosed by the other authors.

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