BRCA1 and BRCA2 Missense Variants of High and Low Clinical Significance Influence Lymphoblastoid Cell Line Post-Irradiation Gene Expression

Nic Waddell¹, Anette Ten Haaf¹, Anna Marsh¹, Julie Johnson¹, Logan C. Walker¹, kConFab Investigators², Milena Gongora³, Melissa Brown⁴, Piyush Grover⁵, Mark Girolami⁶, Sean Grimmond³, Georgia Chenevix-Trench¹, Amanda B. Spurdle²

¹Queensland Institute of Medical Research, Brisbane, Australia, ²Peter MacCallum Cancer Centre, Melbourne, Australia, ³Institute for Molecular Biosciences, University of Queensland, Brisbane, Australia, ⁴School of Molecular and Biobal Micr0s, University of Queensland, Brisbane, Australia, ⁵Department of Computer Science & Engineering, Indian Institute of Technology, Kharagpur, India, ⁶Department of Computing Science, University of Glasgow, Glasgow, United Kingdom

Abstract

The functional consequences of missense variants in disease genes are difficult to predict. We assessed if gene expression profiles could distinguish between BRCA1 or BRCA2 pathogenic truncating and missense mutation carriers and familial breast cancer cases whose disease was not attributable to BRCA1 or BRCA2 mutations (BRCAX cases). 72 cell lines from affected women in high-risk breast ovarian families were assayed after exposure to ionising irradiation, including 23 BRCA1 carriers, 22 BRCA2 carriers, and 27 BRCAX individuals. A subset of 10 BRCAX individuals carried rare BRCA1/2 sequence variants considered to be of low clinical significance (LCS). BRCA1 and BRCA2 mutation carriers had similar expression profiles, with some subclustering of missense mutation carriers. The majority of BRCAX individuals formed a distinct cluster, but BRCA1 individuals with LCS variants had expression profiles similar to BRCA1/2 mutation carriers. Gaussian Process Classifier predicted BRCA1, BRCA2 and BRCAX status, with a maximum of 62% accuracy, and prediction accuracy decreased with inclusion of BRCAX samples carrying an LCS variant, and inclusion of pathogenic missense carriers. Similarly, prediction of mutation status with gene lists derived using Support Vector Machines was good for BRCAX samples without an LCS variant (82–94%), poor for BRCA1 with an LCS (40–50%), and improved for pathogenic BRCA1/2 mutation carriers when the gene list used for prediction was appropriate to mutation effect being tested (71–100%). This study indicates that mutation effect, and presence of rare variants possibly associated with a low risk of cancer, must be considered in the development of array-based assays of variant pathogenicity.

Introduction

Approximately 7% of breast cancer cases occur in women with a strong family history of the disease [1]. Mutations in BRCA1 and BRCA2 account for a considerable proportion of these familial breast cancer cases, with the average cumulative risk in BRCA1 and BRCA2 mutation carriers by age 70 years estimated at 65% and 45%, respectively [2]. The Breast Cancer Information Core (BIC) database (http://research.nhgri.nih.gov/bic/) currently has more than 1400 and 1800 unique sequence variants listed in the BRCA1 and BRCA2 genes, respectively. These include frameshift, nonsense, missense, splice site alterations and polymorphisms. Greater than a third of the BRCA1 and greater than half of the BRCA2 unique variants are “unclassified variants” without compelling evidence of pathogenicity or functional significance. The majority of unclassified variants recorded in the BIC database are predicted missense changes (more than 400 BRCA1 and 800 BRCA2). However other variants which may be categorised as unclassified variants are in-frame deletions or duplications, variants that may disrupt splicing, or variants in the 3'UTR that may affect RNA stability (www.kconfab.org). BRCA1/2 unclassified variants represent a problem in the clinical setting as it is not known which variants are associated with the high risk of disease reported for classical truncating mutations.

Several functional assays may be used to determine the significance of unclassified variants, including transcription activation and complementation assays [3–9], but a disadvantage of biochemical assays is that they rely on the functions of specific domains of the protein, require specialized laboratory skills, and are time-consuming to perform. Other methods for classifying variants include the analysis of clinical and histopathological data
Author Summary

Inherited mutations in the genes BRCA1 and BRCA2 increase risk of breast cancer and contribute to a proportion of breast cancer families. However, more than half of the reported sequence alterations in BRCA1 and BRCA2 are currently of unknown clinical significance. We analysed gene expression in lymphoblastoid cell lines derived from blood of patients with sequence alterations in BRCA1 and BRCA2 and compared these to lymphoblastoid cells from familial breast cancer patients without such alterations. We then classified these lymphoblastoid cells based on their gene profiles. We found that BRCA1 and BRCA2 samples were more similar to each other than to familial breast cancer patients without BRCA1/2 mutations, and that the type of sequence change in BRCA1 and BRCA2 (missense or truncating) influenced gene expression. We included in the study ten familial breast cancer samples, which carried sequence changes in BRCA1 or BRCA2, that are believed to be of little clinical significance. Interestingly these samples were distinct from other familial breast cancer cases without any sequence alteration in BRCA1 or BRCA2, indicating that further work needs to be performed to determine the possible association of these “low clinical significance” sequence changes with a low to moderate risk of cancer.

[10], loss of heterozygosity analysis [11] and bioinformatic analysis to predict the effect of the amino acid change on structure and multiple sequence alignment strategies [12]; [13–15]. Integrated evaluation of unclassified variants which combines several approaches, such as the analysis of co-segregation of the mutation with disease, co-occurrence of the variant with a deleterious mutation, sequence conservation of the amino acid change, severity of amino acid change, tumor loss of heterozygosity, and tumor histopathology classification, provides a quantitative tool for the classification of variants [16–22]. This multifactorial method was developed to classify such rare unclassified variants into two categories, variants with features of classical high-risk mutations (termed pathogenic), and variants that do not have the features of a high-risk mutation (termed neutral or low clinical significance (LCS)). While the availability of appropriate biopspecimens (e.g. number of families and tumors) for inclusion in likelihood prediction is a major factor determining the classification of any single variant, another major caveat of the multifactorial approach is that it is not appropriate for the evaluation of possible moderate or low risk variants, since it uses high-risk mutations as reference for the underlying assumptions [16,19,20]. Therefore, the current multifactorial method cannot exclude the possibility that rare variants classified to be of low clinical significance may be associated with a moderate or low risk of cancer.

Gene expression profiling has increased our understanding of the molecular events in breast tumor development, has been used to predict prognosis, and has characterised breast tumors into subtypes [23–27]. The value of expression profiling for identifying underlying high-risk gene mutation status is indicated by a number of studies. A distinct gene expression profile has been reported for breast tumors of BRCA1 mutation carriers [23,28,29], expected to be homozygous for loss of BRCA1 function at the somatic level. In addition, the existence of distinct gene expression profiles for heterozygous loss of BRCA1 and BRCA2 function is supported by accurate separation of short-term cultures of fibroblasts carrying a germline mutation in the BRCA1 or BRCA2 genes, compared to healthy women undergoing reduction mammoplasty surgery with no family or personal history of any cancer or sporadic breast-cancer-affected controls [30,31]. Lymphoblastoid cell lines (LCLs) have also been shown to have distinct mRNA expression phenotypes for heterozygous carriers of ATM mutations, some of which are known to be associated with an increased risk in breast cancer [32,33]. These findings suggest that germline gene expression signatures, including those from fibroblasts or LCLs, may be used to define BRCA1 or BRCA2 mutation status and to assist in assessing the clinical significance of BRCA1 and BRCA2 unclassified variants.

In this study we compared LCL gene expression signatures of breast cancer cases carrying pathogenic mutations in BRCA1 or BRCA2, to familial breast cancer cases with no known BRCA1/2 mutations (BRCAx). We also considered the possibility that BRCAx individuals with a BRCA1 or BRCA2 sequence variant classified to be neutral/low clinical significance (LCS) using multifactorial likelihood analysis may differ in gene expression profile from BRCAx individuals without such sequence variants. In addition, since truncating alterations comprise the majority of known pathogenic mutations but most BRCA1 and BRCA2 unclassified variants are predicted missense alterations, we compared profiles from individuals with known missense or truncating mutations to determine if mutation effect will affect the mutation-associated expression profile for each gene. We derived gene lists to predict mutation status defined by gene and mutation effect, and then tested the efficacy of these gene lists to predict the gene mutation status of LCLs. We provide evidence that gene lists differ according to gene and mutation effect, and according to the presence of sequence variants of low clinical significance. We also demonstrate that the use of appropriately-derived gene lists improves the prediction of pathogenicity of known mutations.

Results

Differences in LCL Post-Irradiation Gene Expression between BRCAx Individuals with or without a Sequence Variant of Low Clinical Significance

The ultimate aim of this experiment was to establish if gene expression profiles could distinguish between BRCA1 or BRCA2 pathogenic mutation carriers and familial breast cancer cases whose disease was not attributable to BRCA1 or BRCA2 mutations (BRCAx cases). BRCAx breast cancer families are likely to result from mutations in several other genes, and thus represent a heterogeneous group. Moreover, included in the BRCAx group were a subset of 10 BRCAx individuals who carried a BRCA1/2 variant previously classified to be of low clinical significance using multifactorial likelihood approaches [8,19,21,22]. Unsupervised hierarchical clustering showed that BRCAx LCLs containing a BRCA1 or BRCA2 variant of low clinical significance clustered away from the majority of remaining BRCAx samples (Figure 1). A t-test with Benjamini and Hochberg multiple testing correction [34] was performed to determine if there were gene expression differences between the BRCAx individuals with an LCS variant and those without an LCS variant. Expression of 631 genes differed between the two BRCAx subgroups (5% of the 631 genes identified would be expected to pass this restriction by chance). For this reason, BRCAx samples were stratified according to the presence of an LCS variant for further analyses.

Gene expression is similar for carriers of BRCA1 and BRCA2 truncating mutations and rare sequence variants of low clinical significance, but differs from BRCA1 and BRCA2 missense mutations and BRCAx non-BRCA1/2 familial cases.

Unsupervised hierarchical clustering (Figure 2) of LCL expression data from all samples revealed that BRCA1 and BRCA2
samples were more similar to each other than BRCA1 samples without an LCS variant. This result suggests that germline effects of heterozygous mutations in BRCA1 and BRCA2 cannot easily be separated using the experimental conditions used in this study. Although BRCA1/2 samples tended to cluster distinctly from BRCA1/2 samples, nine of ten BRCA1 individuals who carried a BRCA1/2 variant previously classified to be of low clinical significance fell within the major BRCA1 or BRCA2 mutation cluster. In contrast, six of the nine pathogenic missense mutations of BRCA1 or BRCA2 fell into a BRCA1/2 outlier group, which clustered closer to the BRCA1/2 samples.

Gaussian Process Classifier Prediction of BRCA1, BRCA2 and BRCAX Mutation Status

To determine the accuracy of using gene expression data from LCLs to predict BRCA1/2 pathogenic carriers and BRCA1/2 individuals, we used a Gaussian Process Classifier (GPC). GPC analysis was used previously in an analysis of microarray profiles from irradiated short-term fibroblasts of BRCA1/2 mutation carriers [31], and allows for multiway comparison of groups. For GPC analysis 2031 genes which were significantly over/under-expressed at the 5% significance level were selected. The GPC was used in a three way comparison to compare BRCA1 truncating mutation carriers to BRCA2 truncating mutation carriers, and to BRCA1/2 samples without an LCS variant. Samples with BRCA1 or BRCA2 pathogenic missense mutations or classified as BRCAX with an LCS variant were then included to determine their affect on the prediction accuracy. A summary of the prediction accuracy is shown in Table 1. The highest prediction accuracy (62.26%) was achieved when the analysis excluded samples classified as BRCAX with an LCS, and samples with BRCA1 or BRCA2 missense mutations. This prediction accuracy is above the expected performance, as a random prediction with three classes comprised of a similar sample number would be 33% accuracy. When BRCA1 and BRCA2 samples were compared to only BRCAX samples with an LCS variant, the prediction dropped to 43.46%, and the addition of the BRCA1/2 samples without an LCS variant improved the accuracy. In all comparisons the inclusion of the pathogenic non-truncating mutations of BRCA1 and BRCA2 lowered the prediction accuracy.

Comparison of Gene Expression Profiles between BRCA1, BRCA2 and BRCAX

In the clinical setting, unclassified sequence variants of BRCA1 or BRCA2 are generally identified after full sequencing of both genes. Therefore the most common clinical question is whether a variant in BRCA1 or BRCA2 is pathogenic or not. We thus performed pair wise analyses to determine if BRCA1/2 samples could be distinguished from those with pathogenic mutations in BRCA1 or BRCA2. Based on observations from hierarchical clustering analyses and the GPC analysis, we also considered the possibility that the effect of pathogenic BRCA1/2 mutations (truncating or missense) affected LCL gene expression. T-tests were performed using the 20,874 detected probes to elucidate gene differences between i) BRCA1 or BRCA2 truncating vs BRCAX without an LCS variant; ii) BRCA1 or BRCA2 missense mutations vs BRCAX without an LCS variant. The number of genes which passed these restrictions and the overlap between them is outlined in Figure 3A and 3C. The comparisons were then repeated with BRCA1 and BRCA2 with an LCS variant (Figure 3B and 3D). As expected when BRCA1 and BRCA2 were compared to BRCA1/2 samples without an LCS variant, a greater number of genes were deemed significant compared to BRCA1 or BRCA2 vs BRCAX samples with an LCS variant.

Support Vector Machines Prediction of BRCA1, BRCA2 and BRCAX Mutation Status

SVM is a widely accepted classification approach for assessing differences in mRNA expression, and was used to compare BRCA1 or BRCA2 individually to BRCAX samples. Since our detailed analysis of gene lists showed that mutation effect (truncating or missense substitution) appears to affect the genes that are differentially expressed in the carriers after IR (Figure 3), we assessed if these gene differences will affect the predictions. We used SVM with the top 200 genes from the comparison of BRCA1 or BRCA2 truncating mutations to BRCAX, and the top 200 genes from the comparison of BRCA1 and BRCA2 missense mutations to BRCAX (Figure 3A and 3C). The genes which differed between
BRCA1 or BRCA2 and BRCAX with an LCS variant were not used in this comparison as too few genes passed the restriction (Figure 3B and 3D). The top 200 genes are listed in Tables S2, S3, S4, and S5 and the overlap of the top 200 genes used for prediction from [BRCA1 (missense) vs BRCAX (no LCS)] and [BRCA1 (truncating) vs BRCAX (no LCS)] was 16 transcripts, with no overlap between the top 200 genes from [BRCA2 (missense) vs BRCAX (no LCS)] and [BRCA2 (truncating) vs BRCAX (no LCS)]. A total of 715 different genes were represented in the four lists of top 200 gene-lists from comparison of BRCAX (no LCS) to the different BRCA1/2 groups above. The results are summarised in Tables 2 and 3. The BRCA2 truncating pathogenic carriers were consistently predicted with higher accuracy compared to BRCA1 truncating pathogenic carriers. The accuracy of prediction was improved when the gene list used for prediction was appropriate to the mutation effect (truncating or missense) being tested. When the missense-associated gene list was used, pathogenic truncating mutations were predicted with 35% and 68% accuracy for BRCA1 and BRCA2, respectively. Predictions increased to 71% and 84% for BRCA1 and BRCA2, respectively, using the truncating-associated genes. Similarly, the pathogenic missense mutation carriers were predicted with 83% and 100% accuracy when the missense-associated gene list is used, but this accuracy was lower or remained the same when the truncating-specific gene list was used (83% and 0%). Prediction of BRCAX samples that did not carry an LCS variant was high in all comparisons (82–94%). In contrast, prediction of BRCAX samples that did carry an LCS variant was poor (40–50%).

When using SVM, the significance of the predictions can also be represented by the distance the prediction is from the plane, where predictions called with greater confidence are further from the plane that separates the BRCA1 (or BRCA2) and BRCAX samples.

The significance of the predictions for the BRCA1 pathogenic missense mutations is summarised in Figure 4. Although both missense and truncating gene lists correctly predicted 5 of 6 missense mutations, the results show that there is much greater

---

**Table 1. Accuracy of Prediction of Mutation Status using a Gaussian Process Classifier**

<table>
<thead>
<tr>
<th>BRCA1 Missense vs BRCAX (no LCS)</th>
<th>BRCA1 Truncating vs BRCAX (no LCS)</th>
<th>BRCA2 Missense vs BRCAX (no LCS)</th>
<th>BRCA2 Truncating vs BRCAX (no LCS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excluding pathogenic missense</td>
<td>62.26%</td>
<td>43.46%</td>
<td>52.36%</td>
</tr>
<tr>
<td>Including pathogenic missense</td>
<td>46.77%</td>
<td>40%</td>
<td>45.83%</td>
</tr>
</tbody>
</table>

2031 genes were used to predict mutation status between groups, as described in the methods section.

doi:10.1371/journal.pgen.1000080.t001

---

**Figure 3. Venn Diagrams of Differences between BRCA1 or BRCA2 and BRCAX LCLs with or without a LCS.** T-tests (p-value<0.05) were performed to determine genes that differed between LCLs as follows: A) BRCA1 Truncating mutations vs BRCAX with no LCS; B) BRCA1 Missense mutations vs BRCAX with no LCS; C) BRCA2 Truncating mutations vs BRCAX with no LCS; D) BRCA2 Missense mutations vs BRCAX with no LCS. For each comparison, the overlap of genes is shown.

doi:10.1371/journal.pgen.1000080.g003
Table 2. Mutation Prediction of BRCA1 and BRCA2 samples based on SVM

<table>
<thead>
<tr>
<th>Gene list used for prediction</th>
<th>Proportion of mutation group correctly predicted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BRCA1 Truncating</td>
</tr>
<tr>
<td>BRCA1 Truncating list</td>
<td>12/17 (71%)</td>
</tr>
<tr>
<td>BRCA1 Missense list</td>
<td>6/17 (35%)</td>
</tr>
</tbody>
</table>

*Lists included the 200 Highest Ranked Genes from the comparison of BRCA1 to BRCA2 samples without an LCS variant, as described in the methods. doi:10.1371/journal.pgen.1000080.t002

Table 3. Mutation Prediction of BRCA2 and BRCA2 samples based on SVM

<table>
<thead>
<tr>
<th>Gene list used for prediction</th>
<th>Proportion of mutation group correctly predicted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BRCA2 Truncating</td>
</tr>
<tr>
<td>BRCA2 Truncating list</td>
<td>16/19 (84%)</td>
</tr>
<tr>
<td>BRCA2 Missense list</td>
<td>13/19 (68%)</td>
</tr>
</tbody>
</table>

*Lists included the 200 Highest Ranked Genes from the comparison of BRCA2 to BRCA2 samples without an LCS variant, as described in the methods. doi:10.1371/journal.pgen.1000080.t003

Pathway Analysis of Genes Associated with Pathogenic Mutations in BRCA1 or BRCA2

Ingenuity Pathway Analysis of genes which differed between the LCLs carrying pathogenic truncating or missense mutations of BRCA1 or BRCA2 compared to BRCA2 samples without an LCS variant was performed to determine the potential functional relevance of the differentially expressed genes. All BRCA1 and BRCA2 pathogenic mutations resulted in gene expression changes relating to cell cycle, cancer and cellular growth and development, while BRCA1 and BRCA2 missense mutations shared some additional similarities (cell death and cell development pathways). There were also alterations in several pathways that were unique to BRCA1 truncating mutations, BRCA2 truncating mutations, BRCA1 missense mutations, or BRCA2 missense mutations (Figure S1).

Discussion

It is difficult to counsel patients with a strong family history of breast cancer who are found to carry an unclassified variant in BRCA1 or BRCA2. While management at the level of the family should remain unchanged from that of a BRCA1 family with no knowledge of a BRCA1/2 mutation, some individuals from high-risk families may nevertheless interpret information about an unclassified variant to alter their choices regarding prophylactic surgery for example, and so require careful counselling. Gene expression profiling can be used to classify samples based on phenotype, and its frequent use in laboratories world-wide holds great promise for clinical application, to the extent that profiling tools are being developed for diagnostic use e.g. Agendia Inc. (http://www.agendia.com/).

Expression profiles of short-term fibroblasts have previously been reported to separate carriers of a heterozygous mutation in the BRCA1 or BRCA2 genes from sporadic breast-cancer-affected controls [30,31]. We wished to determine if expression profiling of LCLs could similarly be used to predict BRCA1 or BRCA2 mutation status, with the ultimate aim of predicting the significance of unclassified variants of BRCA1 or BRCA2. We chose LCLs as a minimally invasive source of germline material that can be maintained as long term cultures, and because previous studies have shown that LCL array profiling is robust to sourcing of LCLs established in different laboratories [33]. We compared expression profiles of irradiated LCLs from BRCA1 and BRCA2 carriers to those of non-BRCA1/2 BRCA1 familial breast cancer patients, an appropriate reference group for the proposed evaluation of unclassified variants identified in familial breast cancer patients. A relatively early time-point of 30 minutes post-irradiation was chosen to capture gene expression initiation, and

Figure 4. Confidence of Predictions for Missense BRCA1 LCLs as Determined by Distance from the SVM Plane. The SVM plane separating BRCA1 from BRCA2 is shown by the red line. If the sample falls over the line (black point) the missense mutation is correctly predicted as pathogenic for BRCA1 mutation. If the sample falls under the line (red point) the missense mutation is incorrectly predicted as BRCA2. The gene lists used for the predictions are the top 200 genes from BRCA1 missense vs BRCA2, and the top 200 genes from BRCA1 Truncating vs BRCA2. doi:10.1371/journal.pgen.1000080.g004
minimize possible downstream compensation effects. It has previously been shown that 10Gy IR treatment of normal LCLs has an effect on the transcriptional response, with greatest change in mRNA levels for most genes within one hour post-treatment [35], and studies of mouse brain gene expression after whole-body low-dose irradiation have shown that a large number of early IR response genes can be measured at the 30 minute time point [36].

A number of BRCAX cases carried BRCA1 or BRCA2 sequence variants that had been previously classified using multifactorial likelihood modelling methods to be neutral or of low clinical significance—that is, these rare variants are extremely unlikely to be a high-risk mutation in either of these genes, but the modelling methods used cannot assess whether they are truly neutral or associated with a much lower risk of disease. We found that the BRCAX samples with such LCS variants were separated from the majority of BRCAX samples without such LCS variants using unsupervised hierarchical clustering. This result indicates that LCS samples differ in expression profile as a result of their BRCA1 or BRCA2 sequence variant, and was substantiated by the class prediction methods: GPC prediction of the BRCAX samples decreased in accuracy when BRCA1 samples with an LCS were included. In addition, SVM to detect BRCA1 or BRCA2 mutation-related gene lists yielded differences in the significant genes for comparisons to BRCAX samples without an LCS variant, compared to BRCA1 samples with an LCS variant. Accordingly, prediction of BRCA1 subgroup status based on the more robust gene list derived from comparisons to BRCA1 individuals without an LCS variant was generally poorer for BRCAX samples with an LCS (40–50%) compared to those without an LCS (82%–94%). These rather provocative results indicate that the possible effect of all rare variants should be considered in development of assays to assess which variants have features of high-risk mutations. Moreover, the similarity in expression profile of these variants to other BRCA1/2 pathogenic mutations suggests that at least some of these LCS variants may confer small-moderate risks of breast cancer, presumably acting in concert with alterations in other genes in the BRCA1/2 pathway to lead to breast cancer. Given the rarity of these variants, alternative statistical approaches will be required to assess the risk of cancer associated with them.

The assay conditions used in this study could not distinguish between samples with pathogenic BRCA1 mutations and those with pathogenic BRCA2 mutations. Ionising radiation has previously been show to separate fibroblast cells which carry BRCA1 or BRCA2 mutations from sporadic cases with 100% accuracy [31], but our experiment differs in several respects. We compared BRCA1 and BRCA2 cases to familial BRCAX cases as an appropriate reference group for familial breast cases likely to be identified as carriers of BRCA1/2 mutations or unclassified variants, we used LCLs instead of fibroblasts, we selected a lower IR exposure (10Gy vs 15Gy), and we chose a relatively early time point of 30 mins after exposure to IR in order to gain a better understanding of the functional differences in response to IR between the BRCA1, BRCA2, and BRCAX cell lines. Some or all of these factors may explain the difference in the ability of this study to distinguish BRCA1 from BRCA2, both of which are involved in DNA damage repair. However, differences in post-irradiation response between BRCA1 individuals and BRCA1/2 mutation carriers are supported by alternative analysis we have conducted of the subset of genes reported to be involved in post-irradiation response, comparing mutation-negative normal female controls to BRCA1 individuals without an LCS variant, or to BRCA1 or BRCA2 truncating mutation carriers. Our results indicate substantial differences in radiation response between normal controls and the patient groups, and also considerable differences between the BRCAX group and BRCA1 and BRCA2 carriers [37].

The ultimate aim of this experiment was to identify array profiles that would be useful for the classification of unclassified sequence variants of BRCA1 or BRCA2. In the clinical setting, individuals generally present with full sequencing of both genes, and presence of a variant in one gene or the other. We thus assessed the ability to distinguish BRCA1 or BRCA2, separately, from BRCA1 individuals. Importantly, since most unclassified variants are predicted to cause amino acid substitutions, we also assessed the relevance of mutation effect for expression profiles. We found that the genes which significantly differed between BRCA1 or BRCA2 and BRCAX LCSs were dependent on mutation effect. Accordingly, the SVM prediction for each mutation effect was best if the appropriate gene list was used, in terms of both accuracy of prediction (BRCA1 or BRCA2 vs BRCAX) and confidence in the classification as determined by the distance of the prediction from the SVM plane. Thus we strongly urge that mutation effect is taken into account if this type of assay is to be developed for use in predicting the clinical significance of BRCA1/2 variants. The current challenge is that few missense variants have been classified with respect to their clinical significance, with the only 23 individual missense variants termed clinically important by BIC, 17 in BRCA1 and six in BRCA2. Moreover, these are restricted in terms of the domains/regions in which they occur, residing in the BRCA1 start site (n = 2), ring finger (n = 4) or transactivation domains (n = 11), and the BRCA2 Cdk2 phosphorylation site (n = 3) or at one codon (2336, n = 3) in a region of unknown function. It will thus be difficult to accrue a panel of known pathogenic missense variants for use in such predictive assays, and will require a concerted collaborative effort. Assuming sufficient pathogenic variants are identified, the successful execution of such a study may eventually distinguish missense-associated gene expression patterns that are generic to missense mutations, and/or those that are specific to the domain location of missense mutations. In addition, a possibly greater challenge will be identifying assay conditions (cell type, perturbation, time-point etc) that can also identify gene expression differences between patients with rare variants of low clinical significance in BRCA1 and/or BRCA2 and those with truly high-risk pathogenic mutations (truncating or missense) in these genes. Our study, using conditions that were not optimal for separating BRCA1 and BRCA2 mutations nevertheless identified gene expression differences between BRCA1/2 pathogenic mutations and LCS variants, suggesting that larger sample sizes and further experimentation may identify a more robust gene list to separate pathogenic mutations, variants of low clinical significance, and individuals with no sequence alterations in BRCA1/2.

Pathway analysis confirming altered expression of cancer, cell proliferation and cell cycle pathways in BRCA1 and BRCA2 mutation carrier groups is consistent with the known functions of BRCA1 and BRCA2 [38,39]. The pathway differences by mutation type such as cell death and development may reflect that the majority of truncating mutations result in activation of the nonsense mediated decay pathway [40] and complete loss of protein, whereas most missense mutations are likely to result in more subtle effects through ablation of individual functional domains. Some pathways identified were unexpected and are only present in a single mutation type, and it is thus likely that at least some of these pathways were generated by chance alone.

In conclusion, we have provided evidence that carriers of BRCA1 and BRCA2 variants considered to be of low clinical
significance have array profiles distinct from other non-BRCA1/2 familial cases, but resembling profiles of pathogenic BRCA1/2 cases, indicating that further work will be required to evaluate their possible association with a low-moderate risk of cancer. We have also shown that it will be important to consider mutation effect when developing array-based assays for predicting the clinical significance of BRCA1 or BRCA2 unclassified variants. Lastly, our findings demonstrate the ability of array profiling of immortalized lines derived from lymphoblastoid cells to detect germline mutations in genes that result in breast and ovarian cancer, and thus have relevance to the investigation of other genetic diseases irrespective of the organs or tissues they affect.

**Materials and Methods**

**Subjects and Lymphoblastoid Cell Lines**

LCLs were derived from breast cancer-affected women recruited into the Kathleen Cunningham Foundation for Research into Breast Cancer (kConFab), a consortium which ascertainment multiple-case breast cancer families [41]. These include families in which one or more carriers of a BRCA1 or BRCA2 mutation have been identified, and families in which no predisposing mutation has been identified (BRCAX). The recruitment criteria for BRCAX families are: 1) at least one member of the family at high-risk according to the National Breast Cancer Centre Category III guidelines (http://www.nbcc.org.au), and four or more cases of breast or ovarian cancer (on one side of the family), and two or more living affected with breast or ovarian cancer, and four or more living first or second degree unaffected female relatives of affected cases, over the age of 18; 2) two or three cases of breast or ovarian cancer (on one side of the family) in same or adjacent generations, if at least one of these cases is ‘high risk’ (i.e. male breast cancer, bilateral breast cancer, breast plus ovarian cancer in the same individual, or breast cancer with onset less than 40 years), and two or more living affected cases with breast or ovarian cancer, and four or more living first or second degree unaffected female relatives of affected cases, over the age of 18.

Classifications for BRCA1 and BRCA2 pathogenic mutations and variants of low clinical significance (LCS) are described on http://www.kconfab.org/Progress/Classification.shtml. Briefly, LCS variants include BRCA1 or BRCA2 variants described in trans with a deleterious mutation in the same gene in an individual and occur at a frequency of less than 1% in unaffected controls, or considered neutral/low clinical significance as measured using multifactorial likelihood approaches [16,19,21,22].

A cohort of 72 LCLs were used in this study. The full listing of mutation details for LCLs is shown in Table S1. In brief, the study included:

(a) 23 LCLs from women carrying a pathogenic mutation in BRCA1, 17 of which are predicted to lead to a truncated protein, and six of which were missense mutations (2 × 300 T>C G61G; 2 × 5242 C>A A1708E; 1 × 5331 G>A G1738R; 1 × 5632 T>A V1838E);

(b) 22 LCLs from women carrying a pathogenic mutation in BRCA2, 19 of which are predicted to lead to a truncated protein, and three of which were missense mutations (3 × 8395 G>C D2723H, one of which also carried the LCS variant 9079 G>C A2951T);

(c) 27 LCLs from women from breast cancer families that have tested negative for pathogenic mutations in BRCA1 or BRCA2 (BRCAX) after complete sequencing and multiplex ligation-dependent probe amplification gene dosage assay (MLPA) large deletion testing of BRCA1 and BRCA2. Ten samples, carried either BRCA1 or BRCA2 sequence germline variants considered from multifactorial likelihood classification to be LCS (BRCA1 3582 G>C D1153H, 1605 C>T R496C, 5236 G>C G1706A (2 samples); BRCA2 333 A>G Y42C, 2834 C>T S869L, 3031 G>A D935N (3 samples), 8795 A>C E2856A) [16,19,21,22] (unpublished data). The remaining 17 samples carried no BRCA1 or BRCA2 sequence variants other than common polymorphisms.

**Gene Expression Profiling**

LCLs were grown in RPMI 1640 media with 15% fetal bovine serum, 1% penicillin-streptomycin and 1% L-glutamine. The cell number was normalised and fresh medium was added to cells 24 hr prior to irradiation with 10 Gy, using a calibrated Cs137 c-source delivering 1 Gy/1.5 min. Total RNA was harvested 30 min later using an RNeasy kit (Qiagen, Doncaster, VIC). The Illumina Totalprep RNA amplification kit (Ambion, Austin, TX) was used to amplify and biotinylate 450 ng of total RNA. Biotinylated RNA was hybridised overnight at 55°C to Illumina Human-6 version 1 BeadChips containing >46,000 probes (Illumina Inc., San Diego, CA). The microarrays were washed, stained with streptavidin-Cy3, and then scanned with an Illumina BeadArray Scanner. Duplicate arrays were performed for eight cell lines across the different groups for quality control purposes, with duplicates performed on different days. All duplicate arrays showed highest correlation with each other (correlation >0.98). Duplicate samples were not included in analysis. Comparative real-time PCR was performed for ten genes on 6–8 samples, using GAPDH to normalise all data, and the comparative cycle threshold method for analysis. Paired student t tests were performed to determine the significance of gene expression changes. Expression differences were validated for 8/10 genes tested.

**Data Analysis**

Raw data was imported into Illumina Beadstudio and then exported into Genespring v7.3 (Agilent Technologies, Forest Hill, VIC) for further analysis. Data was normalised (per chip normalized to 50th percentile and per gene normalized to median) and filtered using an Illumina detection score of >0.99 in at least one sample, which yielded 20,874 probes that were used in all further analyses. The majority of these probes used in the analysis were designed by Illumina to assay the curated portion of the NIH Ref sequence database-16,923 were present in the Ref sequence database, comprising 63% of all Ref sequence-listed probes on the array. Transcripts which had a >2-fold change versus the mean were visualised using unsupervised Hierarchical Clustering (Figures 1 and 2). The clustering method used was a Pearson correlation similarity measure with an average linkage clustering algorithm. Two different methods were used to classify LCLs based on mutation status: [1] A multi comparison Gaussian Process Classifier (GPC) [42] with Leave-One-Out cross-validation to determine the prediction errors, as previously used to predict BRCA1/BRCA2 mutation status of irradiated fibroblasts [31]; [2] A linear classification method commonly used for classification of microarray data, Support Vector Machines (SVM) [43] with Leave-One-Out cross validation. The GPC analysis used 2031 genes which were derived from a t-test to select the genes that were significantly over/under-expressed at the 5% significance, while the SVM used genes from the 20,874 detected probes which differed between groups of LCLs using a t-test p of 0.05. All resulting gene lists are available as supplementary data and all data is available via GEO: Accession number GSE10905.
Ingenuity Pathway Analysis (Ingenuity Systems, www.ingenuity.com) was used for biological interpretation of gene lists. Analysis of the transcripts found to be up- and down-regulated in irradiated LCLs as identified for the different mutation categories identified those biochemical networks most likely to be affected by a BRCA1 and BRCA2 truncating and missense mutation, relative to BRCA2. Those pathways with multiple hits or a significance score ≥20 were then compared.

Supporting Information

Figure S1  Biological Pathways defined by genes dysregulated in BRCA1 and BRCA2 mutation carriers. Pathways identified by Ingenuity pathway analysis of the top 200 genes defined for truncating and missense BRCA1 or BRCA2 mutations compared to BRCA2 without an LCS were compared for overlap. Bold lines and pathways denoted in uppercase indicate biological pathways identified as differentially expressed in both BRCA1 and BRCA2.

Table S1  Detailed Mutation Status of LCLs. Found at: doi:10.1371/journal.pgen.1000080.s001 (0.20 MB TIF)

Table S2  The top 200 significant genes from the comparison of BRCA1 Misseone vs BRCA2 without an LCS. Found at: doi:10.1371/journal.pgen.1000080.s002 (0.03 MB XLS)

Table S3  The top 200 significant genes from the comparison of BRCA1 Truncating vs BRCA2 without an LCS. Found at: doi:10.1371/journal.pgen.1000080.s003 (0.05 MB XLS)

References


Acknowledgments

We thank Sue Healey for providing assistance with BIC nomenclature and classifications. We also wish to thank Heather Thorne, Eveline Niedermayer, Jan Groves, Amanda Williams, the kConFab mutation review committee, kConFab research nurses and staff, the heads and staff of the Family Cancer Clinics, and the Clinical Follow Up Study (funded by NHMRC grants 145604 and 200704) for their contributions to this resource, and the many families who contribute to kConFab.

Author Contributions


