Deletion of Chromosomes 13q and 14q Is a Common Feature of Tumors with BRCA2 Mutations

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Abstract

Introduction: Germline BRCA1 or BRCA2 mutations account for 20–30% of familial clustering of breast cancer. The main indication for BRCA2 screening is currently the family history but the yield of mutations identified in patients selected this way is low.

Methods: To develop more efficient approaches to screening we have compared the gene expression and genomic profiles of BRCA2-mutant breast tumors with those of breast tumors lacking BRCA1 or BRCA2 mutations.

Results: We identified a group of 66 genes showing differential expression in our training set of 7 BRCA2-mutant tumors and in an independent validation set of 19 BRCA2-mutant tumors. The differentially expressed genes include a prominent cluster of genes from chromosomes 13 and 14 whose expression is reduced. Gene set enrichment analysis confirmed that genes in specific bands on 13q and 14q showed significantly reduced expression, suggesting that the affected bands may be preferentially deleted in BRCA2-mutant tumors. Genomic profiling showed that the BRCA2-mutant tumors indeed harbor deletions on chromosomes 13q and 14q. To exploit this information we have created a simple fluorescence in situ hybridization (FISH) test and shown that it detects tumors with deletions on chromosomes 13q and 14q.

Conclusion: Together with previous reports, this establishes that deletions on chromosomes 13q and 14q are a hallmark of BRCA2-mutant tumors. We propose that FISH to detect these deletions would be an efficient and cost-effective first screening step to identify potential BRCA2-mutation carriers among breast cancer patients without a family history of breast cancer.

Introduction

Germline mutations in pathways critical for maintenance of genomic integrity confer an increased risk of developing breast cancer [1]. Inherited mutations in two genes, breast cancer 1 (BRCA1) and BRCA2, are associated with a particularly striking increase in breast cancer risk [2]. Consistent with the Knudson two-hit model, both alleles of BRCA1 and BRCA2 are inactivated in tumors, indicating that the genes behave like classic tumor suppressor genes [3]. Their gene products are implicated in the repair of DNA double-strand breaks [4]; BRCA1 is required for recruitment of repair proteins to sites of breakage [5], whereas BRCA2 nucleates RAD51 filament assembly on single-stranded DNA exposed by resection from the break [6]. Loss of these functions leads to genomic instability [7].
### Table 1. Characteristics of patients and tumors.

| ID  | Tumor set | BRCA status | Sex | Age at surgery (year) | Tumor size (mm) | Tumor cells (%) | Histologic grade | ER   | PR   | ERBB2 |
|-----|-----------|-------------|-----|----------------------|-----------------|-----------------|------------------|------|------|-------|
| 52  | Training  | BRCA2       | F   | 35                   | 17              | 92              | 3                | ++   | --   | --    |
| 86  | Training  | BRCA2       | F   | 46                   | 16              | 90              | 3                | +++  | +    | --    |
| 106 | Training  | BRCA2       | F   | 57                   | 22              | 85              | 3                | +++  | +    | --    |
| 133 | Training  | BRCA2       | F   | 40                   | 15              | 75              | 2                | +    | +    | --    |
| 144 | Training  | BRCA2       | F   | 40                   | 12              | 55              | 2                | ++   | --   | +     |
| 146 | Training  | BRCA2       | F   | 64                   | 25              | 80              | 3                | --   | --   | +     |
| 148 | Training  | BRCA2       | F   | 62                   | 25              | 90              | 3                | ++   | --   | ++    |
| 8   | Training  | BRCA2       | F   | 51                   | 18              | 90              | 3                | --   | --   | --    |
| 9   | Training  | BRCA2       | F   | 51                   | 25              | 95              | 3                | ++   | ++   | --    |
| 14  | Training  | BRCA2       | F   | 56                   | 40              | 78              | 2                | ++   | +++  | --    |
| 16  | Training  | BRCA2       | F   | 45                   | 12              | 90              | 2                | nd   | +++  | --    |
| 22  | Training  | BRCA2       | F   | 50                   | 27              | 95              | 3                | +++  | +++  | --    |
| 24  | Training  | BRCA2       | F   | 64                   | 18              | 90              | 2                | +++  | +    | --    |
| 25  | Training  | BRCA2       | F   | 35                   | 12              | 70              | 1                | ++   | --   | --    |
| 26  | Training  | BRCA2       | F   | 37                   | 12              | 92              | 2                | ++   | +    | --    |
| 33  | Training  | BRCA2       | F   | 42                   | 35              | 73              | 1                | ++   | --   | --    |
| 37  | Training  | BRCA2       | F   | 45                   | 20              | 92              | 2                | +++  | +++  | --    |
| 38  | Training  | BRCA2       | F   | 64                   | 13              | 90              | 3                | +++  | --   | --    |
| 40  | Training  | BRCA2       | F   | 41                   | 12              | 95              | 2                | +    | ++   | --    |
| 41  | Training  | BRCA2       | F   | 38                   | 21              | 92              | 3                | ++   | +    | --    |
| 46  | Training  | BRCA2       | F   | 60                   | 38              | 90              | 2                | --   | --   | --    |
| 66  | Training  | BRCA2       | F   | 73                   | 12              | 90              | 2                | +++  | +++  | --    |
| 75  | Training  | BRCA2       | F   | 58                   | 14              | 80              | 2                | --   | --   | +++   |
| 79  | Training  | BRCA2       | F   | 42                   | 11              | 90              | 3                | ++   | +    | --    |
| 81  | Training  | BRCA2       | F   | 46                   | 28              | 80              | 2                | +    | ++   | --    |
| 82  | Training  | BRCA2       | F   | 50                   | 9               | 85              | 1                | ++   | ++   | --    |
| 84  | Training  | BRCA2       | F   | 47                   | 27              | 92              | 3                | ++   | +    | --    |
| 85  | Training  | BRCA2       | F   | 64                   | 15              | 90              | 1                | --   | +++  | --    |
| 93  | Training  | BRCA2       | F   | 44                   | 18              | 85              | 2                | +++  | +++  | --    |
| 97  | Training  | BRCA2       | F   | 69                   | 40              | 80              | 3                | +++  | +    | --    |
| 111 | Training  | BRCA2       | F   | 73                   | 15              | 80              | 1                | +++  | --   | --    |
| 3   | Validation| BRCA2       | F   | 36                   | 18              | 95              | 3                | --   | +    | +++   |
| 15  | Validation| BRCA2       | F   | 42                   | 15              | 95              | 3                | --   | --   | --    |
| 17  | Validation| BRCA2       | F   | 76                   | 3               | 95              | 1                | +++  | +    | --    |
| 30  | Validation| BRCA2       | F   | 51                   | nd              | 95              | 3                | +++  | --   | nd    |
| 48  | Validation| BRCA2       | F   | 54                   | 20              | 90              | 1                | ++   | +    | --    |
| 49  | Validation| BRCA2       | F   | 49                   | 35              | 66              | 2                | +++  | +    | --    |
| 65  | Validation| BRCA2       | F   | 46                   | 37              | 95              | 3                | --   | --   | --    |
| 71  | Validation| BRCA2       | F   | 43                   | 21              | 73              | 2                | ++   | +++  | +     |
| 83  | Validation| BRCA2       | F   | 50                   | 18              | 50              | 2                | ++   | --   | --    |
| 89  | Validation| BRCA2       | F   | 30                   | 30              | 82              | nd               | ++   | ++   | --    |
| 96  | Validation| BRCA2       | F   | 41                   | 25              | 85              | 3                | --   | --   | +     |
| 99  | Validation| BRCA2       | M   | 63                   | 21              | 90              | 1                | +++  | ++   | --    |
| 43  | Genomic   | BRCA2       | F   | 38                   | 12              | 90              | 2                | ++   | --   | --    |
| 149 | Genomic   | BRCA2       | F   | 76                   | 70              | 60              | 2                | +++  | --   | --    |

Footnote. Tumor set: Training set, tumors used to create the gene expression signature; Validation set, BRCA2 tumors from Bergonie Cancer Institute; Genomic set, tumors only used for CGH and SNP analysis. nd, not determined. There was no statistically significant difference (p > 0.05, Fisher test) between the BRCA2 and BRCA2 groups for the following comparisons: age at surgery < 49 years (median age); tumor size < 18 mm (median tumor size); tumor cell content < 90% (median tumor cell content); +++ vs other ER status; + vs other PR status; -- vs other ERBB2 status.

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Stanford classification but this is not specific enough to be useful clinically to identify tumors with BRCA2 mutations [9].

In this study, we have used gene expression and genomic data to identify specific molecular features that distinguish tumors with BRCA2 mutations from tumors with other breast cancer predisposition mutations. Based on these results we have developed a fluorescent in-situ hybridization (FISH) test that can be used to screen for tumors with an increased risk of containing BRCA2 mutations.

Methods

Patients and Samples

All samples were from the Bergonie Cancer Institute, Bordeaux, except for sample 144 from the Val d’Aurelle Regional Cancer Center, Montpellier; samples 146 and 148 from the Dupuytren Hospital, Limoges; and the BRCA2 tumors in the validation set from the Curie Institute. The microarray data for the validation set were generously provided by the Translational Research Unit at the Curie Institute, Paris. The control group contained BRCA1 tumors, defined as tumors lacking known BRCA1/2 mutations from families with either i) at least three breast cancer-affected first or second-degree relatives; or ii) breast cancer before age 42 or ovarian cancer in two first-degree relatives or two second-degree relatives via a male. All patients agreed to the use of their samples for research purposes, in compliance with the French law on medical genetic diagnosis of suspected breast cancer predisposition, in compliance with the French law on genetic testing (law number 94-654).

Tumor and Mutation Characterization

Clinical, pathological and genetic data for each case are listed in Table 1. Immunohistochemistry for ER, progesterone receptor (PR) and HER2 (ERBB2) were performed as previously described [10]. HER2 expression was scored according to the Herceptest system. ER and PR were scored by multiplying the percentage of positive cells by the intensity (score 0–20: –; score 21–100: +; score 101–200: ++; score 201–300: +++). Screening for germline mutations was made on leucocyte DNA as previously described [10].

Gene Expression and Genomic Chip Hybridization

RNA was extracted from the tumors as described [10] and hybridized to Affymetrix U133 Plus 2.0 genechip microarrays by the Genopole Alsace-Lorraine genomics platform, except for the validation set which was hybridized by the Curie Institute genomics platform. DNA was extracted from the tumors and hybridized to Integrachip V7 bacterial artificial chromosome (BAC) arrays as described [10]. SNP array profiling was performed on Illumina Human610-Quad v1.0 BeadChips (Illumina, Inc., San Diego, CA) by Integrategen (Evry, France). The gene expression and genomic data are available in Array Express under accession numbers E-TABM-654, E-MEXP-3686, E-MEXP-3690 and in GEO under accession number GSE39710.

Data Processing and Statistical Analyses

Given the rarity of the tumors, it was not possible to avoid processing the tumors in batches; the hybridization dates for the Affymetrix chips are given in the CEL files. The 12 BRCA1 controls for the validation set were chosen because they showed the smallest batch effect relative to the Curie Institute tumors. The 12 BRCA1 tumors in the validation set were separate from the 24 BRCA1 tumors in the training set. The gene expression data were normalized with the RMA algorithm in R version 2.13.1 [11–13]. To eliminate redundant genes sharing a gene symbol, the most variable probe set was selected based on the standard deviation across the entire dataset. Differentially expressed genes were identified by moderated t-test in limma [14] (an R script for the expression analysis is available on request). The 66 BRCA2 gene signature genes were combined to make a BRCA2 score by summing the mean-centered expression values weighted by the t values from limma. Gene Set Enrichment Analysis (GSEA) was performed with Broad Institute java software [15,16]: the expression dataset was ranked by t-statistic in limma, then enrichment was scored by GSEA for chromosome bands using the MSigDB positional gene sets [15,16]. Centroid-linkage hierarchical clustering was performed in Cluster 3.0 and visualized in TreeView [17]. Array CGH data was normalized with CAPweb software [18] and genomic alterations were visualized with VAMP software using the same thresholds as previously described [10].

Fluorescence In Situ Hybridization

To detect deletions on chromosomes 13 and 14, FISH was performed with four BAC probes supplied by BlueGnome (Cambridge, UK). Two clones labeled with SpectrumGreen were used to detect the pericentromeric regions of chromosomes 13 and 14: RP11-408E5 on 13q12.11 (hg19 chr13:19700993–19850551); and RP11-98N22 on 14q11.2 (hg19 chr14:20500968–20660726). Two clones labeled with SpectrumOrange (giving red spots in the figures) were used to detect the deletions on chromosomes 13 and 14: RP11-71C5 on 13q14.11 (hg19 chr13:44921196–45086777) and RP11-242P2 on 14q31.1 (hg19 chr14:80030106–80193689). Nuclei obtained by touch imprints were fixed in 3:1 methanol:acetic acid, washed and dried. The BAC probes were mixed, 5 μl of hybridization mix was added per slide, and a coverslip was glued in place to create a hybridization chamber. The sections were denatured at 75°C for 5 minutes and hybridized at 37°C overnight. Stringent washes were performed at 65°C for 10 minutes, then the sections were dehydrated in ethanol and mounted. Images were acquired with a Zeiss Axio Imager Z2 microscope (Gottingen, Germany). The number of red and green spots per nucleus was scored in morphologically intact and non-overlapping nuclei. Deletions were reported when ≥50% of nuclei with the modal number of green spots contained fewer red spots or when they contained single green and red spots.

Results

Identification of Genes Differentially Expressed in BRCA2-mutant Tumors

To gain insight into the biology of BRCA2-mutant breast tumors, we performed a supervised analysis looking for genes differentially expressed in BRCA2-mutant and control tumors. All of the tumors came from patients with a familial clustering of breast cancer potentially caused by germline mutation of a breast cancer predisposition gene. The BRCA2-mutant group included 7 tumors from patients with known germline BRCA2 mutations. The control group (“BRCA1”) contained 24 patients without mutations in BRCA1 or BRCA2 identifiable by conventional screening. RNA
Figure 1. Unsupervised hierarchical clustering of the 66 BRCA2 signature genes in the training set. There are seven BRCA2-mutant tumors and 24 BRCAX tumors (tumors from patients lacking known BRCA1/2 mutations but with a familial history of breast cancer). The upper left quadrant contains many genes on 13q and 14q that show reduced expression in BRCA2 tumors.

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| Affymetrix ID | Gene Symbol | Gene Description | Band | t    | p     |
|---------------|-------------|------------------|------|------|-------|
| 222127_s_at   | EXOC1       | exocyst complex component 1 | 4q12 | −7.05 | 0.0011 |
| 223564_s_at   | GNB1L       | G protein beta polypeptide 1-like | 22q11 | 6.85  | 0.0011 |
| 632_at        | GSK3A       | glycogen synthase kinase 3 alpha | 19q13 | 6.42  | 0.0025 |
| 1555377_at    | OR4D2       | olfactory receptor, family 4, subfamily D, member 2 | 17q22 | 6.13  | 0.0030 |
| 208429_x_at   | HNF4A       | hepatocyte nuclear factor 4, alpha | 20q13 | 6.12  | 0.0030 |
| 207973_x_at   | ACRV1       | acrosomal vesicle protein 1 | 11q23 | 6.21  | 0.0030 |
| 218431_at     | C14orf133   | VPS33B interacting protein | 14q24 | −6.01 | 0.0034 |
| 1552510_at    | SLC34A3     | solute carrier family 34 (sodium phosphate), member 3 | 9q34  | 5.9   | 0.0041 |
| 204690_at     | STX8        | syntaxin 8 | 17p12 | −5.84 | 0.0044 |
| 227630_at     | PPP2R5E     | protein phosphatase 2, regulatory subunit B', epsilon | 14q23 | −5.7  | 0.0047 |
| 205621_at     | ALKBH1      | alkB, alkylation repair homolog 1 (E. coli) | 14q24 | −5.69 | 0.0047 |
| 202569_s_at   | MARK3       | MAP/microtubule affinity-regulating kinase 3 | 12p13 | −5.74 | 0.0047 |
| 203958_s_at   | WBP4        | WW domain binding protein 4 (formin binding protein 21) | 13q14 | −5.51 | 0.0048 |
| 230055_at     | KHDC1       | KH homology domain containing 1 | 6q13  | 5.6   | 0.0048 |
| 221966_at     | GPR137      | G protein-coupled receptor 137 | 11cen | 5.62  | 0.0048 |
| 207733_x_at   | PSOG9       | pregnancy specific beta-1-glycoprotein 9 | 19q13 | 5.59  | 0.0048 |
| 1555614_at    | SUGT1P1     | suppressor of G2 allele of SKP1 (S. cerevisiae) pseudogene | 9p13  | 5.57  | 0.0048 |
| 1552772_at    | CLEC4D      | C-type lectin domain family 4, member D | 12p13 | 5.57  | 0.0048 |
| 203958_s_at   | TPT1        | tumor protein, translationally-controlled 1 | 13q14 | −5.51 | 0.0048 |
| 1563639_a_at  | FHAD1       | forkhead-associated (FHA) phosphopeptide binding domain 1 | 1p36  | 5.54  | 0.0048 |
| 234680_at     | KRTAP17-1   | keratin associated protein 17-1 | 17q12 | 5.52  | 0.0048 |
| 156257_s_at   | C10orf90    | chromosome 10 open reading frame 90 | 10q26 | 5.45  | 0.0055 |
| 236979_at     | BCL2L15     | BCL2-like 15 | 1p13  | 5.39  | 0.0061 |
| 221095_s_at   | KCNE2       | potassium voltage-gated channel, Isk-related family, member 2 | 21q22 | 5.4   | 0.0061 |
| 213239_at     | PIBF1       | progesterone immunomodulatory binding factor 1 | 13q22 | −5.36 | 0.0063 |
| 1567257_at    | OR1L2       | olfactory receptor, family 1, subfamily J, member 2 | 9q34  | 5.34  | 0.0064 |
| 225389_at     | BTBD6       | BTB (POZ) domain containing 6 | 14q32 | −5.31 | 0.0066 |
| 207778_at     | REG1P       | regenerating islet-derived 1 pseudogene | 2p12  | 5.3   | 0.0066 |
| 226005_at     | UBE2G1      | ubiquitin-conjugating enzyme E2G 1 (UBC7 homolog, yeast) | 17p13 | −5.25 | 0.0070 |
| 215424_s_at   | SWN1        | SWN domain containing 1 | 14q24 | −5.23 | 0.0070 |
| 1564112_at    | FATM1A      | Family with sequence similarity 71, member A | 1q32  | 5.25  | 0.0070 |
| 237980_at     | LINCO0347   | hypothetical LOC338864 | 13q21 | 5.24  | 0.0070 |
| 213103_at     | STARD13     | STAR-related lipid transfer (START) domain containing 13 | 13q12 | −5.18 | 0.0071 |
| 237257_at     | RAB4B       | RAB4B, member RAS oncogene family | 19q13 | 5.19  | 0.0071 |
| 201767_s_at   | ELAC2       | elaC homolog 2 (E. coli) | 17p11 | −5.2  | 0.0071 |
| 209944_at     | ZNF410      | zinc finger protein 410 | 14q24 | −5.16 | 0.0071 |
| 1558641_at    | SPATA24     | spermatogenesis associated 24 | 5q31  | 5.2   | 0.0071 |
| 212735_at     | KIAA0226    | Beclin-1 associated RUN domain containing protein | 3p29  | 5.17  | 0.0071 |
| 215449_at     | TSP2       | translocator protein 2 | 6p21  | 5.15  | 0.0071 |
| 1553253_at    | ASB16       | ankyrin repeat and SOCS box-containing 16 | 17q21 | 5.14  | 0.0071 |
| 231625_at     | SLC22A9     | solute carrier family 22 member 9 | 11q13 | 5.2   | 0.0071 |
| 225312_at     | COMM6D      | COMM domain containing 6 | 13q22 | −5.12 | 0.0074 |
| 217187_at     | MUC5AC      | mucin 5AC, oligomeric mucus | 11p15 | 5.1   | 0.0077 |
| 1553728_at    | LRRC43      | leucine rich repeat containing 43 | 12q24 | 5.07  | 0.0079 |
| 1552863_a_at  | CACNG6      | calcium channel, voltage-dependent, gamma subunit 6 | 19q13 | 5.07  | 0.0079 |
| 217095_x_at   | NCR1        | natural cytotoxicity triggering receptor 1 | 19q13 | 5.06  | 0.0079 |
| 223610_at     | SEMA5B      | semaphorin 5b | 3q21  | 5.06  | 0.0079 |
| 203065_s_at   | CAV1        | caveolin 1, caveolae protein, 22 kDa | 7q31  | −5.03 | 0.0080 |
| 202226_s_at   | CRK         | v-crk sarcoma virus CT10 oncogene homolog (avian) | 17p13 | −5.04 | 0.0080 |
from these 31 tumors was tested on Affymetrix gene expression chips. Sixty-six genes were differentially expressed in the BRCA2 and BRCAX groups at a false discovery rate, 0.01 after Benjamini Hochberg correction for multiple testing (Table 2). Hierarchical clustering confirmed, as expected, that the differentially expressed genes cleanly split the tumors into two groups (Figure 1). The BRCA2 group in the heatmap contains five BRCAX tumors that may represent tumors whose BRCA2 mutations were missed by screening or tumors that phenocopy BRCA2.

**Validation of a Putative BRCA2 Signature**

We combined the differentially expressed genes in Table 2 to make a potential BRCA2 gene expression signature. Receiver operating characteristic (ROC) analysis showed that the area under the curve (AUC) for classification of the training set was 1.0 with the BRCA2 signature genes, indicating perfect classification of the tumors. This is not surprising given the small size of the dataset. To test for overfitting, we analyzed an independent validation set of 19 BRCA2-mutant tumors from the Curie Institute genetics clinic and 12 BRCAX from the Bergonie Cancer Institute. Given the rarity of the disease it is unfortunately difficult to avoid batch effects that might confound the result. Nevertheless, the AUC of the ROC curve was 0.76 in the validation set (Figure 2), indicating that the BRCA2 signature was able to classify BRCA2-mutant tumors reasonably well. Hierarchical clustering confirmed that the BRCA2 signature genes were differentially expressed in the validation set (Figure 3). While this suggests that the BRCA2 signature has discriminant value in our tumors and in the validation set from the Curie Institute we note that this is not generally the case because the signature does not identify BRCA2-mutant tumors in some published datasets. For example, the AUC in the Waddell dataset [19] was 0.64, perhaps because of differences in the technology or in the populations studied. We conclude that the BRCA2 signature may have discriminant value in tumors processed according to our protocol.

**Table 2. Cont.**

| Affymetrix ID | Gene Symbol | Gene Description | Band | t   | p     |
|---------------|-------------|------------------|------|-----|-------|
| 235416_at     | LOC643201   | centrosomal protein 192 kDa pseudogene | 5q35 | 5.03| 0.0080|
| 1557827_at    | C10orf103   | chromosome 10 open reading frame 103 | 10q22| 5.03| 0.0080|
| 225187_at     | KIAA1967    | DBC1 deleted in breast cancer 1 | 8p22 | −4.98| 0.0082|
| 212936_at     | FAM172A     | family with sequence similarity 172, member A | 5q15 | −4.99| 0.0082|
| 215898_at     | TTLL5       | tubulin tyrosine ligase-like family, member 5 | 14q24| −4.98| 0.0082|
| 212778_at     | PAC52       | phosphofurin acidic cluster sorting protein 2 | 14q32| −5  | 0.0082|
| 1562914_a_at  | FLJ25328    | hypothetical LOC148231 | 19p13| 5  | 0.0082|
| 215826_x_at   | ZNF835      | zinc finger protein 835 | 19q13| 4.97| 0.0084|
| 238158_at     | MEIG1       | meiosis expressed gene 1 homolog (mouse) | 10p13| 4.97| 0.0084|
| 219499_at     | SEC61A2     | Sec61 alpha 2 subunit (S. cerevisiae) | 10p14| 4.94| 0.0087|
| 207650_x_at   | PTGER1      | prostaglandin E receptor 1 (subtype EP1), 42 kDa | 19p13| 4.94| 0.0087|
| 237388_x_at   | SUN5        | Sad1 and UNC84 domain containing 5 | 20q11| 4.92| 0.0091|
| 1557679_at    | C8orf68     | chromosome 8 open reading frame 68 | 8p23 | 4.91| 0.0092|
| 224256_at     | LOC100129449| PRO2055 | 2q23 | 4.89| 0.0095|
| 1564362_x_at  | ZNF843      | zinc finger protein 843 | 16p11| 4.88| 0.0097|
| 205970_at     | MT3         | metallothionein 3 | 16q13| 4.87| 0.0098|
| 1569095_at    | LOC731424   | hypothetical LOC731424 | 4q35 | 4.87| 0.0098|

Footnote. t: moderated t-statistic for 66 genes that best discriminate between BRCA2 and BRCAX tumors. p: p-value after Benjamini Hochberg correction (all genes had an unadjusted p-value < 0.0001).

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**Gene Set Enrichment Analysis (GSEA) Reveals the Mechanism Behind the BRCA2 Signature**

The striking feature of the heatmap in Figure 1 is the cluster of 22 genes showing reduced expression in BRCA2-mutant tumors.

**Figure 2.** ROC analysis of the BRCA2 signature in the validation set. Each tumor was given a score that was a weighted sum of the mean centered gene expression levels for each gene in the signature. The validation set contained 19 BRCA2 and 12 BRCAX tumors. The AUC was 0.76.

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Figure 3. Unsupervised hierarchical clustering of the 66 BRCA2 signature genes in the validation set. There are 19 BRCA2-mutant tumors and 12 BRCAX tumors. The lower left quadrant contains many genes on 13q and 14q that show reduced expression in BRCA2 tumors. doi:10.1371/journal.pone.0052079.g003
These genes show a correlation of 0.90 in the heatmap. To exclude fortuitous hybridization as an explanation for this strong clustering we verified that the probe sequences were different and that they were labeled by Affymetrix as valid, non-cross-hybridizing probes for the indicated genes. Fourteen of the 22 BRCA2 signature genes showing reduced expression are from chromosomes 13 and 14. To determine whether this was due to chance, we ranked the dataset by moderated t statistic (BRCA2 vs control), then performed GSEA with gene sets derived from individual chromosomal bands. The bands most frequently lost are shown in Table 3. The enrichment for bands on 13q and 14q was highly significant (p<0.001 for the family-wise error rate, the most stringent criterion in the Broad Institute implementation of GSEA). The most likely explanation for underexpressed genes to be derived from specific chromosomal bands is deletion of those bands in the corresponding tumors.

CGH and SNP Analysis of BRCA2-mutant Tumors

To test directly for loss of the regions containing the BRCA2 signature genes we measured DNA copy number on CGH and SNP chips. The resulting CGH and SNP profiles confirmed that the incriminated regions are indeed deleted in the BRCA2-mutant tumors (Figure 4). The common region of overlap of the deletions extends from 13q13.3 to 13q14.3 and from 14q24.2 to 14q32.2. The cumulative rates of gain and loss for the BRCA2 and BRCAX tumors are shown in Figure 5. This shows that the long arms of both chromosomes 13 and 14 contain large regions that are preferentially deleted in the BRCA2-mutant tumors. We conclude that the BRCA2 signature genes are differentially expressed because they are deleted in the BRCA2 tumors.

Table 3. GSEA for loss of chromosomal bands.

| Band | Genes | ES | NES |
|------|-------|----|-----|
| 13q14 | 67    | -0.63 | -2.75 |
| 13q31 | 22    | -0.81 | -2.71 |
| 13q13 | 22    | -0.74 | -2.45 |
| 14q24 | 77    | -0.54 | -2.43 |
| 17p13 | 185   | -0.44 | -2.3  |
| 14q32 | 105   | -0.48 | -2.28 |
| 10q26 | 72    | -0.51 | -2.27 |
| 4p16  | 91    | -0.49 | -2.25 |

Footnote. The genes column shows the number of genes used to score the band. The nominal, FDR and FWER p-values were all <0.001. ES, enrichment score; NES normalized enrichment score.

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Figure 4. Genomic profiles in the training set. Upper panels: BAC-CGH profiles of BRCA2-mutant tumors showing gains in red, losses in green and modal copy number in yellow. Lower panels: BAF profiles of BRCA2-mutant tumors on Illumina SNP arrays. The boundaries of the common regions of deletion on chromosomes 13 and 14 are marked by vertical red lines.

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Identification of Deletions by FISH

If the signature works by detecting large deletions on chromosomes 13 and 14, it would be better to screen tumors in a clinical setting by FISH rather than by gene expression or CGH/SNP profiling. FISH is ideally suited to detecting small changes in copy number. To test whether it would be feasible to screen for BRCA2-mutant tumors in this way, we performed FISH with probes mapping to the regions commonly deleted on chromosomes 13 and 14 (Figure 6). We tested nine BRCA2 tumors and nine control BRCAX tumors, of which five BRCA2 and eight BRCAX were not previously characterized by CGH. The results are expressed as the percentage of nuclei with less than the modal number of spots for the centromeric probes or with a ploidy of one for both probes (Table 4). The tumors were scored as “loss” when the percentage was ≥50%, and “other” when it was <50%. Contingency tables for the chromosomes individually or for both chromosomes together are shown in Table 5. For both chromosomes scored together, the sensitivity and specificity for detection of BRCA2-mutant tumors were 78% and 89%, respectively. We conclude that FISH provides a simple technique to screen tumors for deletions on 13q and 14q that may be associated with BRCA2 mutations.

Discussion

The main conclusion from our study is that deletions on chromosomes 13q and 14q are a common feature of BRCA2-mutant tumors. We initially set out to identify a gene expression signature that would distinguish these tumors from other tumors in patients presenting to our genetics clinics. Hierarchical clustering of the genes in the signature split the tumors into two groups in both the training and the validation sets, suggesting that the signature detects a signal that is useful for classification of the tumors. Given the GSEA and SNP/CGH results we strongly suspect that the reduced expression of the genes in the signature is caused by a reduction in the DNA copy number of the deleted regions. It is more difficult to detect deletion than amplification in gene expression data, because the former may further decrease a barely detectable signal whereas the latter can increase expression 100-fold. This probably explains why the genes in the signature are a minority of the genes in the deleted regions. It is more difficult to detect deletion than amplification in gene expression data, because the former may further decrease a barely detectable signal whereas the latter can increase expression 100-fold. This probably explains why the genes in the signature are a minority of the genes in the deleted regions. In contrast, deletion of these regions...
was noted in several previous DNA copy number and SNP studies [7,21–25]. In addition to published studies, we examined the GISTIC database (Tumorscape Release 1.6) [26] to determine whether loss of chromosomes 13 and 14 is a common event in breast cancer. Several regions are reported as harboring deletions on chromosome 13 (hg18 chr13:44680312–57088104, 57088104–114059427, 18097312–46301361 and 50901262–114059427), as expected given the presence of \textit{BRCA2} and \textit{RB1} on 13 q. In contrast, GISTIC reports no regions as being deleted on chromosome 14 in breast cancer at above the background rate (q >0.25).

There are several possible explanations for selective deletion of specific genomic regions in \textit{BRCA2} tumors. The commonly deleted region on chromosome 13 is distal to the \textit{BRCA2} gene, but we can not altogether exclude that \textit{BRCA2} itself may be a driver gene in some cases, for example if there were complex genomic rearrangements on 13 q. \textit{BRCA2} was not part of the gene signature, probably because the Affymetrix probes for \textit{BRCA2} are not sensitive enough (the measured level was close to background and showed minimal variation). The best reporters for copy

| Table 4. FISH with probes in the region of common deletion on chromosomes 13 and 14. |
| ID | BRCA status | chr 13 | chr 14 |
|-----|-------------|--------|--------|
| 52  | BRCA2       | 84     | 89     |
| 86  | BRCA2       | 90     | 86     |
| 106 | BRCA2       | 100    | 87     |
| 133 | BRCA2       | 93     | 89     |
| A   | BRCA2       | 84     | 83     |
| B   | BRCA2       | 100    | 0      |
| C   | BRCA2       | 87     | 7      |
| D   | BRCA2       | 100    | 62     |
| E   | BRCA2       | 100    | 73     |
| 16  | BRCA2       | 0      | 0      |
| F   | BRCA2       | 0      | 2      |
| G   | BRCA2       | 0      | 0      |
| H   | BRCA2       | 100    | 100    |
| I   | BRCA2       | 4      | 0      |
| J   | BRCA2       | 0      | 0      |
| K   | BRCA2       | 2      | 0      |
| L   | BRCA2       | 7      | 3      |
| M   | BRCA2       | 10     | 0      |

Footnote. The table shows the percentage of nuclei with less than the modal ploidy or with ploidy = 1 for both the centromeric and the deletion probes. Tumours A-M were not characterized by CGH. doi:10.1371/journal.pone.0052079.t004

| Table 5. Contingency table summarizing the FISH data for deletions on chromosomes 13 and 14. |
|-----|--------|--------|--------|
| Chr 13 and 14 | Other | Loss |
| BRCA2 | 2 | 7 |
| BRCA2 | 8 | 1 |
| p = 0.015 | |
| Chr 13 | Other | Loss |
| BRCA2 | 0 | 9 |
| BRCA2 | 8 | 1 |
| p = 0.0004 | |
| Chr 14 | Other | Loss |
| BRCA2 | 2 | 7 |
| BRCA2 | 8 | 1 |
| p = 0.015 | |

Footnote. "Loss" refers to cases where \( \geq 50\% \) of nuclei had less than the modal ploidy or had ploidy = 1. "Other" refers to cases where the value was \(< 50\% \). The p value is for a Fisher exact test. The values for \("\text{Chr}13 \text{ and } 14\)" refer to cases where both chromosomes were affected. doi:10.1371/journal.pone.0052079.t005

Figure 6. FISH with probes in the region of common deletion in a \textit{BRCA2}-mutant tumor. A, chromosome 13; B, chromosome 14. Red: probe in the deleted region; Green, pericentromeric probe. Each nucleus contains two green spots and one red spot, indicating that the tumor is diploid for chromosomes 13 and 14 but has heterozygous deletions in the regions tested by the red probes. doi:10.1371/journal.pone.0052079.g006
number are housekeeping genes that lack feedback or exogenous regulation. By their nature these genes shed no light on the mechanism driving deletion. An alternative explanation is that loss of BRCA2 function generates repair intermediates or triggers checkpoint responses that are toxic in the presence of specific genes located in the deleted regions. Loss of these genes would allow the cell to resume division and form a tumor. This model predicts that the driver genes in the deleted regions should be DNA repair or checkpoint genes. \textit{ALK1/R1} could have this effect, but few other genes in the \textit{BRCA2} signature are obvious candidates for these roles. Another possibility is that the deleted regions contain fragile sites that are more difficult to repair in the absence of BRCA2. Fragile sites are prone to replication fork collapse, a process that often leads to the formation of double strand breaks that require repair by homologous recombination. BRCA2 is required for loading of RAD51 to initiate homologous recombination \cite{[27,28]} so increased breakage at fragile sites in the affected regions is certainly a possibility.

Screening for \textit{BRCA2} mutations is widely performed in genetics laboratories to explain familial clustering of breast cancer. Our study design focused on patients referred to genetics clinics because this is the context in which the need to distinguish \textit{BRCA2}-mutant from other tumors most commonly arises. Because of the size of the \textit{BRCA2} gene it can take many months to identify mutations. This is rarely a problem in the context of genetic counseling because some interventions can be undertaken without knowledge of the mutation (for example, more frequent screening with imaging techniques) and others may even benefit from the delay by giving patients more time for reflection (for example, prophylactic mastectomy and oophorectomy). The same can not be said of medical treatment of established tumors, which must be delivered without delay. The advent of medical treatments specific for \textit{BRCA2}-mutant tumors has created a need to identify these tumors on a more rapid time scale than has hitherto been considered necessary. In particular, \textit{BRCA2} defects are synthetic lethal with inhibition of poly-ADP-ribose polymerase 1 (PARP1) \cite{[27,28]}. We note that the \textit{BRCA2} group in the training set contains five \textit{BRCA2}A tumors which presumably either phenocopy \textit{BRCA2} mutation or contain \textit{BRCA2} mutations that evaded detection by sequencing. It would be interesting to know whether tumors that phenocopy \textit{BRCA2} mutation are also sensitive to PARP inhibitors.

In the long term it is likely that diagnostic laboratories will routinely use next generation sequencing (NGS) to identify mutations in \textit{BRCA2} and other relevant genes in the diagnostic biopsy when the patient initially presents with cancer. This is technically feasible but rarely performed outside major centers at present because of the cost and the complexity of the downstream bioinformatic analysis. To bridge the gap while waiting for NGS to become more widely available we propose to use FISH to screen breast tumors for deletions on 13q and 14q in order to identify tumors potentially associated with \textit{BRCA2}. The technology for FISH is very well established for diagnosis of \textit{ERBB2} amplification in sporadic breast tumors. It would require only a small modification of existing protocols to screen for loss of 13q and 14q in centers that already screen for \textit{ERBB2} amplification by FISH. Patients whose tumors harbor deletions in those regions could then be screened by sequencing to identify either germline or somatic \textit{BRCA2} mutations, followed by treatment with PARP inhibitors, if appropriate.

Conclusion

We have shown that breast tumors arising in patients with germline \textit{BRCA2} mutations have a higher frequency of deletions on 13q and 14q than is seen in other breast tumors. We propose that FISH for deletions on these chromosomes would be a rapid and technically feasible first step to enrich for tumors worth screening for \textit{BRCA2} mutations. This would greatly facilitate the selection of patients for PARP inhibitor therapy.

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Author Contributions

Conceived and designed the experiments: ML, RI, NS. Performed the experiments: AR, GB. Analyzed the data: GM, RI, AR, EL, AdR, NE. Performed mutation screening: NJ, FB, NS. Performed experiments: AR, GB. Analyzed the data: GM, RI, AR, EL, AdR, NE. Contributed reagents/materials/analysis tools: EBS, ML, LV, IC. Wrote the paper: RI, ML, AR. Performed mutation screening: NJ, FB, NS.

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