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Identification of a BRCA2-Specific Modifier Locus at 6p24 Related to Breast Cancer Risk


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Common genetic variants contribute to the observed variation in breast cancer risk for BRCA2 mutation carriers; those known to date have all been found through population-based genome-wide association studies (GWAS). To corroborate genomically identified breast cancer riskSNPs in BRCA2 mutation carriers, we conducted a deep sequencing analysis of an ongoing GWAS discovery study. Using the ranked P-values of the breast cancer associations with the imputed genotype of 1.4 M SNPs, 19,029 SNPs were selected and designed for inclusion on a custom Illumina array that included a total of 211,155 SNPs as part of a multi-consortial project. DNA samples from 3,881 breast cancer affected and 4,330 genotyped and available for analysis. We replicated previously reported breast cancer susceptibility alleles in these mutation carriers from 47 studies belonging to the Consortium of Investigators of Modifiers of BRCA1/2 (CIBERNE), HEF2, Heidelberg, Germany, 79 Inserm (National Institute of Health and Medical Research), CESP (Center for Research in Epidemiology and Population Health), U1018, Environmental Epidemiology of Cancer, Villejuif, France, 80 University of Paris-Sud, UMR-S 1018, Villejuif, France, 81 Copenhagen General Population Study and Department of Clinical Biochemistry, Herlev Hospital, Copenhagen University Hospital, University of Copenhagen, Copenhagen, Denmark, 82 Division of Clinical Epidemiology and Aging Research, German Cancer Research Center, Heidelberg, Germany, 83 Dr. Margarete Fischer-Bosch-Institute of Clinical Pharmacology, Stuttgart, Germany, 84 University of Tubingen, Tubingen, Germany, 85 Molecular Genetics of Breast Cancer, Deutsches Krebsforschungszentrum (DKFZ), Heidelberg, Germany, 86 Institute for Prevention and Occupational Medicine of the German Social Accident Insurance (IfP), Böchum, Germany, 87 Institute and Outpatient Clinic of Occupational Medicine, Saarland University Medical Center and Saarland University Faculty of Medicine, Homburg, Germany, 88 Institute of Pathology, Medical Faculty of the University of Bonn, Bonn, Germany, 89 Department of Internal Medicine, Evangelische Kliniken Bonn gGmbH, Johanniter Krankenhaus, Bonn, Germany, 90 Department of Clinical Genetics, Helsinki University Central Hospital, Helsinki, Finland, 91 Department of Obstetrics and Gynecology, Hannover Medical School, Hannover, Germany, 92 Department of Oncology and Pathology, Karolinska Institute, Stockholm, Sweden, 93 School of Medicine, Institute of Clinical Medicine, Pathology, and Forensic Medicine, Biocenter Kuopio, Cancer Center of Eastern Finland, University of Eastern Finland, Kuopio, Finland, 94 Imaging Center, Department of Clinical Pathology, Kuopio University Hospital, Kuopio, Finland, 95 Vesalius Research Center, VIB, Leuven, Belgium, 96 Laboratory for Translational Genetics, Department of Oncology, University of Leuven, Belgium, 97 Division of Cancer Epidemiology, German Cancer Research Center (DKFZ), Heidelberg, Germany, 98 Cancer Epidemiology Centre, The Cancer Council Victoria, Melbourne, Australia, 99 Centre for Molecular, Environmental, Genetic, and Analytic Epidemiology, The University of Melbourne, Melbourne, Australia, 100 Department of Preventive Medicine, Keck School of Medicine, University of Southern California, Los Angeles, California, United States of America, 101 Laboratory of Cancer Genetics and Tumor Biology, Department of Clinical Genetics and Biocenter Oulu, University of Oulu, Oulu University Hospital, Oulu, Finland, 102 Department of Human Genetics and Department of Pathology, Leiden University Medical Center, Leiden, The Netherlands, 103 Division of Genetics and Epidemiology of Breast Cancer Research, The Institute of Cancer Research, Sutton, United Kingdom, 104 Division of Breast Cancer Research, Breast Cancer Research Centre, Institute of Cancer Research, London, United Kingdom, 105 Medical Epidemiology and Biostatistics, Karolinska Institutet, Stockholm, Sweden, 106 Department of Medical Oncology, Erasmus University Medical Center, Rotterdam, The Netherlands, 107 CRUK/CHR Sheffield Cancer Research Centre, Department of Oncology, University of Sheffield, Sheffield, United Kingdom, 108 Department of Genetics and Pathology, Pomeranian Medical University, Szczecin, Poland, 109 Human Genotyping–CEugen Human Cancer Genetics Program, Spanish National Cancer Research Centre (CNIO), Madrid, Spain, 110 Department of Molecular Biology and Medicine, Massachusetts General Hospital, Boston, Massachusetts, United States of America, 111 Program in Medical and Population Genetics, Broad Institute of Harvard and MIT, Cambridge, Massachusetts, United States of America, 112 Departments of Genetics and Medicine, Harvard Medical School, Boston, Massachusetts, United States of America

Abstract

Common genetic variants contribute to the observed variation in breast cancer risk for BRCA2 mutation carriers; those known to date have all been found through population-based genome-wide association studies (GWAS). To corroborate genomically identified breast cancer riskSNPs in BRCA2 mutation carriers, we conducted a deep sequencing analysis of an ongoing GWAS discovery study. Using the ranked P-values of the breast cancer associations with the imputed genotype of 1.4 M SNPs, 19,029 SNPs were selected and designed for inclusion on a custom Illumina array that included a total of 211,155 SNPs as part of a multi-consortial project. DNA samples from 3,881 breast cancer affected and 4,330 genotyped and available for analysis. We replicated previously reported breast cancer susceptibility alleles in these mutation carriers from 47 studies belonging to the Consortium of Investigators of Modifiers of BRCA1/2 (CIBERNE), HEF2, Heidelberg, Germany, 79 Inserm (National Institute of Health and Medical Research), CESP (Center for Research in Epidemiology and Population Health), U1018, Environmental Epidemiology of Cancer, Villejuif, France, 80 University of Paris-Sud, UMR-S 1018, Villejuif, France, 81 Copenhagen General Population Study and Department of Clinical Biochemistry, Herlev Hospital, Copenhagen University Hospital, University of Copenhagen, Copenhagen, Denmark, 82 Division of Clinical Epidemiology and Aging Research, German Cancer Research Center, Heidelberg, Germany, 83 Dr. Margarete Fischer-Bosch-Institute of Clinical Pharmacology, Stuttgart, Germany, 84 University of Tubingen, Tubingen, Germany, 85 Molecular Genetics of Breast Cancer, Deutsches Krebsforschungszentrum (DKFZ), Heidelberg, Germany, 86 Institute for Prevention and Occupational Medicine of the German Social Accident Insurance (IfP), Böchum, Germany, 87 Institute and Outpatient Clinic of Occupational Medicine, Saarland University Medical Center and Saarland University Faculty of Medicine, Homburg, Germany, 88 Institute of Pathology, Medical Faculty of the University of Bonn, Bonn, Germany, 89 Department of Internal Medicine, Evangelische Kliniken Bonn gGmbH, Johanniter Krankenhaus, Bonn, Germany, 90 Department of Clinical Genetics, Helsinki University Central Hospital, Helsinki, Finland, 91 Department of Obstetrics and Gynecology, Hannover Medical School, Hannover, Germany, 92 Department of Oncology and Pathology, Karolinska Institute, Stockholm, Sweden, 93 School of Medicine, Institute of Clinical Medicine, Pathology, and Forensic Medicine, Biocenter Kuopio, Cancer Center of Eastern Finland, University of Eastern Finland, Kuopio, Finland, 94 Imaging Center, Department of Clinical Pathology, Kuopio University Hospital, Kuopio, Finland, 95 Vesalius Research Center, VIB, Leuven, Belgium, 96 Laboratory for Translational Genetics, Department of Oncology, University of Leuven, Belgium, 97 Division of Cancer Epidemiology, German Cancer Research Center (DKFZ), Heidelberg, Germany, 98 Cancer Epidemiology Centre, The Cancer Council Victoria, Melbourne, Australia, 99 Centre for Molecular, Environmental, Genetic, and Analytic Epidemiology, The University of Melbourne, Melbourne, Australia, 100 Department of Preventive Medicine, Keck School of Medicine, University of Southern California, Los Angeles, California, United States of America, 101 Laboratory of Cancer Genetics and Tumor Biology, Department of Clinical Genetics and Biocenter Oulu, University of Oulu, Oulu University Hospital, Oulu, Finland, 102 Department of Human Genetics and Department of Pathology, Leiden University Medical Center, Leiden, The Netherlands, 103 Division of Genetics and Epidemiology of Breast Cancer Research, The Institute of Cancer Research, Sutton, United Kingdom, 104 Division of Breast Cancer Research, Breast Cancer Research Centre, Institute of Cancer Research, London, United Kingdom, 105 Medical Epidemiology and Biostatistics, Karolinska Institutet, Stockholm, Sweden, 106 Department of Medical Oncology, Erasmus University Medical Center, Rotterdam, The Netherlands, 107 CRUK/CHR Sheffield Cancer Research Centre, Department of Oncology, University of Sheffield, Sheffield, United Kingdom, 108 Department of Genetics and Pathology, Pomeranian Medical University, Szczecin, Poland, 109 Human Genotyping–CEugen Human Cancer Genetics Program, Spanish National Cancer Research Centre (CNIO), Madrid, Spain, 110 Department of Molecular Biology and Medicine, Massachusetts General Hospital, Boston, Massachusetts, United States of America, 111 Program in Medical and Population Genetics, Broad Institute of Harvard and MIT, Cambridge, Massachusetts, United States of America, 112 Departments of Genetics and Medicine, Harvard Medical School, Boston, Massachusetts, United States of America

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Introduction

The lifetime risk of breast cancer associated with carrying a **BRCA2** mutation varies from 40 to 84% [1]. To determine whether common genetic variants modify breast cancer risk for **BRCA2** mutation carriers, we previously conducted a GWAS of **BRCA2** mutation carriers from the Consortium of Investigators of Modifiers of **BRCA1/2** (CIMBA) [2]. Using the Affymetrix 6.0 platform, the discovery stage results were based on 899 young (<40 years) affected and 804 unaffected carriers of European ancestry. In a rapid replication stage wherein 65 discovery stage SNPs with the smallest p-values were genotyped in 2,436 additional **BRCA2** mutation carriers, only published loci associated with breast cancer risk in the general population, including **FGFR2** (10q26; rs2981575; P = 1.2×10^-10), were associated with breast cancer risk at the genome-wide significance level among **BRCA2** mutation carriers.
 GWAS discovery stage samples. Details of these samples have been described previously [2]. Data from 899 young (<40 years) affected and 804 older (>40 years) unaffected carriers of European ancestry from 14 countries were used to select SNPs for inclusion on the iCOGS array.

Samples genotyped in the extended replication set. Forty-seven studies from 24 different countries (including two East-Asian countries) provided DNA from a total of 10,048 BRCA2 mutation carriers. All eligible samples were genotyped using COGs, including those from the discovery stage.

Genotyping and quality control

BRCA2 SNP selection for inclusion on iCOGS. The Collaborative Oncological Gene-Environment Study (COGS) consortium developed a custom genotyping array (referred to as the iCOGS array) to provide efficient genotyping of common and rare genetic variants to identify novel loci that are associated with risk of breast, ovarian, and prostate cancers as well as to fine-map known cancer susceptibility loci. SNPs were selected for inclusion on iCOGS separately by each participating consortium: Breast Cancer Association Consortium (BCAC) [6], Ovarian Cancer Association Consortium (OCAC) [7], Prostate Cancer Association Group to Investigate Cancer Associated Alterations in the Genome (PRACTICAL) [8], and CIMBA. SNP lists from a BRCA1 GWAS and SNPs in candidate regions were used together with the BRCA2 GWAS lists to generate a ranked CIMBA SNP list that included SNPs with the following nominal proportions: 55.5% from the BRCA1 GWAS, 41.6% from the BRCA2 GWAS and fine mapping, 2.9% for CIMBA candidate SNPs. Each consortium was given a share of the array: nominally 25% of the SNPs each for BCAC, PRACTICAL and OCAC; 17.5% for CIMBA; and 7.5% for SNPs from commonly researched pathways (e.g., inflammation). For the CIMBA BRCA2 GWAS, we used the iCOGS array as the platform to genotype the extended replication set of the discovery GWAS stage [2]. SNPs were selected on the basis of the strength of their associations with breast cancer risk in the discovery stage [2], using imputed genotype data for 1.4 M SNPs identified through CEU+TSI samples on HapMap3, release 2. A ranked list of SNPs was based on the 1-df trend test statistic, after excluding highly correlated SNPs ($r^2 > 0.4$). The final list included the 39,015 SNPs with the smallest p-values. An additional set of SNPs were selected for fine mapping of the regions surrounding the SNPs found to be associated with breast cancer in the discovery GWAS stage: rs16917302 on 10q21 and a locus on 20q13 (rs311499), which represented the largest possible, international collection of BRCA2 mutation carriers.

**Materials and Methods**

Ethics statement

Each of the host institutions (Table S1) recruited under ethically-approved protocols. Written informed consent was obtained from all subjects.

Study subjects

The majority of BRCA2 mutation carriers were recruited through cancer genetics clinics and some came from population or community-based studies. Studies contributing DNA samples to these research efforts were members of the Consortium of Investigators of Modifiers of BRCA1/2 (CIMBA) with the exception of one study (NICCC). Eligible subjects were women of European descent who carried a pathogenic BRCA2 mutation, had complete phenotype information, and were at least 18 years of age. Harmonized phenotypic data included year of birth, age at cancer diagnosis, age at bilateral prophylactic mastectomy and oophorectomy, age at interview or last follow-up, BRCA2 mutation description, self-reported ethnicity, and breast cancer estrogen receptor status.

mutation carriers. Two other loci, in ZNF365 (rs16917302) on 10q21 and a locus on 20q13 (rs311499), were also associated with breast cancer risk in BRCA2 mutation carriers with P-values $< 10^{-4}$ ($P = 3.8 \times 10^{-5}$ and $6.6 \times 10^{-5}$, respectively). A nearby SNP in ZNF365 was also associated with breast cancer risk in a study of unselected cases [3] and in a study of mammographic density [4]. Additional follow-up replicated the findings for rs16917302, but not rs311499 [5] in a larger set of BRCA2 mutation carriers. To seek additional breast cancer risk modifying loci for BRCA2 mutation carriers, we conducted an extended replication of the GWAS discovery results in a larger set of BRCA2 mutation carriers in CIMBA, which represents the largest, international collection of BRCA2 mutation carriers.
Hardy-Weinberg equilibrium (P)

Quality control of SNPs. Of the 211,155 SNPs on the iCOGS array, we excluded SNPs for the following reasons (Table S2); on the Y-chromosome, call rate <95%, deviations from Hardy-Weinberg equilibrium (P<10^{-7}) using a stratified 1-d.f. test [10], and monomorphic. SNPs that gave discrepant genotypes among known duplicates were also excluded. After quality control filtering, 200,908 SNPs were available for analysis (Table S2); 18,086 of which were selected on the basis of the discovery GWAS [2]. Cluster plots of all reported SNPs were inspected manually for quality (Figure S1).

Description of imputation. Genotypes for SNPs identified through the 1000 Genomes Phase I data (released Jan 2012) [11] were imputed using SNPs on the iCOGS chip in a region of 500 kb around the novel modifier locus at 6p24. The boundaries were determined according to the linkage disequilibrium (LD) structure in the region based on HapMap data. The imputation was carried out using IMPUTE 2.2 [12]. SNPs with imputation information/accuracy r^2<0.30 were excluded in the analyses.

Quality control of DNA samples. Of 10,048 genotyped samples (Table S2), 742 were excluded because they did not meet the phenotypic eligibility criteria or had self-reported non-CEU ethnicity. Samples were then excluded for the following reasons: not female (XXY, XY), call rate <95%, low or high heterozygosity (P<10^{-7}), discordant genotypes from previous CIBRA genotyping efforts, or discordant duplicate samples. For duplicates with concordant phenotypic data, or in cases of cryptic monozygotic twins, only one of the samples was included. Cryptic duplicates for which phenotypic data indicated different individuals were all excluded. Samples of non-European ancestry were identified using multi-dimensional scaling, after combining the BRCA2 mutation carrier samples with the HapMap2 CEU, CHB, JPT and YRI samples using a set of 37,120 uncorrelated SNPs from the iCOGS array. Samples with >19% non-European ancestry were excluded (Figure S2). A total of 4,330 affected and 3,881 unaffected BRCA2 mutation carrier women of European ancestry from 42 studies remained in the analysis (Table S1), including 3,234 breast cancer cases and 3,490 unaffected carriers that were not in the discovery set.

BRCA1 and BCAC samples. Details of the sample collection, genotyping and quality control process for the BRCA1 and BCAC samples, are reported elsewhere [13,14].

Statistical methods

The associations between genotype and breast cancer risk were analyzed within a retrospective cohort framework with time to breast cancer diagnosis as the outcome [15]. Each BRCA2 carrier was followed until the first event: breast or ovarian cancer diagnosis, bilateral prophylactic mastectomy, or age at last observation. Only those with a breast cancer diagnosis were considered as cases in the analysis. The majority of mutation carriers were recruited through genetic counseling centers where genetic testing is targeted at women diagnosed with breast or ovarian cancer and in particular to those diagnosed with breast cancer at a young age. Therefore, these women are more likely to be sampled compared to unaffected mutation carriers or carriers diagnosed with the disease at older ages. As a consequence, sampling was not random with respect to disease phenotype and standard methods of survival analysis (such as Cox regression) may lead to biased estimates of the associations [16]. We therefore conducted the analysis by modelling the retrospective likelihood of the observed genotypes conditional on the disease phenotypes. This has been shown to provide unbiased estimates of the associations [15]. The implementation of the retrospective likelihoods has been described in detail elsewhere [15,17]. The associations between genotype and breast cancer risk were assessed using the 1-degree of freedom score test statistic based on the retrospective likelihood [15]. In order to account for non-independence between relatives, an adjusted version of the score test was used in which the variance of the score was derived taking into account the correlation between the genotypes [18]. P-values were not adjusted using genomic control because there was little evidence of inflation. Inflation was assessed using the genomic inflation factor, λ. Since this estimate is dependent on sample size, we also calculated λ adjusted to 1000 affected and 1000 unaffected samples. Per-allele and genotype-specific hazard-ratios (HR) and 95% confidence intervals (CI) were estimated by maximizing the retrospective likelihood. Calendar-year and cohort-specific breast cancer incidences for BRCA2 were used [1]. All analyses were stratified by country of residence. The USA and Canada strata were further subdivided by self-reported Ashkenazi Jewish ancestry. The assumption of proportional hazards was assessed by fitting a model that included a genotype-by-age interaction term. Between-country heterogeneity was assessed by comparing the results of the main analysis to a model with country-specific log-HRs. A possible survival bias due to inclusion of prevalent cases was evaluated by re-fitting the model after excluding affected carriers that were diagnosed ≥5 years prior to study recruitment. The associations between genotypes and tumor subtypes were evaluated using an extension of the retrospective likelihood approach that models the association with two or more subtypes simultaneously [19]. To investigate whether any of the significant SNPs were associated with ovarian cancer risk for BRCA2 mutation carriers and whether the inclusion of ovarian cancer patients as unaffected subjects biased our results, we also analyzed the data within a competing risks framework and estimated HR simultaneously for breast and ovarian cancer using the methods described elsewhere [15]. Analyses were carried out in R using the GenABEL libraries [20] and custom-written software. The retrospective likelihood was modeled in the pedigree-analysis software MENDEL [21], as described in detail elsewhere [15].

TCGA analysis. Affymetrix SNP 6.0 genotype calls for normal (non-tumor) breast DNA were downloaded for all available individuals from The Cancer Genome Atlas in September 2011. Analyses were limited to the 401 individuals of European ancestry based on principal component analysis. Expression levels in breast tumor tissue were adjusted for the top two principal components, age, gender (there are some male breast cancer cases in TCGA), and average copy number across the gene in the tumor. Linear regression was then used to test for association between the SNP and the adjusted gene expression level for all genes within one megabase.

Gene set enrichment analysis. To investigate enrichment of genes associated with breast cancer risk, the gene-set enrichment approach was implemented using Versatile Gene-based Association Study [22] based on the ranked P-values from retrospective likelihood analysis. Association List Go Annotator was also used to prioritize gene pathways using functional annotation from gene ontology (GO) [23] to increase the power to detect association to a pathway, as opposed to individual genes in the pathway. Both analyses were corrected for LD between SNPs, variable gene size, and interdependence of GO categories,
where applicable, based on imputation. 100,000 Monte Carlo simulations were performed in VEGAS and 5000 replicate gene lists using random sampling of SNPs and 5000 replicate studies (sampling with replacement) were performed to estimate P-values.

**Predicted absolute breast cancer risks by combined SNP profile.** We estimated the absolute risks of developing breast cancer based on the joint distribution of SNPs associated with breast cancer for **BRCA2** mutation carriers. The methods have been described elsewhere [24]. To construct the SNP profiles, we considered the single SNP from each region with the strongest evidence of association in the present dataset. We included all loci that had previously been found to be associated with breast cancer risk through GWAS in the general population and demonstrated associations with breast cancer risk for **BRCA2** mutation carriers, and loci that had GWAS level of significance in the current study. We assumed that all loci in the profile were independent (i.e. they interact multiplicatively on **BRCA2** breast cancer risk). Genotype frequencies were obtained under the assumption of Hardy-Weinberg Equilibrium. For each SNP, the effect of each allele was assumed to be consistent with a multiplicative model (log-additive). We assumed that the average, age-specific breast cancer incidences, over all associated loci, agreed with published breast cancer risk estimates for **BRCA2** mutation carriers [1].

**Results**

The genomic inflation factor (λ) based on the 18,086 **BRCA2** GWAS SNPs in the 6,724 **BRCA2** mutation carriers who were not used in the SNP discovery set was 1.034 (λ adjusted to 1000 affected and 1000 unaffected: 1.010, Figure S3). Multiple variants were associated with breast cancer risk in the combined discovery and replication datasets (Figure S4). SNPs in three independent regions had P-values<5×10⁻⁸; one was a region not previously associated with breast cancer.

The most significant associations were observed for known breast cancer susceptibility regions, rs2420946 (per allele P = 2×10⁻¹⁴) in **FGFR2** and rs3380562 (P = 5.4×10⁻¹¹) near **TOX3** (Table 1). Breast cancer risk associations with other SNPs reported previously for **BRCA2** mutation carriers are summarized in Table 1. In this larger set of **BRCA2** mutation carriers, we also identified novel SNPs in the 12p11 (**PTHLH**), 5q11 (**MAP3K1**), and 9p21 (**CDKN2A/B**) regions with smaller P-values for association than those of previously reported SNPs. These novel SNPs were not correlated with the previously reported SNPs (r²<0.14). For one of the novel SNPs identified in the discovery GWAS [2], ZNF563 rs16917302, there was weak evidence of association with breast cancer risk (P = 0.01); however, an uncorrelated SNP, rs17221319 (r²<0.01), 54 kb upstream of rs16917302 had stronger evidence of association (P = 6×10⁻⁶).

One SNP, rs9348512 at 6p24 not known to be associated with breast cancer, had a combined P-value of association of 3.9×10⁻⁶ amongst all **BRCA2** samples (Table 2), with strong evidence of replication in the set of **BRCA2** samples that were not used in the discovery stage (P = 5.2×10⁻⁶). The minor allele of rs9348512 (MAF = 0.35) was associated with a 15% increased risk of breast cancer among **BRCA2** mutation carriers (per allele HR = 0.85, 95% CI 0.80–0.90) with no evidence of between-country heterogeneity (P = 0.78, Figure S5). None of the genotyped (n = 68) or imputed (n = 3,507) SNPs in this region showed a stronger association with risk (Figure 1; Table S3), but there were 40 SNPs with P<10⁻⁴ (pairwise r²>0.38 with rs9348512, with the exception of rs11526201 for which r² = 0.01, Table S3). The association with rs9348512 did not differ by 6174delT mutation status (P for difference = 0.33), age (P = 0.39), or estrogen receptor (ER) status of the breast tumor (P = 0.41). Exclusion of prevalent breast cancer cases (n = 1,752) produced results (HR = 0.83, 95% CI 0.77–0.89, P = 3.40×10⁻⁵) consistent with those for all cases.

SNPs in two additional regions had P-values<10⁻⁷ for breast cancer risk associations for **BRCA2** mutation carriers (Table 2). The magnitude of associations for both SNPs was similar in the discovery and second stage samples. In the combined analysis of all samples, the minor allele of rs619373, located in **FGF13** (Xq26.3), was associated with higher breast cancer risk (HR = 1.30, 95% CI 1.17–1.43, P = 3.1×10⁻⁶). The minor allele of rs184577, located in **C11P18.1-AS1** (2p22–p21), was associated with lower breast cancer risk (HR = 0.93, 95% CI 0.79–0.91, P = 3.6×10⁻⁴). These findings were consistent across countries (P for heterogeneity between country strata = 0.39 and P = 0.30, respectively; Figure S6). There was no evidence that the HR estimates for rs619373 and rs184577 change with age of the **BRCA2** mutation carriers (P for the genotype-age interaction = 0.80 and P = 0.40, respectively) and no evidence of survival bias for either SNP (rs619373: HR = 1.35, 95% CI 1.20–1.53, P = 1.5×10⁻⁶ and rs184577: HR = 0.86, 95% CI 0.79–0.93, P = 2.0×10⁻⁴, after excluding prevalent cases). The estimates for risk of ER-negative and ER-positive breast cancer were not significantly different (P for heterogeneity between tumor subtypes = 0.79 and 0.67, respectively). When associations were evaluated under a competing risks model, there was no evidence of association with ovarian cancer risk for SNPs rs9348512 at 6p24, rs619373 in **FGF13** or rs184577 at 2p22 and the breast cancer associations were virtually unchanged (Table S4).

Gene set enrichment analysis confirmed that strong associations exist for known breast cancer susceptibility loci and the novel loci identified here (gene-based P<1×10⁻⁷). The pathways most strongly associated with breast cancer risk that contained statistically significant SNPs included those related to ATP binding, organ morphogenesis, and several nucleotide bindings (pathway-based P<0.05).

To begin to determine the functional effect of rs9348512, we examined associations of expression levels of any nearby gene in breast tumors with the minor A allele. Using data from The Cancer Genome Atlas, we found that the A allele of rs9348512 was strongly associated with mRNA levels of **GCNT2** in breast tumors (p = 7.3×10⁻⁵).

The hazard ratios for the percentiles of the combined genotype distribution of loci associated with breast cancer risk in **BRCA2** mutation carriers were translated into absolute breast cancer risks under the assumption that SNPs interact multiplicatively. Based on our results for SNPs in **FGFR2**, **TOX3**, 12p11, 5q11, **CDKN2A/B**, **LSP1**, 8q24, **ESR1**, **2qFGF35**, 3p24, 12q24, 5p12, 11q13 and also the 6p24 locus, the 5% of the **BRCA2** mutation carriers at lowest risk were predicted to have breast cancer risks by age 80 in the range of 21–47% compared to 83–100% for the 5% of mutation carriers at highest risk on the basis of the combined SNP profile distribution (Figure 2). The breast cancer risk by age 50 was predicted to be 4–11% for the 5% of the carriers at lowest risk compared to 29–81% for the 5% at highest risk.

**Discussion**

In the largest assemblage of **BRCA2** mutation carriers, we identified a novel locus at 6q24 that is associated with breast cancer risk, and noted two potential SNPs of interest at Xq26 and 2p22. We also replicated associations with known breast cancer susceptibility SNPs previously reported in the general population and in **BRCA2** mutation carriers. For the 12p11 (**PTHLH**), 5q11 (**MAP3K1**), and 9p21 (**CDKN2A/B**), we found uncorrelated SNPs.
Table 1. Per allele hazard ratios (HR) and 95% confidence intervals (CI) of previously published breast cancer loci among BRCA2 mutation carriers from previous reports and from the iCOGS array, ordered by statistical significance of the region.

<table>
<thead>
<tr>
<th>Chr (Nearby Genes)</th>
<th>Report Status</th>
<th>SNP</th>
<th>r²</th>
<th>MinorAllele</th>
<th>Previously Reported Results</th>
<th>iCOGS Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Per Allele HR (95%CI)</td>
<td>p-value²</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Affected N</td>
<td>Unaffected N</td>
</tr>
<tr>
<td>10q26 (FGFR2)</td>
<td>reported</td>
<td>n2981575</td>
<td>G</td>
<td>[2]</td>
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<td>2,016</td>
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<tr>
<td></td>
<td>novel</td>
<td>n2420946</td>
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<td>0.96</td>
<td>2,162</td>
<td>2,026</td>
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<tr>
<td>16q12 (TOX3)</td>
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<td>[2]</td>
<td>3,798</td>
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<td></td>
<td>novel</td>
<td>n27633</td>
<td>C</td>
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<tr>
<td>12p11 (PTHLH)</td>
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<td>novel</td>
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<td>2,636</td>
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<tr>
<td>5q11 (MAP3K1)</td>
<td>reported</td>
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<td>[24]</td>
<td>3,807</td>
<td>3,316</td>
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<td>n1688611</td>
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<td>0.14</td>
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<tr>
<td>1p11 (ZNF365)</td>
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<td>n16917302</td>
<td>C</td>
<td>[5]</td>
<td>3,807</td>
<td>3,315</td>
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<td></td>
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<td>n17221319</td>
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<td>0.00</td>
<td>3,807</td>
<td>3,315</td>
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<tr>
<td>2q24 (STXBP4, COX1)</td>
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<td>novel</td>
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<td>1q13</td>
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<td>n1214943</td>
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<td>[34]</td>
<td>3,423</td>
<td>2,827</td>
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<td>n11514720</td>
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<td>3,423</td>
<td>2,827</td>
</tr>
<tr>
<td>17q23 (STXBP4, COX1)</td>
<td>reported</td>
<td>n6504950</td>
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<td>[24]</td>
<td>3,401</td>
<td>2,813</td>
</tr>
<tr>
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<td>n8170</td>
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<td>0.55</td>
<td>3,365</td>
<td>3,086</td>
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<tr>
<td>9q31</td>
<td>reported</td>
<td>n1338704a</td>
<td>G</td>
<td>[24]</td>
<td>3,300</td>
<td>2,646</td>
</tr>
<tr>
<td></td>
<td>novel</td>
<td>n865686</td>
<td>C</td>
<td>0.34</td>
<td>3,799</td>
<td>3,312</td>
</tr>
</tbody>
</table>

¹ Reporting status of the SNP is either previously reported or novel to this report.
² p-value was calculated based on the 1-degree of freedom score test statistic.
³ rs311499 could not be designed onto the iCOGS array. A surrogate (rs² = 1.0), rs311499, was included, however, and reported here.
⁴ Stronger associations were originally reported for the SNP, assuming a dominant or recessive model of the ‘risk allele’. doi:10.1371/journal.pgen.1003173.t001
Table 2. Breast cancer hazard ratios (HR) and 95% confidence intervals (CI) of novel breast cancer loci with P-values of association <10\(^{-5}\) among BRCA2 mutation carriers.

<table>
<thead>
<tr>
<th>SNP rs No. Chr (Nearby Genes)</th>
<th>Discovery Stage</th>
<th>Stage 2</th>
<th>Combined</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Genotype</td>
<td>Affected No. (%)</td>
<td>Unaffected No. (%)</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>-----------------</td>
<td>-----------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>rs9348512 Chr6 (TFAP2A, C6orf218)</td>
<td>CC</td>
<td>390 (46.4)</td>
<td>248 (38.3)</td>
</tr>
<tr>
<td></td>
<td>CA</td>
<td>368 (43.8)</td>
<td>299 (46.2)</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>82 (9.8)</td>
<td>100 (15.5)</td>
</tr>
<tr>
<td></td>
<td>per allele</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs619373 ChrX (FGF13)</td>
<td>GG</td>
<td>693 (75.8)</td>
<td>568 (87.8)</td>
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<tr>
<td></td>
<td>GA</td>
<td>143 (15.7)</td>
<td>78 (12.1)</td>
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<tr>
<td></td>
<td>AA</td>
<td>4 (8.5)</td>
<td>1 (0.1)</td>
</tr>
<tr>
<td></td>
<td>per allele</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs1184577 Chr2 (C2orf58)</td>
<td>GG</td>
<td>520 (61.9)</td>
<td>368 (56.9)</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>278 (33.1)</td>
<td>234 (36.2)</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>42 (5.0)</td>
<td>45 (7.0)</td>
</tr>
<tr>
<td></td>
<td>per allele</td>
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</tbody>
</table>

\(^{1}\)P-value was calculated based on the 1-degree of freedom score test.
that had stronger associations than the originally identified SNP in the breast cancer susceptibility region that should be replicated in the general population. In BRCA2 mutation carriers, evidence for a breast cancer association with genetic variants in PTHLH has been restricted previously to ER-negative tumors [25]; however, the novel susceptibility variant we reported here was associated with risk of ER+ and ER- breast cancer.

The novel SNP rs9348512 (6p24) is located in a region with no known genes (Figure 1). C6orf218, a gene encoding a hypothetical protein LOC221718, and a possible tumor suppressor gene, TFAP2A, are within 100 kb of rs9348512. TFAP2A encodes the AP-2α transcription factor that is normally expressed in breast ductal epithelium nuclei, with progressive expression loss from normal, to ductal carcinoma in situ, to invasive cancer [26,27]. AP-2α also acts as a tumor suppressor via negative regulation of MYC [28] and augmented p53-dependent transcription [29]. However, the minor allele of rs9348512 was not associated with gene expression changes of TFAP2A in breast cancer tissues in The Cancer Genome Atlas (TCGA) data; this analysis might not be informative since expression of TFAP2A in invasive breast tissue is low [26,27]. Using the TCGA data and a 1 Mb window, expression changes with genotypes of rs9348512 were observed for GCNT2, the gene encoding the enzyme for the blood group I antigen glucosaminyl (N-acetyl) transferase 2. GCNT2, recently found to be overexpressed in highly metastatic breast cancer cell lines [30] and basal-like breast cancer [31], interacts with TGF-β to promote epithelial-to-mesenchymal transition, enhancing the metastatic potential of breast cancer [31]. An assessment of alterations in expression patterns in normal breast tissue from BRCA2 mutation carriers by genotype are needed to further evaluate the functional implications of rs9348512 in the breast tumorigenesis of BRCA2 mutation carriers.

To determine whether the breast cancer association with rs9348512 was limited to BRCA2 mutation carriers, we compared results to those in the general population genotyped by BCAC and to BRCA1 mutation carriers in CIMBA. No evidence of an associations between rs9348512 and breast cancer risk was observed in the general population (OR = 1.00, 95% CI 0.98–1.02, P = 0.74) [14], nor in BRCA1 mutation carriers (HR = 0.99, 95% CI 0.94–1.04, P = 0.75) [13]. Stratifying cases by ER status, there was no association observed with ER-subtypes in either the general population or among BRCA1 mutation carriers (BCAC: ER positive P = 0.89 and ER negative P = 0.60; CIMBA BRCA1: P = 0.49 and P = 0.99, respectively). For the two SNPs associated with breast cancer with P<10−5, neither rs619373, located in FGF13 (Xq26.3), nor rs184577, located in CYP1B1-AS1 (2p22-p21), was associated with breast cancer risk in the general population [14] or among BRCA1 mutation carriers [13]. The narrow CIs for the overall associations in the general population and in BRCA1 mutation carriers rule out associations of magnitude similar to those observed for BRCA2 mutation carriers. The consistency of the association in the discovery and replication stages and by country, the strong quality control measures and filters, and the clear cluster plot for rs9348512 suggest that our results constitute the discovery of a novel breast cancer susceptibility locus specific to BRCA2 mutation carriers rather than a false positive finding. Replicating this SNP in an even larger population of BRCA2 mutation carriers would be ideal, but not currently...
possible because we know of no investigators with appropriate data and germline DNA from BRCA2 mutation carriers who did not contribute their mutation carriers to iCOGS. However, CIMBA studies continue to recruit individuals into the consortium.

rs9348512 (6p24) is the first example of a common susceptibility variant identified through GWAS that modifies breast cancer risk specifically in BRCA2 mutation carriers. Previously reported BRCA2-modifying alleles for breast cancer, including those in FGFR2, TOX3, MAP3K1, LSP1, 8q24, ESR1, ZNF365, 3p24, 12q24, 5p12, 11q13 and the newly identified BRCA2 modifier locus at 6p24. The figure shows the risks at the 5th and 95th percentiles of the combined genotyped distribution as well as minimum, maximum and average risks.

doi:10.1371/journal.pgen.1003173.g002

Supporting Information

Figure S1 Cluster plots for SNPs (A.) rs9348512, (B.) rs619373, and (C.) rs184577.
(TIF)

Figure S2 Multidimensional scaling plots of the top two principal components of genomic ancestry of all eligible BRCA2 iCOGS samples plotted with the HapMap CEU, ASI, and YRI samples: (A.) samples from Finland and BRCA2 617delT carriers highlighted, and (B.) samples, indicated in red, with >19% non-European ancestry were excluded.
(TIF)

Figure S3 Quantile–quantile plot comparing expected and observed distributions of P-values. Results displayed (A) for the complete sample, (B) after excluding samples from the GWAS discovery stage, and (C) for the complete sample and a set of SNPs from the iCOGS array that were selected independent from the results of the BRCA2 mutation carriers.
(TIF)

Figure S4 Manhattan plot of P-values by chromosomal position for 18,086 SNPs selected on the basis of a previously published genome-wide association study of BRCA2 mutation carriers. Breast cancer associations results based on 4,330 breast cancer cases and 3,881 unaffected BRCA2 carriers.
(TIF)

Figure S5 Forest plot of the country-specific, per-allele hazard ratios (HR) and 95% confidence intervals for the association between breast cancer and rs9348512 genotypes.
(TIF)

Figure S6 Forest plot of the country-specific, per-allele hazard ratios (HR) and 95% confidence intervals for the association with breast cancer for (A.) rs619373 and (B.) rs184577 genotypes.
(TIF)

Table S1 Quality control filtering steps for BRCA2 mutation carriers and SNPs on the COGs array.
(DOC)

Table S2 Description of breast cancer affected and unaffected BRCA2 carriers included in the final analysis of the COGs array SNPs.
(DOC)

Table S3 Breast cancer hazards ratios (HR) and 95% confidence intervals (CI) for all SNPs with P<10^{-5} in a 500 Mb region around rs9348512 on 6p24 among BRCA2 mutation carriers.
(DOC)

Table S4 Associations with SNPs at 6p24, FGFI3 and 2p22 and breast and ovarian cancer risk using a competing risk analysis model.
(DOC)

Acknowledgments

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Consortium of Modifiers of BRCA1/2 Associations (CIMBA): The authors would like to acknowledge the contribution of the staff of the genotyping unit under the supervision of Dr. Sylvie LaBoissière as well as Frédérick Robidoux from the McGill University and Genome Quebec Innovation Centre.

Breast Cancer Association Consortium (BCAC): We thank all the individuals who took part in these studies and all the researchers,

Figure 2. Predicted breast cancer risks for BRCA2 mutation carriers by the combined SNP profile distributions. Based on the known breast cancer susceptibility loci at FGFR2, TOX3, 12p11, 5q11, CDKN2A/B, LSP1, 8q24, ESR1, ZNF365, 3p24, 12q24, 5p12, 11q13 and the newly identified BRCA2 modifier locus at 6p24. The figure shows the risks at the 5th and 95th percentiles of the combined genotyped distribution as well as minimum, maximum and average risks.

Breast Cancer Risk

Age

Figure S1 Cluster plots for SNPs (A.) rs9348512, (B.) rs619373, and (C.) rs184577.
(TIF)

Figure S2 Multidimensional scaling plots of the top two principal components of genomic ancestry of all eligible BRCA2 iCOGS samples plotted with the HapMap CEU, ASI, and YRI samples: (A.) samples from Finland and BRCA2 617delT carriers highlighted, and (B.) samples, indicated in red, with >19% non-European ancestry were excluded.
(TIF)

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(TIF)

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(TIF)

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(TIF)

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