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Citation

Gaudet, Mia M., Karoline B. Kuchenbaecker, Joseph Vijai, Robert J. Klein, Tomas Kirchhoff, Lesley McGuffog, Daniel Barrowdale, et al. 2013. Identification of a BRCA2-specific modifier locus at 6p24 related to breast cancer risk. *PLoS Genetics* 9(3): e1003173.

Published Version

doi:10.1371/journal.pgen.1003173

Permanent link

<http://nrs.harvard.edu/urn-3:HUL.InstRepos:10622988>

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

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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 comprehensively identify breast cancer risk modifying loci for *BRCA2* mutation carriers, we conducted a deep replication 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 unaffected *BRCA2* mutation carriers from 47 studies belonging to the Consortium of Investigators of Modifiers of *BRCA1/2* were genotyped and available for analysis. We replicated previously reported breast cancer susceptibility alleles in these *BRCA2* mutation carriers and for several regions (including *FGFR2*, *MAP3K1*, *CDKN2A/B*, and *PTHLH*) identified SNPs that have stronger evidence of association than those previously published. We also identified a novel susceptibility allele at 6p24 that was inversely associated with risk in *BRCA2* mutation carriers (rs9348512; per allele HR=0.85, 95% CI 0.80–0.90, $P = 3.9 \times 10^{-8}$). This SNP was not associated with breast cancer risk either in the general population or in *BRCA1* mutation carriers. The locus lies within a region containing *TFAP2A*, which encodes a transcriptional activation protein that interacts with several tumor suppressor genes. This report identifies the first breast cancer risk locus specific to a *BRCA2* mutation background. This comprehensive update of novel and previously reported breast cancer susceptibility loci contributes to the establishment of a panel of SNPs that modify breast cancer risk in *BRCA2* mutation carriers. This panel may have clinical utility for women with *BRCA2* mutations weighing options for medical prevention of breast cancer.

Citation: Gaudet MM, Kuchenbaecker KB, Vijai J, Klein RJ, Kirchhoff T, et al. (2013) Identification of a BRCA2-Specific Modifier Locus at 6p24 Related to Breast Cancer Risk. *PLoS Genet* 9(3): e1003173. doi:10.1371/journal.pgen.1003173

Editor: Kent W. Hunter, National Cancer Institute, United States of America

Received August 31, 2012; **Accepted** October 30, 2012; **Published** March 27, 2013

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Funding: This work was supported by the following institutions: iCOGS: The creation of the custom Illumina multiplex chip and the genotyping of the BRCA2 carriers in CIMBA was made possible by grants from the Starr Cancer Consortium I4-A402 (PI: K Offit), the Sandra Taub Memorial Fund of the Breast Cancer Research Foundation (PI: K Offit), the Norman and Carol Stone Cancer Genetics Fund (PI: K Offit), and the European Commission's Seventh Framework Programme grant agreement 223175 (HEALTH-F2-2009-223175). AC Antoniou is a Cancer Research UK Senior Cancer Research Fellow. G Chenevix-Trench is an NHMRC Senior Principal Research Fellow. Consortium of Modifiers of BRCA1/2 Associations: The CIMBA data management and data analysis were supported by Cancer Research UK grants C12292/A11174 and C1287/A10118. S Healey is supported by an NHMRC Program Grant to G Chenevix-Trench. AC Antoniou is a Cancer Research UK Senior Cancer Research Fellow. G Chenevix-Trench is an NHMRC Senior Principal Research Fellow. Amsterdam Breast Cancer Study: The ABCS study was supported by the Dutch Cancer Society [grants NKI 2007-3839; 2009 4363]; BBMRI-NL, which is a Research Infrastructure financed by the Dutch government (NWO 184.021.007); and the Dutch National Genomics Initiative. Bavarian Breast Cancer Cases and Controls: The work of the BBCC was partly funded by ELAN-Fond of the University Hospital of Erlangen. British Breast Cancer Study: The BBCC is funded by Cancer Research UK and Breakthrough Breast Cancer and acknowledges NHS funding to the NIHR Biomedical Research Centre, and the National Cancer Research Network (NCRN). Breast Cancer Family Registry Studies: The Australian Breast Cancer Family Study (ABCFS), New York City (New York Breast CFR), Northern California Breast Cancer Family Registry (NC-BCFR), Ontario Familial Breast Cancer Registry (OFBCR), and Utah (Utah Breast CFR) work was supported by the United States National Cancer Institute, National Institutes of Health (NIH), under RFA-CA-06-503 (P30 CA13696 and P30 ES009089), and through cooperative agreements with members of the BCFR and Principal Investigators, including Cancer Care Ontario (U01 CA69467), Columbia University (U01 CA69398), Cancer Prevention Institute of California (U01 CA69417), Fox Chase Cancer Center (U01 CA69631), Huntsman Cancer Institute (U01 CA69446), and University of Melbourne (U01 CA69638). The ABCFS was also supported by the National Health and Medical Research Council of Australia, the New South Wales Cancer Council, the Victorian Health Promotion Foundation (Australia), and the Victorian Breast Cancer Research Consortium. The New York BCFR site was also supported by NIH grants P30 CA13696 and P30 ES009089. MC Southey is a NHMRC Senior Research Fellow and a Victorian Breast Cancer Research Consortium Group Leader. Baltic Familial Breast Ovarian Cancer Consortium: BFBOCC is partly supported by: Lithuania (BFBOCC-LT), Research Council of Lithuania grant LIG-19/2010, and Hereditary Cancer Association (Paveldimo vėžio asociacija). Latvia (BFBOCC-LV) is partly supported by LSC grant 10.0010.08 and in part by a grant from the ESF Nr.2009/0220/1DP/1.1.1.2.0/09/APIA/VIAA/016. Breast Cancer in Galway Genetic Study: Guy's & St. Thomas' NHS Foundation Trust in partnership with King's College London, United Kingdom. BRCA-gene mutations and breast cancer in South African women: BMBSA was supported by grants from the Cancer Association of South Africa (CANSA) to EJ van Rensburg NIH R01CA74415 and P30 CA033752. Beckman Research Institute of the City of Hope: SL Neuhausen was partially supported by the Morris and Horowitz Families Endowed Professorship. BRICOH was supported by NIH R01CA74415 and NIH P30 CA033752. Breast Cancer Study of the University Clinic Heidelberg: The BSUCH study was supported by the Dietmar-Hopp Foundation, the Helmholtz Society and the German Cancer Research Center (DKFZ). Rigshospitalet: The CBCS study was supported by the NEYE Foundation. CECILE Breast Cancer Study: The CECILE study was funded by Fondation de France, Institut National du Cancer (INCa), Ligue Nationale contre le Cancer, Ligue contre le Cancer Grand Ouest, Agence Nationale de Sécurité Sanitaire (ANSES), Agence Nationale de la Recherche (ANR). Copenhagen General Population Study: The CGPS was supported by the Chief Physician Johan Boserup and Lise Boserup Fund, the Danish Medical Research Council and Herlev Hospital. Spanish National Cancer Centre: The CNIO work was partially supported by Spanish Association against Cancer (AECC08), RTICC 06/0020/1060, FISPI08/1120, Mutua Madrileña Foundation (FMMA) and SAF2010-20493. Spanish National Cancer Centre Breast Cancer Study: The CNIO-BCS was supported by the Genome Spain Foundation, the Red Temática de Investigación Cooperativa en Cáncer and grants from the Asociación Española Contra el Cáncer and the Fondo de Investigación Sanitaria (PI11/00923 and PI081120). City of Hope Cancer Center: The City of Hope Clinical Cancer Genetics Community Research Network is supported by Award Number RC4A153828 (PI: JN Weitzel) from the National Cancer Institute and the Office of the Director, National Institutes of Health. CONSORZIO STUDI ITALIANI SUI TUMORI EREDITARI ALLA MAMMELLA: CONSIT TEAM was funded by grants from Fondazione Italiana per la Ricerca sul Cancro (Special Project "Hereditary tumors"), Italian Association for Cancer Research (AIRC, IG 8713), Italian Ministry of Health (Extraordinary National Cancer Program 2006, "Alleanza contro il Cancro" and "Progetto Tumori Femminili), Italian Ministry of Education, University and Research (Prin 2008) Centro di Ascolto Donne Operate al Seno (CAOS) association and by funds from Italian citizens who allocated the 5 × 1000 share of their tax payment in support of the Fondazione IRCCS Istituto Nazionale Tumori, according to Italian laws (INT-Institutional strategic projects "5 × 1000"). German Cancer Research Center: The DKFZ study was supported by the DKFZ. Genen Omgeving studie van de werkgroep Hereditair Borstkanker Onderzoek Nederland: The DNA HEBON study is supported by the Dutch Cancer Society grants NKI1998-1854, NKI2004-3088, NKI2007-3756, the NWO grant 91109024, the Pink Ribbon grant 110005, and the BBMRI grant CP46/NWO. Epidemiological study of BRCA1 & BRCA2 mutation carriers: EMBRACE is supported by Cancer Research UK Grants C1287/A10118 and C1287/A11990. DG Evans is supported by an NIH grant to the Biomedical Research Centre, Manchester. ESTHER Breast Cancer Study: The ESTHER study was supported by a grant from the Baden Württemberg Ministry of Science, Research and Arts. Additional cases were recruited in the context of the VERDI study, which was supported by a grant from the German Cancer Aid (Deutsche Krebshilfe). German Consortium of Hereditary Breast and Ovarian Cancer: GC-HBOC is supported by the German Cancer Aid (grant no 109076), by the Center for Molecular Medicine Cologne (CMCC), and by Deutsche Krebshilfe (107 352). GC-HBOC is supported by Deutsche Krebshilfe. Genetic Modifiers of cancer risk in BRCA1/2 mutation carriers: The GEMO study was supported by the Ligue Nationale contre le Cancer; the Association "Le cancer du sein, parlons-en!" Award and the Canadian Institutes of Health Research for the "CIHR Team in Familial Risks of Breast Cancer" program. Gene Environment Interaction and Breast Cancer in Germany: The GENICA was funded by the Federal Ministry of Education and Research (BMBF) Germany grants 01KW9975/5, 01KW9976/8, 01KW9977/0 and 01KW0114, the Robert Bosch Foundation, Stuttgart, Deutsches Krebsforschungszentrum (DKFZ), Heidelberg, Institute for Prevention and Occupational Medicine of the German Social Accident Insurance (IPA), Bochum, as well as the Department of Internal Medicine, Evangelische Kliniken Bonn gGmbH, Johanner Krankenhaus, Bonn, Germany. Gynecologic Oncology Group: This study was supported by National Cancer Institute grants to the Gynecologic Oncology Group (GOG) Administrative Office and Tissue Bank (CA 27469), the GOG Statistical and Data Center (CA 37517), and GOG's Cancer Prevention and Control Committee (CA 101165). MH Greene and PL Mai are supported by funding from the Intramural Research Program, NCI. Hospital Clínico San Carlos: HCSC was supported by a grant RD06/0020/0021 from RTICC (ISCIII), Spanish Ministry of Economy and Competitiveness. Helsinki Breast Cancer Study: The HEBCS was financially supported by the Helsinki University Central Hospital Research Fund, Academy of Finland (132473), the Finnish Cancer Society, the Nordic Cancer Union, and the Sigrid Juselius Foundation. Hannover-Minsk Breast Cancer Study: The HMBCS was supported by a grant from the Friends of Hannover Medical School and by the Rudolf Bartling Foundation. Study of Genetic Mutations in Breast and Ovarian Cancer patients in Hong Kong and Asia: HRBCP is supported by The Hong Kong Hereditary Breast Cancer Family Registry and the Dr. Ellen Li Charitable Foundation, Hong Kong. Molecular Genetic Studies of Breast and Ovarian Cancer in Hungary: Hungarian Breast and Ovarian Cancer Study was supported by Hungarian Research Grant KTIA-OTKA CK-80745 and the Norwegian EEA Financial Mechanism HU0115/NA/2008-3/ÖP-9. Institut Català d'Oncologia: The ICO study was supported by the Asociación Española Contra el Cáncer, Spanish Health Research Foundation, Ramón Areces Foundation, Carlos III Health Institute, Catalan Health Institute, and Autonomous Government of Catalonia and contract grant numbers ISCIII/RETIC RD06/0020/1051, PI09/02483, PI10/01422, PI10/00748, 2009SGR290, and 2009SGR283. Icelandic Landspítali-University Hospital: The ILUH group was supported by the Icelandic Association "Walking for Breast Cancer Research" and by the Landspítali University Hospital Research Fund. Interdisciplinary Health Research Internal Team Breast Cancer susceptibility: INHERIT work was supported by the Canadian Institutes of Health Research for the "CIHR Team in Familial Risks of Breast Cancer" program, the Canadian Breast Cancer Research Alliance grant 019511 and the Ministry of Economic Development, Innovation and Export Trade grant PSR-SIIRI-701. J Simard is Chairholder of the Canada Research Chair in Oncogenetics. Istituto Oncologico Veneto: The IOVHBOCS study was supported by Ministero dell'Istruzione, dell'Università e della Ricerca and Ministero della Salute ("Progetto Tumori Femminili" and RFP5 2006-5-341353, ACC2/R6.9"). Karolinska Breast Cancer Study: The KARBAC study was supported by the Swedish Cancer Society, the Gustav V Jubilee Foundation, and the Bert von Kantzow Foundation. Kuopio Breast Cancer Project: The KBCP was financially supported by the special Government Funding (EVO) of Kuopio University

Hospital grants, Cancer Fund of North Savo, the Finnish Cancer Organizations, the Academy of Finland, and by the strategic funding of the University of Eastern Finland. Kathleen Cunningham Consortium for Research into Familial Breast Cancer: kConFab is supported by grants from the National Breast Cancer Foundation and the National Health and Medical Research Council (NHMRC) and by the Queensland Cancer Fund; the Cancer Councils of New South Wales, Victoria, Tasmania, and South Australia; and the Cancer Foundation of Western Australia. G Chenevix-Trench and AB Spurdle are NHMRC Senior Research Fellows. Financial support for the AOCFS was provided by the United States Army Medical Research and Materiel Command [DAMD17-01-1-0729], the Cancer Council of Tasmania and Cancer Foundation of Western Australia, and the NHMRC [199600]. G Chenevix-Trench is supported by the NHMRC. The Clinical Follow Up Study (funded 2001–2009 by NHMRC and currently by the National Breast Cancer Foundation and Cancer Australia #628333) Korean Hereditary Breast Cancer Study: KOHBRA is supported by a grant from the National R&D Program for Cancer Control, Ministry for Health, Welfare and Family Affairs, Republic of Korea (1020350). Leuven Multidisciplinary Breast Centre: LMBC is supported by the 'Stichting tegen Kanker' (232-2008 and 196-2010). D Lambrechts is supported by the FWO and the KULPFV/10/016-SymBioSysll. Mammary Carcinoma Risk Factor Investigation: The MARIE study was supported by the Deutsche Krebshilfe e.V. [70-2892-BR I], the Hamburg Cancer Society, the German Cancer Research Center, and the genotype work in part by the Federal Ministry of Education and Research (BMBF) Germany [01KH0402]. Mayo Clinic: MAYO is supported by NIH grant CA128978, an NCI Specialized Program of Research Excellence (SPORE) in Breast Cancer (CA116201), a U.S. Department of Defence Ovarian Cancer Idea award (W81XWH-10-1-0341), and grants from the Breast Cancer Research Foundation and the Komen Foundation for the Cure. Milan Breast Cancer Study Group: MBCSG was funded by grants from Fondazione Italiana per la Ricerca sul Cancro (Special Project "Hereditary tumors"), Italian Association for Cancer Research (AIRC, IG 8713), Italian Ministry of Health ("Progetto Tumori Femminili"), and by Italian citizens who allocated the 5×1000 share of their tax payment in support of the Fondazione IRCCS Istituto Nazionale Tumori, according to Italian laws (INT-Institutional strategic projects "5×1000"). Melbourne Collaborative Cohort Study: MCCS cohort recruitment was funded by VicHealth and Cancer Council Victoria. The MCCS was further supported by Australian NHMRC grants 209057, 251553 and 504711 and by infrastructure provided by Cancer Council Victoria. McGill University: The McGill Study was supported by Jewish General Hospital Weekend to End Breast Cancer, Quebec Ministry of Economic Development, Innovation and Export Trade. Multi-Ethnic Cohort: The MEC was supported by NIH grants CA63464, CA54281, CA098758, and CA132839. Memorial Sloan-Kettering Cancer Center: The MSKCC was supported by Breast Cancer Research Foundation, Niehaus Clinical Cancer Genetics Initiative, Andrew Sabin Family Foundation, and Lymphoma Foundation. Montreal Gene-Environment Breast Cancer Study: The work of MTLGEBCS was supported by the Quebec Breast Cancer Foundation, the Canadian Institutes of Health Research for the "CIHR Team in Familial Risks of Breast Cancer" program grant CRN-87521 and the Ministry of Economic Development, Innovation and Export Trade grant PSR-SIIRI-701. J Simard is Chairholder of the Canada Research Chair in Oncogenetics. National Cancer Institute: The research of MH Greene and PL Mai was supported by the Intramural Research Program of the US National Cancer Institute, NIH, and by support services contracts NO2-CP-11019-50 and NO2-CP-65504 with Westat, Rockville, MD. National Israeli Cancer Control Center: NICCC is supported by Clalit Health Services in Israel. Some of its activities are supported by the Israel Cancer Association and the Breast Cancer Research Foundation (BCRF), New York. N. N. Petrov Institute of Oncology: The NNPIO study has been supported by the Russian Federation for Basic Research (grants 11-04-00227, 12-04-00928, and 12-04-01490) and the Federal Agency for Science and Innovations, Russia (contract 02.740.11.0780). Oulu Breast Cancer Study: The OBSCS was supported by research grants from the Finnish Cancer Foundation, the Academy of Finland, the University of Oulu, and the Oulu University Hospital. Leiden University Medical Centre Breast Cancer Study: The ORIGO study was supported by the Dutch Cancer Society (RUL 1997-1505) and the Biobanking and Biomolecular Resources Research Infrastructure (BBMRI-NL CP16). The Ohio State University Comprehensive Cancer Center: OSUCCG is supported by the Ohio State University Comprehensive Cancer Center. SEABASS is supported by the Ministry of Science, Technology and Innovation, Ministry of Higher Education (UM.C/HIR/MOHE/06) and Cancer Research Initiatives Foundation. The U.S. National Cancer Institute Polish Breast Cancer Study: The PBCS was funded by Intramural Research Funds of the National Cancer Institute, Department of Health and Human Services, USA. Karolinska Mammography Project for Risk Prediction of Breast Cancer - prevalent cases: The pKARMA study was supported by Märit and Hans Rausing's Initiative Against Breast Cancer. Rotterdam Breast Cancer Study: The RBSCS was funded by the Dutch Cancer Society (DDHK 2004-3124, DDHK 2009-4318). Singapore and Sweden Breast Cancer Study: The SASBAC study was supported by funding from the Agency for Science, Technology and Research of Singapore (A*STAR), the U.S. National Institute of Health (NIH), and the Susan G. Komen Breast Cancer Foundation. Sheffield Breast Cancer Study: The SBSCS was supported by Yorkshire Cancer Research S295, S299, and S305PA. South East Asian Breast Cancer Association Study: SEABASS is supported by the Ministry of Science, Technology and Innovation, Ministry of Higher Education (UM.C/HIR/MOHE/06) and Cancer Research Initiatives Foundation. The Malaysian Breast Cancer Genetic Study is funded by research grants from the Malaysian Ministry of Science, Technology and Innovation; Ministry of Higher Education (UM.C/HIR/MOHE/06); and charitable funding from Cancer Research Initiatives Foundation. Study of Epidemiology and Risk Factors in Cancer Heredity: SEARCH is funded by program grants from Cancer Research UK [C490/A10124][C8197/A10123]. AM Dunning was funded by [C8197/A10865]. Sheba Medical Centre: The SMC study was partially funded through a grant by the Israel Cancer Association and the funding for the Israeli Inherited Breast Cancer Consortium. Swedish Breast Cancer Study: SWE-BRCA collaborators are supported by the Swedish Cancer Society. IHCC-Szczecin Breast Cancer Study: The SZBCS was supported by Grant PBZ_KBN_122/P05/2004. The University of Chicago Center for Clinical Cancer Genetics and Global Health: UCHICAGO is supported by grants from the U.S. National Cancer Institute (NIH/NCI) and by the Ralph and Marion Falk Medical Research Trust, the Entertainment Industry Fund National Women's Cancer Research Alliance, and the Breast Cancer Research Foundation. University of California Los Angeles: The UCLA study was supported by the Jonsson Comprehensive Cancer Center Foundation and the Breast Cancer Research Foundation. University of California San Francisco: The UCSF study was supported by the UCSF Cancer Risk Program and the Helen Diller Family Comprehensive Cancer Center. United Kingdom Breakthrough Generations Study: The UKBGS is funded by Breakthrough Breast Cancer and the Institute of Cancer Research (ICR). ICR acknowledges NHS funding to the NIHR Biomedical Research Centre. United Kingdom Familial Ovarian Cancer Registries: UKFOCR was supported by a project grant from CRUK to PDP Pharoah. University of Pennsylvania: The UPENN study was supported by the National Institutes of Health (NIH) (R01-CA102776 and R01-CA083855), Breast Cancer Research Foundation, Rooney Family Foundation, Susan G. Komen Foundation for the Cure, and the Facionald Family Foundation. Victorian Familial Cancer Trials Group: The VFCTG study was supported by the Victorian Cancer Agency, Cancer Australia, and National Breast Cancer Foundation. Women's Cancer Program: The WCP at the Samuel Oschin Comprehensive Cancer Institute is funded by the American Cancer Society Early Detection Professorship (SIOP-06-258-01-COUN). Genetic Modifiers of Cancer Risk in *BRCA1/2* Mutation Carriers (GEMO) study: The study was supported by the Ligue Nationale Contre le Cancer, the Association "Le cancer du sein, parlons-en!" Award, and the Canadian Institutes of Health Research for the "CIHR Team in Familial Risks of Breast Cancer" program. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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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 85 discovery stage SNPs with the smallest P-values were genotyped in 2,486 additional *BRCA2* mutation carriers, only published loci associated with breast cancer risk in the general population, including *FGFR2* (10q26; rs2981575; $P = 1.2 \times 10^{-8}$), were associated with breast cancer risk at the genome-wide significance level among *BRCA2*

Author Summary

Women who carry *BRCA2* mutations have an increased risk of breast cancer that varies widely. To identify common genetic variants that modify the breast cancer risk associated with *BRCA2* mutations, we have built upon our previous work in which we examined genetic variants across the genome in relation to breast cancer risk among *BRCA2* mutation carriers. Using a custom genotyping platform with 211,155 genetic variants known as single nucleotide polymorphisms (SNPs), we genotyped 3,881 women who had breast cancer and 4,330 women without breast cancer, which represents the largest possible, international collection of *BRCA2* mutation carriers. We identified that a SNP located at 6p24 in the genome was associated with lower risk of breast cancer. Importantly, this SNP was not associated with breast cancer in *BRCA1* mutation carriers or in a general population of women, indicating that the breast cancer association with this SNP might be specific to *BRCA2* mutation carriers. Combining this *BRCA2*-specific SNP with 13 other breast cancer risk SNPs also known to modify risk in *BRCA2* mutation carriers, we were able to derive a risk prediction model that could be useful in helping women with *BRCA2* mutations weigh their risk-reduction strategy options.

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 6.6×10^{-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.

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.

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 rs311499 on 20q13, including SNPs with a MAF > 0.05 located 500 kb in both directions of the SNP, based on HapMap 2 data. The final combined list of SNPs for the iCOGS array comprised 220,123 SNPs. Of these, 211,155 were successfully manufactured onto the array. The present analyses are based on the 19,029 SNPs selected on the basis of *BRCA2* GWAS and fine mapping that were included on the iCOGS array.

Genotyping. The genotyping was performed on DNA samples from 10,048 *BRCA2* mutation carriers at the McGill University and Génome Québec Innovation Centre (Montreal, Canada). As a quality control measure, each plate included DNA samples from six individuals who were members of two CEPH trios. Some plates also contained three duplicate pairs of quality control samples. Genotypes were called using GenCall [9]. Initial calling was based on a cluster file generated using 270 samples from Hapmap2. To generate the final calls, we first selected a subset of 3,018 individuals, including samples from each of the genotyping centers in the iCOGS project, each of the participating

consortia, and each major ethnicity. Only plates with a consistent high call rate in the initial calling were used. We also included 380 samples of European, African, and Asian ethnicity genotyped as part of the Hapmap and 1000 Genomes project, and 160 samples that were known positive controls for rare variants on the array. This subset was used to generate a cluster file that was then applied to call the genotypes for the remaining samples.

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 *BRCA2* 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 all 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 ($P < 10^{-6}$), discordant genotypes from previous CIMBA 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 $< 5 \times 10^{-8}$; 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 $P = 2 \times 10^{-14}$) in *FGFR2* and rs3803662 ($P = 5.4 \times 10^{-11}$) 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 ($r^2 < 0.14$). For one of the novel SNPs identified in the discovery GWAS [2], *ZNF365* rs16917302, there was weak evidence of association with breast cancer risk ($P = 0.01$); however, an uncorrelated SNP, rs17221319 ($r^2 < 0.01$), 54 kb upstream of rs16917302 had stronger evidence of association ($P = 6 \times 10^{-3}$).

One SNP, rs9348512 at 6p24 not known to be associated with breast cancer, had a combined P-value of association of 3.9×10^{-8} 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 ($P = 5.2 \times 10^{-5}$). The minor allele of rs9348512 (MAF = 0.35) was associated with a 15% decreased 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 that region showed a stronger association with risk (Figure 1; Table S3), but there were 40 SNPs with $P < 10^{-4}$ (pairwise $r^2 > 0.38$ with rs9348512, with the exception of rs11526201 for which $r^2 = 0.01$, 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, $P = 3.40 \times 10^{-7}$) consistent with those for all cases.

SNPs in two additional regions had P-values $< 10^{-5}$ 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 1.17–1.45, $P = 3.1 \times 10^{-6}$). The minor allele of rs184577, located in *CYP11B-AS1* (2p22–p21), was associated with lower breast cancer risk (HR = 0.85, 95% CI 0.79–0.91, $P = 3.6 \times 10^{-6}$). 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, $P = 1.5 \times 10^{-6}$ and rs184577: HR = 0.86, 95% CI 0.79–0.93, $P = 2.0 \times 10^{-4}$, 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 $P < 1 \times 10^{-5}$). 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 ($\rho = 7.3 \times 10^{-5}$).

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, *ESRI*, *ZNF365*, 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.

Chr (Nearby Genes)	Report Status ¹	SNP	r ²	MinorAllele	Previously Reported Results			iCOGS Results			P-value ²		
					Ref	Affected N	Unaffected N	Per Allele HR (95%CI)	Affected N	Unaffected N		Unaffected MAF	Per Allele HR (95%CI)
10q26 (FGFR2)	reported	rs2981575		G	[2]	2,155	2,016	1.28 (1.18, 1.39)	4,326	3,874	0.40	1.27 (1.19, 1.34)	2 × 10 ⁻¹⁴
16q12 (TOX3)	novel	rs2420946	0.96	A					4,328	3,877	0.39	1.24 (1.16, 1.32)	5 × 10 ⁻¹¹
12p11 (PTHLH)	reported	rs3803662		A	[2]	2,162	2,026	1.20 (1.10, 1.31)	4,330	3,880	0.27	0.89 (0.81, 0.98)	0.02
	reported	rs10771399		G	[34]	3,798	3,314	0.93 (0.84, 1.04)	4,330	3,880	0.11	1.14 (1.07, 1.21)	4 × 10 ⁻⁵
	novel	rs27633	0.05	C					4,252	3,841	0.39	1.04 (0.98, 1.11)	0.20
5q11 (MAP3K1)	reported	rs889312		C	[24]	2,840	2,282	1.10 (1.01, 1.19)	4,330	3,881	0.29	1.24 (1.11, 1.38)	1 × 10 ⁻⁴
	novel	rs16886113	0.14	C					4,330	3,881	0.06	1.03 (0.95, 1.11)	0.51
9p21 (CDKN2A/B)	reported	rs1011970		A	[34]	3,807	3,316	1.09 (1.00, 1.18)	4,330	3,881	0.17	0.84 (0.77, 0.93)	8 × 10 ⁻⁴
	novel	rs10965163	0.00	A					4,329	3,880	0.10	1.11 (1.04, 1.18)	9 × 10 ⁻⁴
11p15 (LSP1)	reported	rs3817198		G	[24]	3,266	2,636	1.14 (1.06, 1.23)	4,316	3,870	0.33	1.03 (0.97, 1.09)	0.31
8q24	reported	rs13281615		G	[24]	3,338	2,723	1.06 (0.98, 1.13)	4,248	3,810	0.43	1.10 (1.04, 1.17)	2 × 10 ⁻³
	novel	rs4733664	0.00	A					4,329	3,879	0.41	0.95 (0.84, 1.06)	0.31
20q13	reported	rs311498 ³		A ⁴	[5]	3,808	3,318	0.95 (0.84, 1.07)	4,330	3,880	0.07	0.90 (0.84, 0.97)	5 × 10 ⁻³
	novel	rs13039229	0.00	C					4,326	3,877	0.21	1.12 (1.00, 1.25)	0.03
6q25 (ESR1)	reported	rs9397435		G	[35]	3,809	3,316	1.14 (1.01, 1.27)	4,330	3,881	0.08	0.92 (0.86, 0.98)	5 × 10 ⁻³
	novel	rs2253407	0.01	A					4,330	3,881	0.47	0.88 (0.80, 0.98)	0.01
10q21 (ZNF365)	reported	rs16917302		C	[5]	3,807	3,315	0.83 (0.75, 0.93)	4,330	3,881	0.11	1.09 (1.02, 1.15)	6 × 10 ⁻³
	novel	rs17221319	0.00	A					4,330	3,881	0.46	1.09 (1.02, 1.15)	7 × 10 ⁻³
3p24 (SLC447, NEK10)	reported	rs4973768		A	[24]	3,370	2,783	1.10 (1.03, 1.18)	4,322	3,875	0.49	0.92 (0.87, 0.98)	0.01
12q24	reported	rs1292011 ⁴		G	[34]	2,530	2,342	0.94 (0.87, 1.01)	4,313	3,875	0.42	1.07 (1.01, 1.15)	0.04
5p12	reported	rs10941679 ⁴		G	[24]	3,263	2,591	1.09 (1.01, 1.19)	4,320	3,875	0.24	1.08 (1.00, 1.17)	0.04
11q13	reported	rs614367		A	[34]	3,789	3,307	1.03 (0.95, 1.13)	4,330	3,880	0.14	1.05 (0.99, 1.12)	0.10
1p11 (NOTCH2)	reported	rs11249433		G	[35]	3,423	2,827	1.09 (1.02, 1.17)	4,328	3,881	0.40	1.04 (0.97, 1.11)	0.23
17q23 (STXB4, COX11)	reported	rs6504950		A	[24]	3,401	2,813	1.03 (0.95, 1.11)	4,329	3,881	0.26	0.98 (0.91, 1.06)	0.62
19p13 (MERIT40)	reported	rs8170		A	[5]	3,665	3,086	0.98 (0.90, 1.07)	4,327	3,876	0.19	0.99 (0.93, 1.05)	0.66
2q35	reported	rs13387042 ⁴		G	[24]	3,300	2,646	1.05 (0.98, 1.13)	4,326	3,880	0.48	0.99 (0.93, 1.05)	0.77
9q31	reported	rs865686		C	[34]	3,799	3,312	0.95 (0.89, 1.01)	4,330	3,880	0.36	1.01 (0.95, 1.07)	0.91
10q22 (ZMIZ1)	reported	rs704010		A	[34]	3,761	3,279	1.01 (0.95, 1.08)	4,328	3,878	0.38		

¹Reporting status of the SNP is either previously reported or novel to this report.

²p-value was calculated based on the 1-degree of freedom score test statistic.

³rs311499 could not be designed onto the iCOGS array. A surrogate (r² = 1.0), rs311498, was included, however, and reported here.

⁴Stronger 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.

SNP rs No. Chr. (Nearby Genes)	Genotype	Discovery Stage				Stage 2				Combined				
		Affected No. (%)	Unaffected No. (%)	HR (95% CI)	P-value ¹	Affected No. (%)	Unaffected No. (%)	HR (95% CI)	P-value ¹	Affected No. (%)	Unaffected No. (%)	MAF	HR (95% CI)	P-value ¹
rs9348512 Chr6 (TFAP2A, C6orf218)	CC	390 (46.4)	248 (38.3)	1.00		1392 (43.0)	1640 (42.3)	1.00		1883 (43.5)	1731 (44.6)	0.35	1.00	
	CA	368 (43.8)	299 (46.2)	0.81 (0.67–0.96)		1515 (43.4)	1432 (44.3)	0.92 (0.83–1.01)		1883 (43.5)	1731 (44.6)		0.89 (0.82–0.97)	
	AA	82 (9.8)	100 (15.5)	0.55 (0.42–0.74)		368 (10.5)	410 (12.7)	0.72 (0.62–0.84)		450 (10.4)	510 (12.1)		0.68 (0.59–0.78)	
	per allele			0.76 (0.67–0.87)	2.6×10^{-5}			0.87 (0.81–0.93)	5.2×10^{-5}				0.85 (0.80–0.90)	3.9×10^{-8}
rs619373 ChrX (FGF13)	GG	693 (75.8)	568 (87.8)	1.00		2882 (82.7)	2784 (86.1)	1.00		3575 (82.6)	3352 (86.4)	0.07	1.00	
	GA	143 (15.7)	78 (12.1)	1.43 (1.13–1.80)		583 (16.7)	439 (13.6)	1.25 (1.10–1.43)		726 (16.8)	517 (13.3)		1.29 (1.15–1.45)	
	AA	4 (8.5)	1 (0.1)	2.01 (0.50–8.06)		21 (0.6)	11 (0.3)	2.09 (1.09–4.03)		25 (0.6)	12 (0.3)		1.99 (1.16–3.41)	
	per allele			1.43 (1.15–1.78)	3.0×10^{-3}			1.27 (1.12–1.44)	2.0×10^{-4}				1.30 (1.17–1.45)	3.1×10^{-6}
rs184577 Chr2 (C2orf58)	GG	520 (61.9)	368 (56.9)	1.00		2104 (60.3)	1824 (56.4)	1.00		2624 (60.6)	2192 (56.5)	0.25	1.00	
	GA	278 (33.1)	234 (36.2)	0.86 (0.71–1.03)		1212 (34.7)	1231 (38.1)	0.83 (0.75–0.92)		1490 (34.4)	1465 (37.8)		0.83 (0.76–0.91)	
	AA	42 (5.0)	45 (7.0)	0.67 (0.46–0.96)		174 (5.0)	179 (5.5)	0.80 (0.64–0.99)		216 (5.0)	224 (5.8)		0.77 (0.64–0.93)	
	per allele			0.84 (0.73–0.97)	1.5×10^{-2}			0.86 (0.79–0.93)	8.6×10^{-5}				0.85 (0.79–0.91)	3.6×10^{-6}

¹P-value was calculated based on the 1-degree of freedom score test. doi:10.1371/journal.pgen.1003173.t002

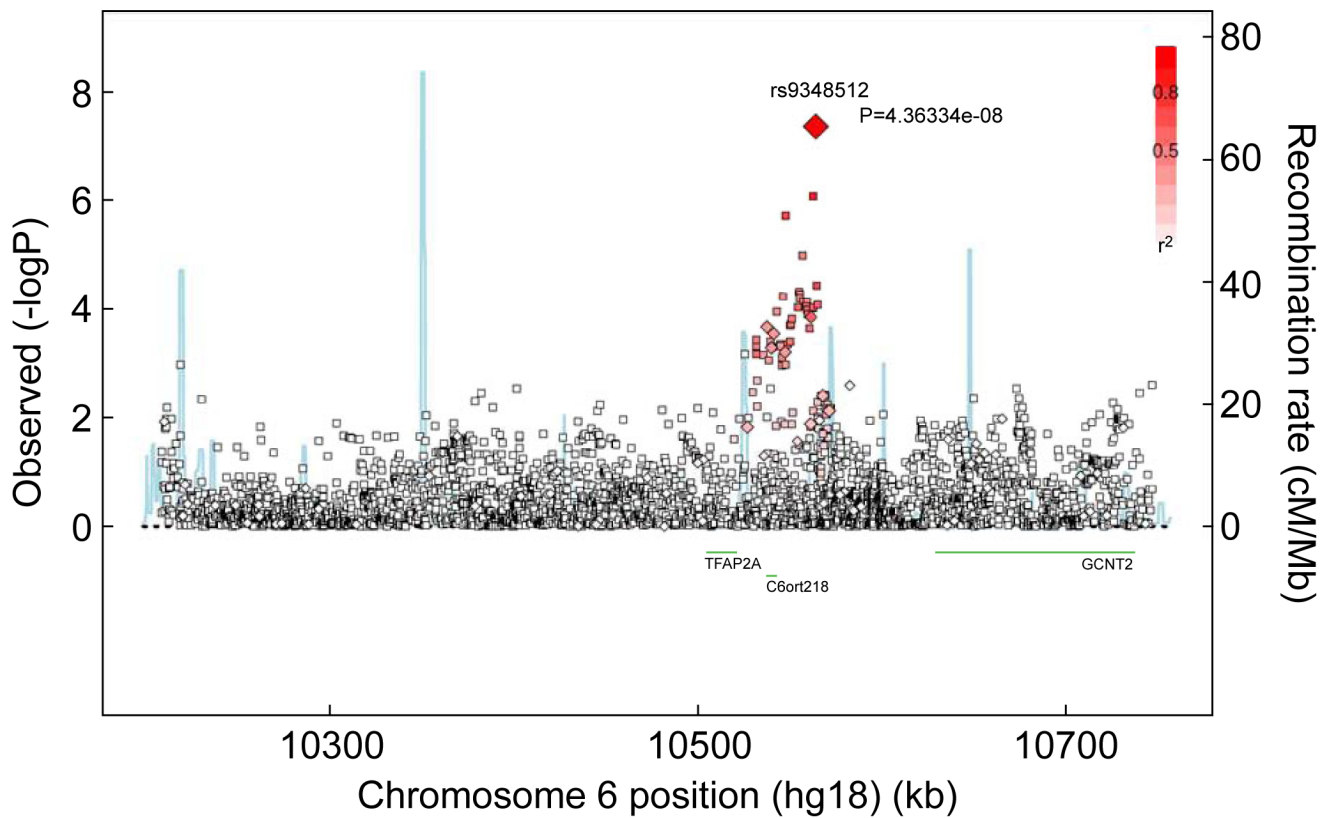


Figure 1. Associations between SNPs in the region surrounding rs9348512 on chromosome 6 and breast cancer risk. Results based on imputed and observed genotypes. The blue spikes indicate the recombination rate at each position. Genotyped SNPs are represented by diamonds and imputed SNPs are represented by squares. Color saturation indicates the degree of correlation with the SNP rs9348512. doi:10.1371/journal.pgen.1003173.g001

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-2 α 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-2 α 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 association 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 *CYP11B-ASI* (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

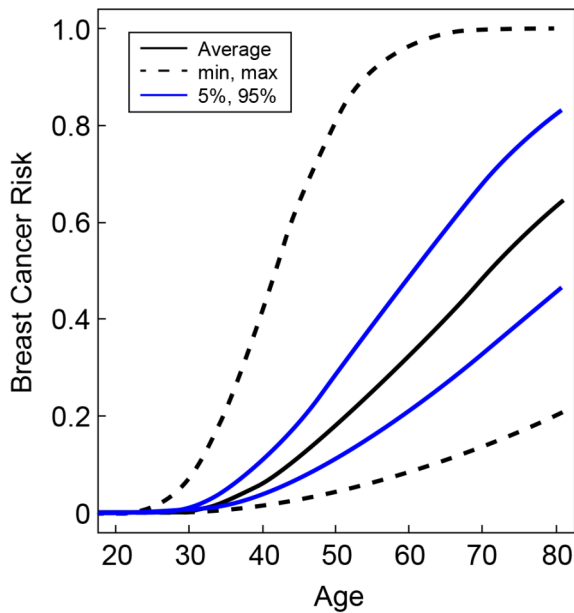


Figure 2. Predicted breast cancer risks for *BRCA2* mutation carriers by the combined SNP profile distributions. Based on the known breast cancer susceptibility loci at *FGFR2*, *TOX3*, 12p11, 5q11, *CDKN2A/B*, *LSP1*, 8q24, *ESR1*, *ZNF365*, 3p24, 12q24, 5p12, 11q13 and the newly identified *BRCA2* modifier locus at 6p24. The figure shows the risks at the 5th and 95th percentiles of the combined genotyped distribution as well as minimum and average risks. doi:10.1371/journal.pgen.1003173.g002

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*, 2q35, *SLCAA7*, 5p12, 1p11.2, *ZNF365*, and 19p13.1 (ER-negative only) [18,32,33], are also associated with breast cancer risk in the general population and/or *BRCA1* mutation carriers. Knowledge of the 6p24 locus might provide further insights into the biology of breast cancer development in *BRCA2* mutation carriers. Additional variants that are specific modifiers of breast cancer risk in *BRCA2* carriers may yet be discovered; their detection would require assembling larger samples of *BRCA2* mutation carriers in the future.

While individually each of the SNPs associated with breast cancer in *BRCA2* mutation carriers are unlikely to be used to guide breast cancer screening and risk-reducing management strategies, the combined effect of the general and *BRCA2*-specific breast cancer susceptibility SNPs might be used to tailor manage subsets of *BRCA2* mutation carriers. Taking into account all loci associated with breast cancer risk in *BRCA2* mutation carriers from the current analysis, including 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. These results might serve as a stimulus for prospective trials of the clinical utility of such modifier panels.

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* 6174delT 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 $P < 10^{-3}$ in a 500 Mb region around rs9348512 on 6p24 among *BRCA2* mutation carriers. (DOC)

Table S4 Associations with SNPs at 6p24, *FGF13* and 2p22 and breast and ovarian cancer risk using a competing risk analysis model. (DOC)

Acknowledgments

iCOGS: We acknowledge the contributions of Kyriaki Michailidou, Jonathan Tyrer, and Ali Amin Al Olama to the iCOGS statistical analyses and Shahana Ahmed, Melanie J. Maranian, and Catherine S. Healey for their contributions to the iCOGS genotyping quality control process.

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 Frédéric Robidoux from the McGill University and Génome Québec Innovation Centre.

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

clinicians, technicians, and administrative staff who have enabled this work to be carried out.

Amsterdam Breast Cancer Study (ABCS): We thank Annegien Broeks, Sten Cornelissen, Richard van Hien, Linde Braaf, Senno Verhoef, Laura van 't Veer, Emiel Rutgers, Ellen van der Schoot, and Femke Atsma.

Bavarian Breast Cancer Cases and Controls (BBCC): We thank Lothar Haerberle, Sonja Oeser, Silke Landrith, and Reiner Strick.

British Breast Cancer Study (BBCS): We thank Eileen Williams, Elaine Ryder-Mills, and Kara Sargus.

Breast Cancer Family Registry (BCFR) Studies: Samples from the NC-BCFR were processed and distributed by the Coriell Institute for Medical Research. We wish to thank members and participants in the Breast Cancer Family Registry for their contributions to the study. The ABCFS would like to also thank Maggie Angelakos, Judi Maskiell, and Gillian Dite. The content of this manuscript does not necessarily reflect the views or policies of the National Cancer Institute or any of the collaborating centers in the BCFR, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government or the BCFR.

Baltic Familial Breast Ovarian Cancer Consortium (BFBOCC): BFBOCC-LT acknowledges Vilius Rudaitis, Laimonas Griškevičius, and Ramūnas Janavičius. BFBOCC-LV acknowledges oncologists Janis Eglitis, Anna Krilova, and Aivars Stengrevics.

Breast Cancer in Galway Genetic Study (BIGGS): We thank Niall McInerney, Gabrielle Collieran, Andrew Rowan, and Angela Jones.

BRCA-gene mutations and breast cancer in South African women (BMBSA): We wish to thank the families who contribute to the BMBSA study.

Beckman Research Institute of the City of Hope (BRICOH): We wish to thank Greg Wilhoite, Yuan Chun Ding, Linda Steele, and Marie Pinto for their work in participant enrollment and biospecimen and data management.

Breast Cancer Study of the University Clinic Heidelberg (BSUCH): We thank Peter Bugert, Medical Faculty Mannheim.

Copenhagen General Population Study (CGPS): We appreciate the staff and participants of the Copenhagen General Population Study. For the excellent technical assistance, we thank Dorthe Uldall Andersen, Maria Birna Arnadottir, Anne Bank, and Dorthe Kjeldgård Hansen.

Spanish National Cancer Centre (CNIO): We thank Alicia Barroso, Rosario Alonso, and Guillermo Pita for their assistance.

Spanish National Cancer Centre Breast Cancer Study (CNIO-BCS): We thank Charo Alonso, Guillermo Pita, Nuria Álvarez, Daniel Herrero, Primitiva Menendez, José Ignacio Arias Pérez, Pilar Zamora, the Human Genotyping-CEGEN Unit (CNIO).

CONSORZIO STUDI ITALIANI sui Tumori Ereditari Alla Mammella (CONSIT TEAM): Bernard Peissel, Daniela Zaffaroni, and Giulia Melloni of the Fondazione IRCCS Istituto Nazionale Tumori (INT), Milan; Bernardo Bonanni of Istituto Europeo di Oncologia (IEO), Milan; Alessandra Viel and Riccardo Dolcetti of the Centro di Riferimento Oncologico (CRO) IRCCS, Aviano (PN); Liliana Varesco of the IRCCS AOU San Martino-IST Istituto Nazionale per la Ricerca sul Cancro, Genoa; Laura Papi of University of Florence, Florence; Laura Ottini and Giuseppe Giannini of "La Sapienza" University, Rome; Adele Patrini of the Ospedale di Circolo-Università dell'Insubria, Varese; Antonella Savarese and Aline Martayain of the Istituto Nazionale Tumori Regina Elena (IRE), Rome; and Stefania Tommasi of the Istituto Nazionale Tumori "Giovanni Paolo II", Bari, and the personnel of the CGT-lab at IFOM-IEO Campus, Milan, Italy.

Dana Farber Cancer Institute (DFCI): We thank the study staff and participants.

Genen Omgeving studie van de werkgroep Hereditaire Borstkanker Onderzoek Nederland (DNA HEBON): DNA HEBON consists of the following Collaborating Centers: Coordinating center: Netherlands Cancer Institute, Amsterdam, NL: M.A. Rookus, F.B.L. Hogervorst, F.E. van Leeuwen, S. Verhoef, M.K. Schmidt, J.L. de Lange; Erasmus Medical Center, Rotterdam, NL: J.M. Collée, A.M.W. van den Ouweland, M.J. Hoening, C. Seynaeve, C.H.M. van Deurzen; Leiden University Medical Center, NL: C.J. van Asperen, J.T. Wijnen, R.A. Tollenaar, P. Devilee, T.C.T.E.F. van Cronenburg; Radboud University Nijmegen Medical Center, NL: C.M. Kets, A.R. Mensenkamp; University Medical Center Utrecht, NL: M.G.E.M. Ausems, R.B. van der Luijt; Amsterdam Medical Center, NL: C.M. Aalfs, T.A.M. van Os; VU

University Medical Center, Amsterdam, NL: J.J.P. Gille, Q. Waisfisz, H.E.J. Meijers-Heijboer; University Hospital Maastricht, NL: E.B. Gómez-García, M.J. Blok; University Medical Center Groningen, NL: J.C. Oosterwijk, A.H. van der Hout, M.J. Mourits, G.H. de Boek. The Netherlands Foundation for the detection of hereditary tumours, Leiden, NL: H.F. Vasen.

Epidemiological study of BRCA1 & BRCA2 mutation carriers (EMBRACE): Douglas F. Easton is the PI of the study. EMBRACE Collaborating Centres are: Coordinating Centre, Cambridge: Susan Peock, Debra Frost, Steve Ellis, Elena Fineberg, Radka Platte. North of Scotland Regional Genetics Service, Aberdeen: Zosia Miedzobrodzka, Helen Gregory. Northern Ireland Regional Genetics Service, Belfast: Patrick Morrison, Lisa Jeffers. West Midlands Regional Clinical Genetics Service, Birmingham: Trevor Cole, Kai-ren Ong, Jonathan Hoffman. South West Regional Genetics Service, Bristol: Alan Donaldson, Margaret James. East Anglian Regional Genetics Service, Cambridge: Marc Tischkowitz, Joan Paterson, Amy Taylor. Medical Genetics Services for Wales, Cardiff: Alexandra Murray, Mark T. Rogers, Emma McCann. St James's Hospital, Dublin & National Centre for Medical Genetics, Dublin: M. John Kennedy, David Barton. South East of Scotland Regional Genetics Service, Edinburgh: Mary Porteous, Sarah Drummond. Peninsula Clinical Genetics Service, Exeter: Carole Brewer, Emma Kivuva, Anne Searle, Selina Goodman, Kathryn Hill. West of Scotland Regional Genetics Service, Glasgow: Rosemarie Davidson, Victoria Murday, Nicola Bradshaw, Lesley Snadden, Mark Longmuir, Catherine Watt, Sarah Gibson, Eshika Haque, Ed Tobias, Alexis Duncan. South East Thames Regional Genetics Service, Guy's Hospital London: Louise Izatt, Chris Jacobs, Caroline Langman. North West Thames Regional Genetics Service, Harrow: Angela Brady, Huw Dorkins, Athalie Melville, Kashmir Randhawa. Leicestershire Clinical Genetics Service, Leicester: Julian Barwell. Yorkshire Regional Genetics Service, Leeds: Julian Adlard, Gemma Serra-Feliu. Cheshire & Merseyside Clinical Genetics Service, Liverpool: Ian Ellis, Catherine Houghton. Manchester Regional Genetics Service, Manchester: D. Gareth Evans, Fiona Lalloo, Jane Taylor. North East Thames Regional Genetics Service, NE Thames, London: Lucy Side, Alison Male, Cheryl Berlin. Nottingham Centre for Medical Genetics, Nottingham: Jacqueline Eason, Rebecca Collier. Northern Clinical Genetics Service, Newcastle: Fiona Douglas, Oonagh Claber, Irene Jobson. Oxford Regional Genetics Service, Oxford: Lisa Walker, Diane McLeod, Dorothy Halliday, Sarah Durell, Barbara Stayner. The Institute of Cancer Research and Royal Marsden NHS Foundation Trust: Rosalind A. Eeles, Susan Shanley, Nazneen Rahman, Richard Houlston, Elizabeth Bancroft, Elizabeth Page, Audrey Ardern-Jones, Kelly Kohut, Jennifer Wiggins, Elena Castro, Emma Killick, Sue Martin, Gillian Rea, Anjana Kulkarni. North Trent Clinical Genetics Service, Sheffield: Jackie Cook, Oliver Quarrell, Cathryn Bardsley. South West Thames Regional Genetics Service, London: Shirley Hodgson, Sheila Goff, Glen Brice, Lizzie Winchester, Charlotte Eddy, Vishakha Tripathi, Virginia Attard, Anna Lehmann. Wessex Clinical Genetics Service, Princess Anne Hospital, Southampton: Diana Eccles, Anneke Lucassen, Gillian Crawford, Donna McBride, Sarah Smalley.

ESTHER Breast Cancer Study (ESTHER): Additional cases were recruited in the context of the VERDI study. We thank Hartwig Ziegler, Sonja Wolf, and Volker Hermann.

German Consortium of Hereditary Breast and Ovarian Cancer (GC-HBOC): We are very thankful to all family members who participated in this study; Wolfram Heinritz, Center Leipzig, and Dieter Schäfer, Center Frankfurt, for providing DNA samples; and Juliane Köhler for excellent technical assistance; as well as Heide Hellebrand, Stefanie Engert, and GC-HBOC.

Genetic Modifiers of Cancer Risk in BRCA1/2 Mutation Carriers (GEMO): National Cancer Genetics Network «UNICANCER Genetic Group», France. We wish to thank all the GEMO collaborating groups for their contribution to this study. GEMO Collaborating Centers are: Coordinating Centres, Unité Mixte de Génétique Constitutionnelle des Cancers Fréquents, Hospices Civils de Lyon - Centre Léon Bérard, & Equipe «Génétique du cancer du sein», Centre de Recherche en Cancérologie de Lyon: Olga Similnikova, Sylvie Mazoyer, Francesca Damiola, Laure Barjhoux, Carole Vernet-Pierre, Sophie Giraud, Mélanie Léone; and Service de Génétique Oncologique, Institut Curie, Paris: Dominique Stoppa-Lyonnet, Marion Gauthier-Villars, Bruno Buecher, Claude Houdayer, Virginie Moncoutier, Muriel Belotti, Carole Tirapo, Antoine de Pauw. Institut Gustave Roussy, Villejuif: Brigitte Bressac-de-

Paillerets, Olivier Caron. Centre Jean Perrin, Clermont-Ferrand; Yves-Jean Bignon, Nancy Uhrhammer. Centre Léon Bérard, Lyon; Christine Lasset, Valérie Bonadonna, Sandrine Handallou. Centre François Baclesse, Caen; Agnès Hardouin, Pascaline Berthet. Institut Paoli Calmettes, Marseille; Hagay Sobol, Violaine Bourdon, Tetsuro Noguchi, Audrey Remenieras, François Eisinger. CHU Arnaud-de-Villeneuve, Montpellier; Isabelle Coupier, Pascal Pujol. Centre Oscar Lambret, Lille; Jean-Philippe Peyrat, Joëlle Fournier, Françoise Révillon, Philippe Vennin, Claude Adenis. Hôpital René Huguenin/Institut Curie, St Cloud; Etienne Rouleau, Rosette Lidereau, Liliane Demange, Catherine Nogues. Centre Paul Strauss, Strasbourg; Danièle Muller, Jean-Pierre Fricker. Institut Bergonié, Bordeaux; Emmanuelle Barouk-Simonet, Françoise Bonnet, Virginie Bubien, Nicolas Sevenet, Michel Longy. Institut Claudius Regaud, Toulouse; Christine Toulas, Rosine Guimbaud, Laurence Gladiéff, Viviane Feillel. CHU Grenoble; Dominique Leroux, Hélène Dreyfus, Christine Rebeschun, Magalie Peysselon. CHU Dijon; Fanny Coron, Laurence Faivre. CHU St-Etienne; Fabienne Prieur, Marine Lebrun, Caroline Kientz. Hôtel Dieu Centre Hospitalier, Chambéry; Sandra Fert Ferrer. Centre Antoine Lacassagne, Nice; Marc Frénaux. CHU Limoges; Laurence Vénat-Bouvet. CHU Nantes; Capucine Delnatte. CHU Bretonneau, Tours; Isabelle Mortemousque. Groupe Hospitalier Pitié-Salpêtrière, Paris; Florence Coulet, Chrystelle Colas, Florent Soubrier. CHU Vandoeuvre-les-Nancy; Johanna Sokolowska, Myriam Bronner. Creighton University, Omaha, USA; Henry T. Lynch, Carrie L. Snyder.

Gene Environment Interaction and Breast Cancer in Germany (GENICA): The GENICA network: Dr. Margarete Fischer-Bosch-Institute of Clinical Pharmacology, Stuttgart, and University of Tübingen, Germany; [CJ, Hiltrud Brauch], Department of Internal Medicine, Evangelische Kliniken Bonn gGmbH, Johanniter Krankenhaus, Bonn, Germany [Yon-Dschun Ko, Christian Baisch], Institute of Pathology, University of Bonn, Bonn, Germany [Hand-Peter Fischer], Molecular Genetics of Breast Cancer, Deutsches Krebsforschungszentrum (DKFZ), Heidelberg, Germany [UH]; and Institute for Prevention and Occupational Medicine of the German Social Accident Insurance (IPA), Bochum, Germany [Thomas Bruening, Beate Pesch, Sylvia Rabstein, Anne Spickenheuer, VH].

Hospital Clinico San Carlos (HCSC): We acknowledge Alicia Tosar for her technical assistance.

Helsinki Breast Cancer Study (HEBCS): HEBCS would like to thank Drs. Kristiina Aittomäki, Carl Blomqvist and Kirsimari Aaltonen, and Taru A. Muranen and RN Irja Erkkilä for their help with the HEBCS data and samples.

Hannover-Minsk Breast Cancer Study (HMBCS): We thank Natalia Bogdanova, Natalia Antonenkova, Hans Christiansen, and Peter Hillemanns.

Study of Genetic Mutations in Breast and Ovarian Cancer patients in Hong Kong and Asia (HRBCP): We wish to thank Hong Kong Sanatorium and Hospital for their continual support.

Molecular Genetic Studies of Breast- and Ovarian Cancer in Hungary (HUNBOCS): We wish to thank the Hungarian Breast and Ovarian Cancer Study Group members (Janos Papp, Aniko Bozsik, Kristof Arvai, Judit Franko, Maria Balogh, Gabriella Varga, Judit Ferenczi, Department of Molecular Genetics, National Institute of Oncology, Budapest, Hungary), and the clinicians and patients for their contributions to this study.

University Hospital Vall d'Hebron (HVH): We thank the study staff and participants.

Interdisciplinary Health Research Internal Team Breast Cancer susceptibility (INHERIT): We would like to thank Dr Martine Dumont, Martine Tranchant for sample management and skillful technical assistance.

Kuopio Breast Cancer Project (KBCP): We thank Eija Myöhänen and Helena Kemiläinen.

Kathleen Cunningham Consortium for Research into Familial Breast Cancer (kConFab/AOCS): We thank Heather Thorne, Eveline Niedermayr, all the kConFab research nurses and staff, the heads and staff of the Family Cancer Clinics, and the Clinical Follow Up Study for their contributions to this resource, and the many families who contribute to kConFab.

Leuven Multidisciplinary Breast Centre (LMBC): We thank Gilian Peuteman, Dominiek Smeets, Thomas Van Brussel, and Kathleen Corthouts.

Mammary Carcinoma Risk Factor Investigation (MARIE): We thank Dieter Flesch-Janys, Rebecca Hein, Stefan Nickels, Muhabbet Celik, Sabine Behrens, and Ursula Eilber.

Milan Breast Cancer Study Group (MBCSG): We thank Daniela Zaffaroni of the Fondazione Istituto Nazionale Tumori, Milan, Italy and the personnel of the CGT laboratory at IFOM-IEO Campus, Milan, Italy.

Montreal Gene-Environment Breast Cancer Study (MTLGEBCS): We thank Martine Tranchant (Cancer Genomics Laboratory, CRCHUQ), Marie-France Valois, Annie Turgeon, and Lea Heguy (McGill University Health Center, Royal Victoria Hospital; McGill University) for DNA extraction, sample management, and skillful technical assistance.

General Hospital Vienna (MUV): We thank the study staff and participants.

National Israeli Cancer Control Center (NICCC): We wish to thank the NICCC National Familial Cancer Consultation Service team led by Sara Dishon, the lab team led by Dr. Flavio Lejbkowitz, and the research field operations team led by Dr. Mila Pinchev.

Oulu Breast Cancer Study (OBCS): We thank Katri Pylkäs, Arja Jukkola-Vuorinen, Mervi Grip, Saira Kauppila, Meeri Otsukka, and Kari Mononen.

Ontario Cancer Genetics Network (OCGN): We thank the study staff and participants.

Leiden University Medical Centre Breast Cancer Study (ORIGO): We thank E. Krol-Warmerdam, and J. Blom for patient accrual, administering questionnaires, and managing clinical information. The LUMC survival data were retrieved from the Leiden hospital-based cancer registry system (ONCDOC) with the help of Dr. J. Molenaar.

The Ohio State University Comprehensive Cancer Center (OSUCCG): Kevin Sweet, Caroline Craven, and Michelle O'Conor were instrumental in accrual of study participants, ascertainment of medical records and database management. Samples were processed by the OSU Human Genetics Sample Bank.

Odense University Hospital (OUH): We thank the study staff and participants.

Università di Pisa (PBCS): We thank the study staff and participants.

The U.S. National Cancer Institute Polish Breast Cancer Study (PBCS): We thank the study collaborators Drs. Louise Brinton, Mark Sherman, Stephen Chanock, Neonila Szeszenia-Dabrowska, Beata Peplonska, and Witold Zatonski, as well as Pei Chao and Michael Stagner, for their data management support.

Rotterdam Breast Cancer Study (RBCS): We thank Petra Bos, Jannet Blom, Ellen Crepin, Elisabeth Huijskens, Annette Heemskerk, and the Erasmus MC Family Cancer Clinic.

Sheffield Breast Cancer Study (SBCS): We thank Sue Higham, Helen Cramp, and Dan Connley.

South East Asian Breast Cancer Association Study (SEABASS): We would like to thank Yip Cheng Har, Nur Aishah Mohd Taib, Phuah Sze Yee, Norhashimah Hassan, and all the research nurses, research assistants, and doctors involved in the MyBrCa Study for assistance in patient recruitment, data collection, and sample preparation. In addition, we thank Philip Iau, Sng Jen-Hwei, and Sharifah Nor Akmal for contributing samples from the Singapore Breast Cancer Study and the HUKM-HKL Study respectively.

Study of Epidemiology and Risk Factors in Cancer Heredity (SEARCH): We thank the SEARCH and EPIC teams.

Sheba Medical Centre (SMC): SMC team wishes to acknowledge the assistance of the Meirav Comprehensive breast cancer center team at the Sheba Medical Center for assistance in this study.

Swedish Breast Cancer Study (SWE-BRCA): Swedish scientists participating as SWE-BRCA collaborators are: from Lund University and University Hospital: Åke Borg, Håkan Olsson, Helena Jernström, Karin Henriksson, Katja Harbst, Maria Soller, Niklas Loman, Ulf Kristoffersson; from Gothenburg Sahlgrenska University Hospital: Anna Öfverholm, Margareta Nordling, Per Karlsson, Zakaria Einbeigi; from Stockholm and Karolinska University Hospital: Anna von Wachenfeldt, Annelie Liljegren, Annika Lindblom, Brita Arver, Gisela Barbany Bustinza, Johanna Rantala; from Umeå University Hospital: Beatrice Melin, Christina Edwindsdotter Ardnor, Monica Emanuelsson; from Uppsala University: Hans Ehren-crona, Maritta Hellström Pigg, Richard Rosenquist; from Linköping University Hospital: Marie Stenmark-Askmal, Sigrun Liedgren.

The University of Chicago Center for Clinical Cancer Genetics and Global Health (UCHICAGO): We wish to thank

Cecilia Zvocec, Qun Niu, physicians, genetic counselors, research nurses and staff of the Cancer Risk Clinic for their contributions to this resource, and the many families who contribute to our program.

University of California Los Angeles (UCLA): We thank Joyce Seldon MSGC and Lorna Kwan MPH for assembling the data for this study.

University of California San Francisco (UCSF): We would like to thank Ms. Salina Chan for her data management and the following genetic counselors for participant recruitment: Beth Crawford, Nicola Stewart, Julie Mak, and Kate Lamvik.

United Kingdom Breakthrough Generations Study (UKBGS): We thank Breakthrough Breast Cancer and the Institute of Cancer Research for support of the Breakthrough Generations Study, and the study participants, study staff, and the doctors, nurses, and other health care providers and health information sources who have contributed to the study.

United Kingdom Familial Ovarian Cancer Registries (UKFOCR): We thank Simon Gayther, Susan Ramus, Carole Pye, Patricia Harrington, and Eva Wozniak for their contributions towards the UKFOCR.

Victorian Familial Cancer Trials Group (VFCTG): We acknowledge Geoffrey Lindeman, Marion Harris, Martin Delatycki of the Victorian Familial Cancer Trials Group. We thank Sarah Sawyer and Rebecca Driessen for assembling this data and Ella Thompson for performing all DNA amplification.

Author Contributions

Conceived and designed the experiments: P Hall, FJ Couch, J Simard, D Altshuler, DF Easton, G Chenevix-Trench, AC Antoniou, K Offit.

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- Performed the experiments: MM Gaudet, KB Kuchenbaecker, J Vijai, RJ Klein, T Kirchoff. Analyzed the data: MM Gaudet, KB Kuchenbaecker, J Vijai, RJ Klein, L McGuffog, D Barrowdale, AM Dunning, J Simard, D Altshuler, DF Easton, AC Antoniou, K Offit. Contributed reagents/materials/analysis tools: L McGuffog, D Barrowdale, AM Dunning, A Lee, J Dennis, S Healey, E Dicks, P Soucy, OM Similnikova, VS Pankratz, X Wang, RC Eldridge, DC Tessier, D Vincent, F Bacot, FBL Hogervorst, S Peock, D Stoppa-Lyonnet, P Peterlongo, RK Schmutzler, KL Nathanson, M Piedmonte, CF Singer, M Thomassen, TvO Hansen, SL Neuhausen, I Blanco, MH Greene, J Garber, JN Weitzel, IL Andrulis, DE Goldgar, E D'Andrea, T Caldes, H Nevanlinna, A Osorio, EJ van Rensburg, A Arason, G Rennett, AMW van den Ouweland, AH van der Hout, CM Kets, CM Aalfs, JT Wijnen, MGEM Ausems, D Frost, S Ellis, E Fineberg, R Platte, DG Evans, C Jacobs, J Adlard, M Tischkowitz, ME Porteous, F Damiola, L Golmard, L Barjhoux, M Longy, M Belotti, SF Ferrer, S Mazoyer, AB Spurdle, S Manoukian, M Barile, M Genuardi, N Arnold, A Meindl, C Sutter, B Wappenschmidt, SM Domchek, G Pfeiler, E Friedman, UB Jensen, M Robson, S Shah, C Lazaro, PL Mai, J Benitez, MC Southey, MK Schmidt, PA Fasching, J Peto, MK Humphreys, Q Wang, H Michailidou, EJ Sawyer, B Burwinkel, P Guénel, SE Bojesen, RL Milne, H Brenner, M Lochmann, K Aittomäki, T Dörk, S Margolin, A Mannermaa, D Lambrechts, J Chang-Claude, P Radice, G Giles, CA Haiman, R Winqvist, P Devilee, M Garcia-Closas, N Schoof, MJ Hooning, A Cox, PDP Pharoah, A Jakubowska, N Orr, A González-Neira, G Pita, MR Alonso, P Hall, FJ Couch, DF Easton, G Chenevix-Trench, AC Antoniou, K Offit. Wrote the paper: MM Gaudet, KB Kuchenbaecker, J Vijai, RJ Klein, AC Antoniou, K Offit.
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