Assessing Associations between the AURKA-HMMR-TPX2-TUBG1 Functional Module and Breast Cancer Risk in BRCA1/2 Mutation Carriers

Vidal, Holger Heyn, Dominique Stoppa-Lyonnet, Melanie Leone, Laure Barjhoux, Marion Fassy-Colcombet, Antoine de Pauw, Christine Lasset, Sandra Fert Ferrer, Laurent Castera, Pascaline Berthet, Francois Cornelis, Yves-Jean Bignon, Francesca Damiola, Sylvie Mazoyer, Olga M. Sinilnikova, Christopher A. Maxwell, Joseph Vijai, Mark Robson, Noah Kauff, Marina J. Corines, Danylko Villano, Julie Cunningham, Adam Lee, Noralane Lindor, Conxi Lazaro, Douglas F. Easton, Kenneth Offit, Georgia Chenevix-Trench, Fergus J. Couch, Antonis C. Antoniou and Miguel Angel Pujana

Linköping University Post Print

N.B.: When citing this work, cite the original article.

Original Publication:


Copyright: Public Library of Science
http://www.plos.org/

Postprint available at: Linköping University Electronic Press
http://urn.kb.se/resolve?urn=urn:nbn:se:liu:diva-117653
Assessing Associations between the AURKA-HMMR-TPX2-TUBG1 Functional Module and Breast Cancer Risk in BRCA1/2 Mutation Carriers

Foundation Trust. RE and EB are supported by Cancer Research United Kingdom Grant C5047/A8385. FCCC: The authors acknowledge support from The University of Kansas Cancer Center (P30 CA165824) and the Kansas Bioscience Authority Eminent Scholar Program. AKG was funded by 5U01CA113916, R01CA140323, and by the Chancellor's Distinguished Chair in Biomedical Sciences Professorship. GC-HBC: The German Consortium of Hereditary Breast and Ovarian Cancer (GHC-HBC) is supported by the German Cancer Aid (grant no 109076, RKS) and by the Center for Molecular Medicine Cologne (CMMC). GEMO: The study was supported by the Ligue Nationale Contre le Cancer; the Association “Le cancer du sein, par bons-ent” Award; and the Canadian Institutes of Health Research for the "CIHR Team in Familial Risks of Breast Cancer" program. G-FAST: TVM is a postdoctoral researcher funded by the Fund for Scientific Research Flanders (FWO). GO3: 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 11165). MHG, PLM and Dr. Savage were supported by funding from the Intramural Research Program, NCI. HCSC: Was supported by a grant DK12/0036/0008 and 12/00539 from ISICII (Spain), partially supported by European Regional Development FEDER funds. HEBCS: The HEBCS was financially supported by the Helsinki University Central Hospital Research Fund, Academy of Finland (132473), the Finnish Cancer Society and the Sigrid Juselius Foundation. HEBON: The HEBON study is supported by the Dutch Cancer Society grants NKI988-1854, NKI2004-3088, NKI2007-3756, the Netherlands Organization of Scientific Research grant NWO 9110902. The Pink ribbon grant 10005 and the BBMRI grant NWO 184.021.007/CP46. HUNBUCS: 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. ICO: The ICO-IDIBELL study was supported by grants from the Spanish Ministry of Health ISCIII FIS (PI10/ 01422, PI120152, and PI1300285) and RTICC (RD12/0036/008); the Ramón Areces (XV), Eugenio Rodrigo, and Roses contra el Cancer (2012) Foundations; the Spanish Association Against Cancer (AECC 2010); and the AGAUR Generalitat de Catalunya (2009-SGR290 and 2009-SGR293). IHCC: The IHCC was supported by Grant PBZ_MBN_122/P05/2004; Katarzyna Jaworska is a fellow of International PhD program, Postgraduate School of Molecular Medicine, Warsaw Medical University, supported by the Polish Foundation of center, Nijmegen, The Netherlands, Department of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands, Department of Pathology, Leiden University Medical Center, Leiden, The Netherlands, Department of Clinical Genetics, Leiden University Medical Center, Leiden, The Netherlands, Department of Epidemiology, Netherlands Cancer Institute, Amsterdam, The Netherlands, Department of Clinical Genetics, Vrije Universiteit (VU) University Medical Center, Amsterdam, The Netherlands, Department of Clinical Genetics, Maastricht University Medical Center, Maastricht, The Netherlands, Department of Clinical Genetics, Erasmus University Medical Center, Rotterdam, The Netherlands, Department of Clinical Genetics, Academic Medical Center, Amsterdam, The Netherlands, Division of Gynecologic Oncology, NorthShore University HealthSystem, University of Chicago, Chicago, Illinois, United States of America, Division of Cancer Medicine, Peter MacCallum Cancer Centre, East Melbourne, Victoria, Australia, Gynecologic Oncology Group Statistical and Data Center, Roswell Park Cancer Institute, Buffalo, New York, United States of America, Central Connecticut Cancer Consortium, Hartford Hospital/Helen and Harry Gray Cancer Center, Hartford, Connecticut, United States of America, Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, University of North Carolina, Chapel Hill, North Carolina, United States of America, Division of Gynecologic Oncology, Ohio State University, Columbus Cancer Council, Hilliard, Ohio, United States of America, Division of Gynecologic Oncology, Yale University School of Medicine, New Haven, Connecticut, United States of America, Centre of Familial Breast and Ovarian Cancer and Centre for Integrated Oncology (CIO), University Hospital of Cologne, Cologne, Germany, Institute for Medical Informatics, Statistics and Epidemiology, University of Leipzig, Leipzig, Germany, Department of Gynecology and Obstetrics, Division of Tumor Genetics, Klinikum Rechts der Isar, Technical University Munich, Munich, Germany, Department of Gynecology and Obstetrics, Ludwig-Maximilian University Munich, Munich, Germany, Department of Gynecology and Obstetrics, Christian-Albrechts-University of Kiel University Medical Center Schleswig-Holstein, Kiel, Germany, Institute of Human Genetics, Christian-Albrechts-University of Kiel University Medical Center Schleswig-Holstein, Kiel, Germany, Department of Gynecology and Obstetrics, University Hospital Düsseldorf, Heinrich-Heine University Düsseldorf, Düsseldorf, Germany, Institute of Human Genetics, Department of Human Genetics, University Hospital Heidelberg, Heidelberg, Germany, Department of Gynecology and Obstetrics, University Hospital Ulm, Ulm, Germany, Institute of Cell and Molecular Pathology, Hannover Medical School, Hannover, Germany, Institute of Human Genetics, University of Münster, Münster, Germany, Department of Gynecology and Obstetrics, University Hospital Carl Gustav Carus, Technical University Dresden, Dresden, Germany, Institute of Human Genetics, Campus Virchow Klinikum, Charite Berlin, Berlin, Germany, Centre of Familial Breast and Ovarian Cancer, Department of Medical Genetics, Institute of Human Genetics, University Würzburg, Würzburg, Germany, Department of Clinical Genetics, Veje Hospital, Veje, Denmark, Section of Molecular Diagnostics, Department of Biochemistry, Aalborg University Hospital, Aalborg, Denmark, Department of Clinical Genetics, Aarhus University Hospital, Aarhus, Denmark, Department of Clinical Genetics, Odense University Hospital, Odense, Denmark, Department of Cancer Epidemiology and Genetics, Masaryk Memorial Cancer Institute, Brno, Czech Republic, Fondazione Istituto di Oncologia Molecolare (IFOM), Fondazione Italiana per la Ricerca sul Cancro (FIRC), Milan, Italy, Department of Experimental Oncology, Istituto Europeo di Oncologia (IEO), Cogenetech Cancer Genetic Test Laboratory, Milan, Italy, Unit of Medical Genetics, Department of Preventive and Predictive Medicine, Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Fondazione Istituto Nazionale Tumori (INT), Milan, Italy, Unit of Molecular Bases of Genetic Risk and Genetic Testing, Department of Preventive and Predictive Medicine, Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Fondazione Istituto Nazionale Tumori (INT), Milan, Italy, Department of Molecular Medicine, “Sapienza” University, Rome, Italy, Immunology and Molecular Oncology Unit, Istituto Oncologico Veneto (IOV), Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Padua, Italy, Laboratoire de Diagnostic Génétique et Service d’Onco-Hématologie, Hopitaux Universitaires de Strasbourg, Centre Hospitalier Régional Universitaire (CHRU) Nouvel Hôpital Civil, Strasbourg, France, Cancer Genomics Laboratory, Centre Hospitalier Universitaire de Québec Research Center and Laval University, Quebec City, Canada, Department of Gynecology and Obstetrics, and Comprehensive Cancer Center, Medical University of Vienna, Vienna, Austria, Breast Cancer Family Registry (BCFR), Cancer Prevention Institute of California, Fremont, California, United States of America, Department of Epidemiology, Cancer Prevention Institute of California, Fremont, California, United States of America, Department of Cancer Biology, Dana-Farber Cancer Institute, Boston, Massachusetts, United States of America, Department of Population Sciences, Beckman Research Institute of City of Hope, Duarte, California, United States of America, Department of Epidemiology, Columbia University, New York, New York, United States of America, Departments of Pediatrics and Medicine, Columbia University Medical Center, New York, New York, United States of America, Department of Clinical Genetics, Fox Chase Cancer Center, Philadelphia, Pennsylvania, United States of America, Department of Dermatology, University of Utah School of Medicine, Salt Lake City, Utah, United States of America, Vilnius University Hospital Santariskiu Clinics, Hematology, Oncology and Transfusion Medicine Center, Department of Molecular and Regenerative Medicine, State Research Centre Institute for Innovative medicine, Vilnius, Lithuania, Cancer Genetics Laboratory, Department of Genetics, University of Pretoria, Arcadia, South Africa.
Abstract

While interplay between BRCA1 and AURKA-RHAMM-TPX2-TUBG1 regulates mammary epithelial polarization, common genetic variation in HMMR (gene product RHAMM) may be associated with risk of breast cancer in BRCA1 mutation carriers. Following on these observations, we further assessed the link between the AURKA-HMMR-TPX2-TUBG1 functional module and risk of breast cancer in BRCA1 or BRCA2 mutation carriers. Forty-one single nucleotide polymorphisms (SNPs) were genotyped in 15,252 BRCA1 and 8,211 BRCA2 mutation carriers and subsequently analyzed using a retrospective likelihood approach. The association of HMMR rs299290 with breast cancer risk in BRCA1 mutation carriers
was confirmed: per-allele hazard ratio (HR) = 1.10, 95% confidence interval (CI) 1.04 – 1.15, p = 1.9 x 10^{-4} (false discovery rate (FDR)-adjusted p = 0.043). Variation in CSTF1, located next to AURKA, was also found to be associated with breast cancer risk in BRCA2 mutation carriers: rs2426618 per-allele HR = 1.10, 95% CI 1.03 – 1.16, p = 0.005 (FDR-adjusted p = 0.045). Assessment of pairwise interactions provided suggestions (FDR-adjusted pInteraction values > 0.05) for deviations from the multiplicative model for rs299290 and CSTF1 rs6064391, and rs299290 and TUBG1 rs11649877 in both BRCA1 and BRCA2 mutation carriers. Following these suggestions, the expression of HMMR and AURKA or TUBG1 in sporadic breast tumors was found to potentially interact, influencing patients’ survival. Together, the results of this study support the hypothesis of a causative link between altered function of AURKA-HMMR-TPX2-TUBG1 and breast carcinogenesis in BRCA1/2 mutation carriers.

Introduction

An integrative genomics study generated a breast cancer network model that predicted novel genetic and molecular relationships for breast cancer tumor suppressors [1]. Among the predictions, the product of the hyaluronan-mediated motility receptor (HMMR) gene, RHAMM, was found to be biochemically and functionally linked to the breast cancer gene, early onset 1 gene product (BRCA1) [1]. Analysis of common genetic variation in HMMR suggested an association with breast cancer risk in Ashkenazi Jewish women, with a greater increased risk in younger individuals [1]. However, this association was not observed either in a European case-control study [2] or in a genome-wide association study in postmenopausal women of European ancestry [3].

Following the initial functional evidence, a molecular mechanism involving RHAMM and BRCA1 was found to regulate mammary epithelial apicobasal polarization and, possibly, differentiation [4]. The results from this study indicated that RHAMM and BRCA1 play a central role in the cytoskeletal reorganization necessary for epithelial polarization. This functional interplay included interactions with the product of the proto-oncogene aurora kinase A (AURKA) and its major regulator, targeting protein for Xklp2 (TPX2), in addition to g-tubulin (TUBG1) [4]. In this scenario, cell proliferation is endorsed by activated AURKA while polarization and differentiation are mediated by activation of BRCA1 and degradation of RHAMM. Intriguingly, the same HMMR variation as originally detected in the Ashkenazi Jewish population was suggested to be associated with breast cancer risk in BRCA1, but not in BRCA2 mutation carriers [4]. This observation was endorsed by complementary analyses in breast cancer tissue; specifically, loss of cell polarity was revealed in in situ breast tissue lesions of BRCA1 mutation carriers and, accordingly, increased staining of phospho-T703-RHAMM (target of AURKA) was preferentially detected in estrogen receptor α (ERα)-negative and BRCA1-mutated tumors [4].

While the HMMR association study in BRCA1/2 mutation carriers drew on a partial dataset from the Consortium of Investigators of Modifiers of BRCA1/2 (CIMBA), the depicted mechanistic model highlighted additional gene candidates for breast cancer risk; i.e., AURKA, TPX2, and TUBG1 [4]. In a previous CIMBA study, no evidence of association was found between functional variation in AURKA and breast cancer risk among BRCA1/2 mutation carriers [5]. However, these results were based on a more limited CIMBA dataset (4,935 BRCA1 and 2,241 BRCA2 mutation carriers) and did not comprehensively assess variation in the AURKA...
genomic region. Furthermore, variation in *TUBG1* was found to be associated with breast cancer risk in a hospital-based case-control study [6], but has not been assessed in *BRCA1/2* mutation carriers.

In addition to multiplicative allele effects, systematic analyses in model organisms have shown that a given phenotype may be substantially determined by genetic interactions (GxG); that is, “epistasis” in statistical terms, defined as deviation from additivity for a quantitative phenotype arising from the effect of genetic variants or mutations in another locus [7]. Importantly, GxG significantly overlap with other types of gene and/or protein relationships [8–10]. Therefore, the functional interplay between the aforementioned genes/proteins in a key mammary epithelial cell process could support the existence of genetic interactions that influence cancer risk.

In the present study, given previous evidence of 1) the functional interplay between BRCA1 and AURKA-RHAMM-TPX2-TUBG1 in mammary epithelial polarization [4], and 2) the potential modification of breast cancer risk in *BRCA1* mutation carriers by common genetic variation in *HMMR* [4], we further assessed the association between variants in *AURKA-HMMR-TPX2-TUBG1* and breast cancer risk in *BRCA1* and *BRCA2* mutation carriers. Genotyped variants from the custom Illumina iSelect array of the Collaborative Oncological Gene-environment Study (iCOGS) were analyzed in a large series of *BRCA1/2* mutation carriers [11,12].

**Materials and Methods**

**Study Subjects and Ethics Statement**

*BRCA1/2* mutation carriers were recruited under the CIMBA initiative following approval of the corresponding protocol by the institutional review board or ethics committee at each participating center, and written informed consent was obtained from the patients when required [11,12]. Sixty CIMBA study centers recruited 15,252 *BRCA1* and 8,211 *BRCA2* mutation carriers that passed quality control assessment in this study. Most of these individuals were recruited through cancer genetics clinics and enrolled into national or regional studies. The remaining carriers were identified by population-based sampling or community recruitment. Eligibility in CIMBA was restricted to female carriers of pathogenic *BRCA1* and *BRCA2* mutations who were ≥ 18 years old at recruitment. Information collected included year of birth, mutation description, self-reported ethnicity, age at last follow-up, ages at breast or ovarian cancer diagnosis, and age at bilateral prophylactic mastectomy or oophorectomy. Information regarding tumor characteristics, including ERα status, was collected for 3,458 *BRCA1* and 1,924 *BRCA2* mutation carriers. Related individuals were identified by a unique family identifier.

**iCOGS Design**

The iCOGS array, genotyping and quality controls for the CIMBA *BRCA1/2* mutation carrier samples have been described recently [11,12]. The final array design included 211,155 manufactured SNPs that were selected on the basis of primary evidence from genome-wide association studies (GWASs) of breast, ovarian and prostate cancer, for fine mapping of known cancer susceptibility loci, and included functional candidate variants of interest [11–15] (also see http://www.nature.com/icogs/primer/cogs-project-and-design-of-the-icogs-array/ and http://ccge.medschl.cam.ac.uk/research/consortia/icogs/). Details of the iCOGS array design have been described elsewhere [11–15]. The genotype data used in this study are available upon request from the CIMBA Data Access Coordinating Committee (contact A.C.A.).
Association Study

Based on previous GWAS results for BRCA1 [16] and BRCA2 [17] mutation carriers, and on the selection of gene candidates, the iCOGS array included SNPs in the AURKA (n = 15), HMMR (n = 14), TPX2 (n = 3) and TUBG1 (n = 4) loci (defined as ± 20 kilobases (kb) from the genomic structure of each gene), and these were analyzed in the present study (S1 Table). In addition, we analyzed five SNPs proximal to the HMMR locus that provided some suggestion of association with breast cancer risk in Ashkenazi Jewish women [1] (S1 Table). In total, these SNPs represented 32 partially independent variants (pairwise r^2 < 0.85). To account for multiple testing, we used a FDR approach for the 41 genotyped SNPs that were evaluated for their associations with breast cancer risk in BRCA1 and BRCA2 mutation carriers; significant results are reported for FDR < 5%. The main analyses focused on evaluating associations between each genotype and breast cancer or ovarian cancer risk separately, in a survival analysis framework. In the breast cancer analysis, the phenotype of each individual was defined by age at breast cancer diagnosis or age at last follow-up. Individuals were followed until the age of the first breast or ovarian cancer diagnosis or bilateral prophylactic mastectomy, whichever occurred first, or until age at last observation. Mutation carriers censored at ovarian cancer diagnosis were considered to be unaffected. For the ovarian cancer analysis, the primary endpoint was the age at ovarian cancer diagnosis, and mutation carriers were followed until the age of ovarian cancer diagnosis or risk-reducing salpingo-oophorectomy, or until age at last observation. In order to maximize the number of ovarian cancer cases, breast cancer was not considered to be a censoring event in this analysis, and mutation carriers who developed ovarian cancer after breast cancer diagnosis were considered affected in the ovarian cancer analysis. To adjust for the non-random sampling of mutation carriers with respect to their disease status, data were analyzed by modeling the retrospective likelihood of the observed genotypes conditional on the disease phenotypes [18]. The associations were assessed using the 1-degree of freedom score test statistic based on this retrospective likelihood. To allow for the non-independence among related individuals, the correlation between the genotypes was taken into account using a kinship-adjusted version of the score test statistic [16]. The p values presented were based on the adjusted score test. To estimate the HRs, the effect of each SNP was modeled as either a per-allele or genotype on the log-scale by maximizing the retrospective likelihood. The evidence of heterogeneity in the associations between countries/study-centers was also evaluated. Associations with breast and ovarian cancer risks were assessed simultaneously within a competing risk analysis framework [11,18]. The significant FDR-adjusted associations (for rs299290 in HMMR and for rs2426618 in AURKA/CSTF1) were subsequently explored using imputed genotypes based on data from the 1,000 Genomes project (March 2012 version [19]). The IMPUTE2 software [20] was used for imputation of non-genotyped SNPs. Associations of each marker with cancer risk were assessed using a similar score test to that used for the genotyped SNPs, but based on the posterior genotype probabilities at each imputed marker for each individual. In all analyses, only those SNPs with an imputation information/accuracy of r^2 > 0.30 and a minor allele frequency (MAF) > 0.3 were considered. The haplotypes and their posterior probabilities were estimated using the expectation-maximization algorithm [21]. Only the four HMMR haplotypes with the highest probabilities were considered; the rest were grouped into a single rare haplotype. Each carrier was assigned the most likely haplotype and the association between haplotypes and age at breast cancer diagnosis was evaluated using a standard Cox proportional hazards model. All possible pairwise gene interactions including rs299290 or rs2426618 were evaluated using a standard Cox proportional hazards model that considered the main effects and the interaction term.
Expression Analysis

The association between gene expression and survival after breast cancer diagnosis was assessed using the NKI-295 dataset of sporadic primary breast tumors [22,23] and a standard Cox proportional hazards model. All possible pairwise gene interactions including HMMR or AURKA microarray probes (2 and 1 probes, respectively) were evaluated using this model. The quantitative analysis of HMMR expression isoforms was carried out using mRNA extracted from lymphoblastoid cell lines of nine rs299290-TT and six rs299290-CC BRCA2 mutation carriers, and the following TaqMan (Applied Biosystems) probes in real-time PCR assays: hs01063269 for total HMMR expression; hs0106328 for the inclusion of exon 4; and hs00234864 for the inclusion of exon 11.

Genome Analyses

Data for formaldehyde-assisted isolation of regulatory elements (FAIREs) that marked transcriptionally active regions in normal human mammary epithelial cells (HMECs) were downloaded from the Gene Expression Omnibus (GEO) reference GSE46074 [24]. Sequence reads were trimmed for the adaptor, masked for low-complexity and low-quality sequences/reads and subsequently aligned to the genome version hg19 using TopHat [25] with default parameters. Peaks were called using HOMER [26], applying a triangle-based distribution, a median length of 150 base pairs, and an α value of 0.01 (99.0% CI). Replicates were analyzed individually and uniquely merged using BEDTools [27]. Chromatin immunoprecipitation data of ERα were downloaded from the GEO reference GSE32222 and analyzed with MACS (version 2.0.9; macs2diff function) [28]. Significance was defined as a false discovery rate < 1%, using default values for all other parameters. Differentially bound genomic regions were assigned to the closest ENSEMBL (version 62) annotated gene using the R-Bioconductor package ChIPpeakAnno [29]. Histone modification and chromatin segmentation data in HMECs were obtained from the UCSC Genome Browser (hg19) and correspond to the GEO references GSE29611 and GSE38163, respectively, deposited by the ENCODE project [30].

Evolutionary analysis of BRCA1 and RHAMM

The full-length nucleotide and protein sequences from 20 (for BRCA1/BRCA1) and 26 (for HMMR/RHAMM) mammalian species, which included human and naked mole rat, were downloaded from the OrthoMaM 2.0 database [31] (S2 Table). For evolutionary analysis, a multiple sequence alignment (MSA) of the corresponding amino acid sequences was generated using the algorithm implemented in PRANK v.140110 [32]. To prevent the inclusion of incorrectly aligned positions, all MSA positions with low statistical support (posterior probabilities < 0.99) in the PRANK alignment were excluded. Next, the high-quality protein MSAs were used as guide in the alignment of the corresponding coding sequences (CDS MSAs). The level of functional constraints acting on the coding regions of both genes was analyzed using the maximum likelihood method implemented in the codeml program of PAML v.4 [33]; this approach allows to estimate the non-synonymous (dN) to synonymous (dS) ratio (ω) in a particular coding region by using a codon-based evolutionary model under a phylogenetic framework, allowing comparison of their fit to the data by the likelihood ratio test (LRT). In particular, the goodness-of-fit of two nested evolutionary models was compared: the M7 model, which assumes a β distribution of ω across sites between 0 and 1 (0 ≤ ω ≤ 1); and the M8 model, which adds to M7 an extra category of positively selected sites (ω > 1). To reduce the probability of false positive results from the M7-M8 comparison, we also estimated the likelihood of the data under the model M8a [34], in which ω was set to 1. The posterior probabilities for each site of belonging to the positively selected class were computed using the Bayes
empirical Bayes approach in codeml [35]. In all models, the topology of the mammalian phylogenetic tree assumed in OrthoMaM database was used (S2 Table).

Results

HMMR rs299290 Association

The product of the HMMR gene, RHAMM, interacts with BRCA1 in the control of mammary epithelial polarization and this function may be at the basis of a modification of breast cancer risk in BRCA1 mutation carriers [4]. In this iCOGS BRCA1/2 study, 14 SNPs (11 with pairwise $r^2 < 0.85$) at the HMMR locus were genotyped in 15,252 BRCA1 and 8,211 BRCA2 mutation carriers from 60 participating centers. Among these variants, the strongest evidence of association with breast cancer risk in BRCA1 mutation carriers was observed for the originally reported SNP rs299290 [4] (MAF = 0.25): BRCA1 per-allele HR = 1.10, 95% CI 1.04–1.15, p = 1.9 x 10$^{-4}$ (FDR-adjusted p = 0.043, accounting for 41 genotyped SNPs used in the association analyses in BRCA1 and BRCA2 mutation carriers). In contrast, no evidence of association was obtained between HMMR variation and breast cancer risk in BRCA2 mutation carriers; specifically, rs299290 per-allele HR = 0.98, 95% CI 0.92–1.05, p = 0.57. The effect among BRCA1 mutation carriers was consistent across most participating countries (Fig. 1A) and no heterogeneity was detected ($\chi^2$ = 0.30). Importantly, the BRCA1 association remained after excluding the centers participating in the original study [4]: n = 5,039, rs299290 per-allele HR = 1.13, 95% CI 1.04–1.22, p = 0.005.

Although the original Ashkenazi Jewish population study suggested associations involving SNPs proximal to HMMR (~450 kb proximal) [1], no evidence of association in BRCA1/2 mutation carriers was obtained for five correlated variants in this region (S1 Table). With respect to rs299290 and ovarian cancer risk, no evidence of association was found under the single disease risk model or the competing risks model (p > 0.65; only breast cancer risk in BRCA1 mutation carriers was significant in this model: p = 2.5 x 10$^{-4}$). Together, these results corroborate the association between variation at the HMMR locus and breast cancer risk in BRCA1 mutation carriers.

Mapping the HMMR locus association

Allelic imputation within ~60 kb centered on HMMR (fully including the proximal genes CCNG1 and NUDCD2) did not detect substantially stronger associations than those identified for rs299290: a variant located in HMMR intron 7 (rs61292050; Fig. 1B) was found to be similarly associated (p = 2.7 x 10$^{-3}$), but this was correlated with rs299290 ($r^2 = 0.95$). Haplotype analyses were then carried out to demarcate the HMMR genomic region potentially harboring a causative variant or mutation. Using the 14 SNPs genotyped in iCOGS, two haplotypes, both characterized by the minor allele of rs299290, were found to be associated with breast cancer risk in BRCA1 mutation carriers (S3 Table). Based on these haplotypes, the minimal region harboring a mutation could be delimited to ~28 kb between rs299284 and rs10038157 (S3 Table).

Analysis of the potential causative variant

The rs299290 variant represents a missense amino-acid change in HMMR exon 11 that is predicted to be benign/neutral/tolerated by several algorithms: Valine 369 to Alanine in accession number NP_001136028.1; MutationAssessor score = 0; Polyphen score = 0.005; and SIFT score = 0.73. Subsequent examination of the splicing of exon 11 and of exon 4, the latter of which is known to be differentially spliced in different conditions and cell types [36], did not
reveal alterations or differences between mRNA samples with different rs299290 genotypes (S1 Fig.). Nonetheless, rs299290 is located \(~14\) kb from the \textit{HMMR} promoter region that is active in mammary epithelial cells, as detected by the analysis of data from genome occupancy profiling [24] (Fig. 1B). In addition, analysis of data for ER\(\alpha\) binding plasticity [37] revealed significant binding of this factor at the \textit{HMMR} promoter in poor-prognosis breast tumors (Fig. 1B).

Causal alleles for different common diseases have shown evidence of positive selection [38]. Notably, a recent report suggested the action of positive selection on the evolution of BRCA1 and RHAMM orthologs in the naked mole rat, which is an exceptionally cancer-resistant species [39]. Following on from this suggestion, we identified footprints of positive selection in the evolution of some amino acids of both proteins. In both cases, model M8 (selection model) better fits the protein alignment data than model M7 (null model): \(p\) values = \(6.69 \times 10^{-14}\) and \(1.55 \times 10^{-5}\), for BRCA1 and RHAMM, respectively. Moreover, the likelihood of the data is significantly higher under model M8 than under the nested model M8a: \(p\) values = \(6.22 \times 10^{-10}\)
and 0.014 for BRCA1 and RHAMM, respectively, confirming the presence of positively selected sites in these alignments. However, Valine 369 RHAMM was not identified in these analyses; the predicted amino acid sites under selection were only linked to rare variants (MAFs < 0.01) (S4 Table). Nonetheless, as suggested in the analysis of the naked mole rat sequence, Valine 369 is within a region with a potential excess of selected positions (Fig. 2A).

Analysis of BRCA1 also showed multiple potential selection sites (Fig. 2B), but the specific region or domain mediating the interaction with RHAMM remains unknown [1,4,40].

### Evaluation of HMMR association by ERα tumor status and BRCA1 mutation class

The original study suggested an association between the rs299290 risk allele and ERα-negative breast cancer for BRCA1 mutation carriers [4]. In the present study, no difference was found in the rs299290 effect between ERα-negative and ERα-positive cases: ERα-negative, per-allele HR = 1.09, 95% CI 1.03–1.15, p = 2.3 x 10^{-3}; ERα-positive, per-allele HR = 1.09, 95% CI 0.98–1.21, p = 0.13; p\_difference = 0.96. Interestingly, there was a suggestion of an rs299290 association with ERα-negative breast cancer for BRCA2 mutation carriers, but in the opposite direction to that observed for BRCA1 mutation carriers: ERα-negative BRCA2 mutation carriers n = 434, per-allele HR = 0.83, 95% CI 0.70–0.97, p = 0.022. In addition, there was no evidence of an rs299290 association with ERα-positive breast cancer in BRCA2 mutation carriers (n = 1,490, p = 0.40, ERα-negative effect p\_difference = 0.019).

Regarding BRCA1 mutation classes, the original study [4] suggested a rs299290 association in carriers of mutations expected to result in a reduced transcript or protein level due to nonsense-mediated RNA decay (Class 1), but not in carriers of mutations likely to generate stable proteins with a potential residual or dominant-negative function (Class 2). The current study indicated a similar association, although the estimations were not significantly different: Class 1, rs299290 per-allele HR = 1.11, 95% CI 1.05–1.18, p = 4.6 x 10^{-4}; Class 2, rs299290 per-allele HR = 1.03, 95% CI 0.94–1.14, p = 0.51. Regarding Ashkenazi Jewish BRCA1 mutation carriers (n = 1,231), there were no significant associations in this population or with founder mutations (185delAG HR = 0.94, p = 0.17; and 5382insC HR = 0.85, p = 0.10). Larger sample series may
be required to assess associations in these settings and their consistency with previous observations in the Ashkenazi Jewish population [1].

**AURKA/CSTF1 association with breast cancer risk in BRCA2 mutation carriers**

As the *HMMR* and *AURKA-TPX2-TUBG1* gene products are functionally related in the regulation of mammary epithelial polarization [4], the associations between variants at these loci that were included on the iCOGS array and cancer risk in *BRCA1/2* mutation carriers were assessed (S1 Table). No associations were observed for *TPX2* and *TUBG1*, but there was an indication of an association for a variant relatively close to *AURKA*; rs2426618 and breast cancer risk in *BRCA2* mutation carriers, per-allele HR = 1.10, 95% CI 1.03–1.16, p = 0.005 (FDR-adjusted p = 0.045; Fig. 3A). There was no evidence of association between this variant and breast cancer risk in *BRCA1* mutation carriers or between the same variant and ovarian cancer risk in *BRCA2* mutation carriers.

![Fig 3. The AURKA/CSTF1 locus and breast cancer risk in BRCA2 mutation carriers.](image-url)
either BRCA1 or BRCA2 mutation carriers (p > 0.30). Consistent with the main analysis, the competing risk model showed an rs2426618 association with breast cancer risk only in BRCA2 mutation carriers (p = 0.002).

The rs2426618 variant is located in intron 5 of a neighboring (distal) gene, CSTF1 (Fig. 3B). Analogous to the HMMR setting, rs2426618 is relatively close (~ 30 kb) to the promoter region of AURKA/CSTF1, which is active in mammary epithelial cells and differentially ERα-regulated in poor-prognosis breast tumors (Fig. 3B). Association analysis with imputed SNPs within ~ 50 kb centered on rs2426618 (fully including AURKA and CSTF1) did not reveal stronger evidence than that of rs2426618: a variant located in AURKA intron 8 (rs187966577, Fig. 3B) was found to be similarly associated (p = 0.002). However, this variant was rare (MAF = 0.009) and poorly imputed (r^2 = 0.37). Finally, the analysis of rs2426618 by ERα tumor status in BRCA2 mutation carriers did not reveal specific associations: ERα-positive, per-allele HR = 1.10, 95% CI 1.02–1.17, p = 0.010; ERα-negative, per-allele HR = 1.11, 95% CI 0.97–1.26, p = 0.15, p_difference = 0.89.

Potential genetic interactions in the AURKA-HMMR-TPX2-TUBG1 module

Given the above associations at HMMR and AURKA/CSTF1, the influence of GxG on breast cancer risk in BRCA1/2 mutation carriers was assessed between rs299290 and the genotyped variants in AURKA/TPX2/TUBG1 (n = 22), and between rs2426618 and the genotyped variants in TPX2/TUBG1 (n = 7). No GxG was detected for rs2426618-TPX2/TUBG1, but potential interactions (unadjusted p_interaction values < 0.05) between rs299290 and AURKA/CSTF1 or TUBG1 variants were suggested (Table 1). An interaction between rs299290 and rs6064391 (in intron 2 of CSTF1, Fig. 3B) could reduce breast cancer risk in both BRCA1 and BRCA2 mutation carriers (interaction HRs = 0.87 and 0.73, respectively; Table 1). In the main effect analysis, rs6064391 showed a suggestion of association with reduced breast cancer risk in BRCA2 mutation carriers: HR = 0.87, unadjusted p = 0.036. Conversely, an interaction between rs299290 and rs11649877 (3’ region of TUBG1) could increase breast cancer risk in both

<table>
<thead>
<tr>
<th>Genotype(s) (gene locus)</th>
<th>HR*</th>
<th>p_interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BRCA1 mutation carriers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs299290-A/G (HMMR)</td>
<td>1.16</td>
<td></td>
</tr>
<tr>
<td>rs6064391-A/A (CSTF1)</td>
<td>1.09</td>
<td></td>
</tr>
<tr>
<td>rs299290-A/G—rs6064391-A/A</td>
<td>0.87</td>
<td>0.047</td>
</tr>
<tr>
<td>rs299290-A/G (HMMR)</td>
<td>1.07</td>
<td></td>
</tr>
<tr>
<td>rs11649877-A/G (TUBG1)</td>
<td>0.94</td>
<td></td>
</tr>
<tr>
<td>rs299290-A/G—rs11649877-A/G</td>
<td>1.33</td>
<td>0.014</td>
</tr>
<tr>
<td><strong>BRCA2 mutation carriers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs299290-G/G (HMMR)</td>
<td>1.32</td>
<td></td>
</tr>
<tr>
<td>rs6064391-A/C (CSTF1)</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td>rs299290-A/G—rs6064391-A/C</td>
<td>0.73</td>
<td>0.034</td>
</tr>
<tr>
<td>rs299290-A/G (HMMR)</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td>rs11649877-A/A (TUBG1)</td>
<td>0.87</td>
<td></td>
</tr>
<tr>
<td>rs299290-A/G—rs11649877-A/A</td>
<td>1.21</td>
<td>0.041</td>
</tr>
</tbody>
</table>

*Each estimate is derived from the interaction term of a Cox regression model.

Table 1. Potential GxG associated with breast cancer risk in BRCA1/2 mutation carriers.

doi:10.1371/journal.pone.0120020.t001
BRCA1 and BRCA2 mutation carriers (HRs = 1.33 and 1.21, respectively; Table 1). In the main effect analysis, there was no evidence of rs11649877 association with breast cancer risk in BRCA1 or BRCA2 mutation carriers (p = 0.49). Genetic interactions frequently represent complex molecular relationships and, thus, can be explained by multiple models of phenotypic differences across genetic backgrounds [41], including those identified here. However, additional studies are required to corroborate the findings.

Integrative studies in model organisms have shown that experimentally identified GxG overlap with other types of gene and/or protein interactions (e.g., gene co-expression) to a degree that is significantly higher than expected by chance [8]. Thus, we assessed the GxGs suggested above for their equivalence with interactions between gene expression profiles across breast tumors, considering breast cancer survival as the outcome. There are no large tumor series from BRCA1/2 mutation carriers that allow gene expression interactions in these mutation backgrounds to be assessed; therefore our analysis was restricted to sporadic cases and aimed to explore complex relationships between HMMR and AURKA or TUBG1. Using the NKI-295 dataset [22], HMMR expression was found to be significantly associated with overall survival: two microarray probes gave identical results: HR = 1.78 and p = 0.001. AURKA expression was also found to be associated with overall survival (single probe HR = 2.07, p = 3.3 x 10^{-9}) but, notably, the combined model with HMMR indicated a protective interaction: the HRs for both HMMR and AURKA probe combinations were 0.57 and 0.55, p_{interaction} values = 0.026, respectively (Fig. 4A). The combined HMMR and TUBG1 expressions also suggested an interaction, but in this case “aggravating”: the HRs for both HMMR probe combinations with a TUBG1 probe were 1.84 and 2.16, p_{interaction} = 0.041 and 0.009, respectively (Fig. 4B). The expression of TUBG1 alone was not found to be associated with overall survival (p = 0.12). Together, these results could indicate a functional correspondence for interactions influencing both breast cancer development and progression. However, the precise effects of the corresponding risk alleles remain to be elucidated.

**Discussion**

The results of this study expand on the previous suggestion that variation in HMMR is specifically associated with breast cancer risk in BRCA1 mutation carriers [4]. By analyzing a much larger number of carriers—genotyped using the high-quality iCOGS approach [11,12]—we
were able to confirm the association of this locus, and to demonstrate that the strongest signal corresponded to rs299290. No specific effect was revealed when analyzing this association by ERα tumor status. Larger carrier series may be required to comprehensively evaluate associations by tumor subtype or, conversely, to establish that variation in HMMR interacts with Class I BRCA1 mutations to give rise to any type of tumor. In this context, mapping of the protein and/or functional domains interacting between BRCA1 and RHAMM would be necessary to decipher the role of rs299290, if any, and of the observed potential positive selection. With regard to the results obtained for BRCA2 mutation carriers, the opposite direction of the potential association with ERα-negative breast cancer compared with the association observed for BRCA1 mutation carriers is intriguing. Opposite effects between cancer subtypes have been observed for other modifier loci [16] and, thus, potentially inform on opposing functional roles in biological processes influencing carcinogenesis. In other words, alteration of RHAMM function in mammary epithelial differentiation may have a differential effect on breast cancer risk depending on whether it occurs in a BRCA1- or BRCA2-mutated background.

The study of gene loci functionally related to HMMR suggests an association between variation in AURKA/CSTF1 and risk of breast cancer among BRCA2 mutation carriers. While there were initial conflicting results for AURKA associations with breast cancer risk [5,42], it is interesting to note that a population case-control study [43] identified variants in the AURKA promoter region associated with breast cancer risk in the same direction as detected in our analysis. The minor allele of rs6064389 was shown to be protective in the general population [43], and a similar association was observed in the analysis of BRCA2 mutation carriers: HR = 0.93, p = 0.021; not significant in BRCA1 mutation carriers, p = 0.25. This variant is partially correlated with rs2426618 (r² = 0.63), and more strongly correlated with rs6064391 (r² = 0.86), which might interact with rs299290.

In addition to the main effects, interaction between HMMR and AURKA, and between HMMR and TUBG1 could influence breast cancer risk in BRCA1 and BRCA2 mutation carriers. The interactions did not remain statistically significant after corrections for multiple testing and, therefore, additional studies are warranted to investigate these findings further. Combined expression of HMMR and AURKA, and HMMR and TUBG1 in sporadic breast tumors appeared to influence patients’ survival differentially. These results could be analogous to those observed for breast cancer risk, but it remains unknown how the corresponding risk alleles may alter gene expression and/or protein function. In this context, over-expression of HMMR, AURKA or TUBG1 impairs mammary epithelial polarization [4] and the rs299290 risk allele could be associated with relatively higher levels of HMMR expression [1,4], which would be consistent with the observed interactions. However, other studies on the potential regulatory impact of this variant and/or the link to selective constraints are needed. It is also important to note that the altered gene products may not be those included in the depicted functional module, but rather other products from the corresponding chromosomal regions.

In summary, centered on the AURKA-HMMR-TPX2-TUBG1 functional module that regulates mammary epithelial polarization, this study confirms previous association results for HMMR rs299290 and suggests novel associations (for AURKA/CSTF1 and HMMR-rs299290 interactions) with breast cancer risk in BRCA1 and/or BRCA2 mutation carriers.

Supporting Information

S1 Fig. Results of the quantification of the expression of HMMR exons 4 and 11 according to the rs299290 major and minor genotypes. (TIF)
S1 Table. Genotyped variants and breast cancer association results in *BRCA1* and *BRCA2* mutation carriers.
(XLSX)

S2 Table. Species and genes used in functional constraints analyses.
(XLSX)

S3 Table. Variation in *HMMR* and haplotype association study for risk of breast cancer in *BRCA1* mutation carriers.
(PDF)

S4 Table. Evolutionary selection tests and results.
(XLS)

S5 Table. Details of acknowledgments and funding support.
(XLSX)

Acknowledgments
This work is dedicated to the memory of Olga M. Sinilnikova, who inspired the CIMBA group and led studies on BRCA1 modifiers. We wish to thank all study participants and clinicians for their valuable contributions to the iCOGS study. We also wish to thank the individual contributors from the following groups: BCFR, EMBRACE, GEMO, HEBON, kConFab, and SWE-BRCA (listed in S5 Table). Additional acknowledgments are also detailed in S5 Table.

Author Contributions

References


