

## Modification of Ovarian Cancer Risk by *BRCA1/2*-Interacting Genes in a Multicenter Cohort of *BRCA1/2* Mutation Carriers

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### Abstract

**Inherited *BRCA1/2* mutations confer elevated ovarian cancer risk. Knowledge of factors that can improve ovarian cancer risk assessment in *BRCA1/2* mutation carriers is important because no effective early detection for ovarian cancers exists. A cohort of 1,575 *BRCA1* and 856 *BRCA2* mutation carriers was used to evaluate haplotypes at *ATM*, *BARD1*, *BRIP1*, *CTIP*, *MRE11*, *NBS1*, *RAD50*, *RAD51*, and *TOPBP1* in ovarian cancer risk. In *BRCA1* carriers, no associations were observed with *ATM*, *BARD1*, *CTIP*, *RAD50*, *RAD51*, or *TOPBP1*. At *BRIP1*, an association was observed for one haplotype with a multiple testing corrected  $P$  ( $P_{\text{corr}}$ ) = 0.012, although no individual haplotype was significant. At *MRE11*, statistically significant associations were observed for one haplotype ( $P_{\text{corr}}$  = 0.007). At *NBS1*, we observed a  $P_{\text{corr}}$  = 0.024 for haplotypes. In *BRCA2* carriers, no associations were observed with *CTIP*, *NBS1*, *RAD50*, or *TOPBP1*. Rare haplotypes at *ATM* ( $P_{\text{corr}}$  = 0.044) and *BARD1* ( $P_{\text{corr}}$  = 0.012) were associated with ovarian cancer risk. At *BRIP1*, two common haplotypes were significantly associated with ovarian cancer risk ( $P_{\text{corr}}$  = 0.011). At *MRE11*, we observed a significant haplotype association ( $P_{\text{corr}}$  = 0.012), and at *RAD51*, one common haplotype was significantly associated with ovarian cancer risk ( $P_{\text{corr}}$  = 0.026). Variants in genes that interact biologically with *BRCA1* and/or *BRCA2* may be associated with modified ovarian cancer risk in women who carry *BRCA1/2* mutations. [Cancer Res 2009;69(14):5801–10]**

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

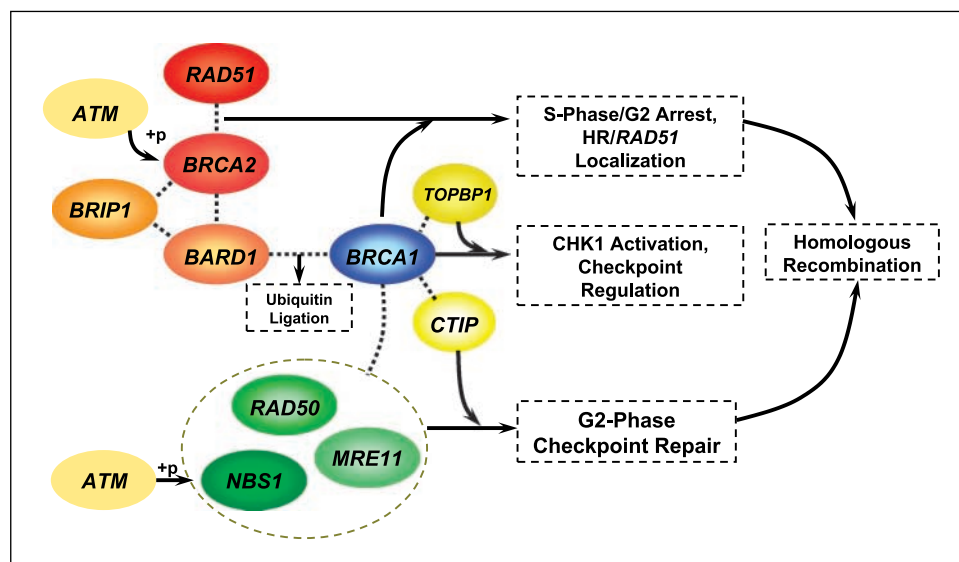
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doi:10.1158/0008-5472.CAN-09-0625

### Introduction

Mutations in *BRCA1* and *BRCA2* (*BRCA1/2*) are associated with an increased risk of developing breast and ovarian cancer. However, there is substantial interindividual variability in the age at diagnosis and site of cancer occurrence in *BRCA1/2* mutation carriers. These observations imply that *BRCA1/2* mutations may be necessary to explain the Mendelian pattern of cancer in some families but are not sufficient to describe interindividual variability in age- and site-specific cancer risk. Proper assessment of ovarian cancer risk in *BRCA1/2* mutation carriers is of clinical significance because no effective strategies for early detection of ovarian cancer exist, and most ovarian tumors are diagnosed at a late stage with poor prognosis (1). Thus, women are counseled to strongly consider risk-reducing salpingo-oophorectomy (RRSO). Although RRSO reduces ovarian and breast cancer risk and mortality (2, 3), the induction of surgical menopause is associated with menopausal symptoms, which may affect quality of life, osteoporosis, and cardiovascular disease. The goal of this research is to identify factors that modify ovarian cancer risk in *BRCA1/2* mutation carriers to improve risk assessment and disease prevention.

Modifying factors may influence cancer risk in *BRCA1/2* mutation carriers. Begg (4) reported that biases may exist in estimates of lifetime cancer risk if relevant covariates are ignored and concluded that modifiers are likely to exist that affect *BRCA1/2*-associated cancer penetrance. Lee and colleagues (5) examined the lifetime risk of cancer in first-degree relatives of *BRCA1/2* mutation carriers with breast or ovarian cancers and concluded that there was more similarity in risks within families than would be expected by chance alone. Several reports suggest that environmental exposures (e.g., oral contraceptives and smoking) affect ovarian cancer penetrance in women who carry a germ-line *BRCA1/2* mutation (6). Few genetic risk modifiers for *BRCA1/2*-associated ovarian cancer have been studied (7, 8).



**Figure 1.** Biological interactions of candidate proteins and *BRCA1* or *BRCA2*.

Genetic modifiers of ovarian cancer risk in *BRCA1/2* mutation carriers can be identified from our knowledge of *BRCA1/2* function (9, 10). Several proteins and protein complexes that interact with *BRCA1/2* have been identified (refs. 11, 12; Fig. 1). *BRCA1* has been found to interact with many DNA repair proteins including the *RAD50-MRE11-NBS1* (*MRN*) protein complex (11, 13). The proteins associated with *BRCA1* respond to aberrant DNA structures in several ways, including acting as DNA damage sensors, signal transducers, and repair effectors. *BRCA1* has been hypothesized to work as a coordinator of the various functions of DNA damage, recognition, response and repair, and double-strand break repair (11). Although the functions of *BRCA1* are not yet completely elucidated, we can hypothesize that the genes encoding the proteins that interact with *BRCA1* could act as candidate modifiers of *BRCA1*-associated cancer penetrance.

Fewer proteins are known to interact with *BRCA2* (14). *BRCA2* interacts with *RAD51*, which is involved in meiotic and mitotic recombination and in the repair of double-strand DNA breaks. The *RAD51* protein interacts directly with the *BRCA2* protein by binding to a series of repeats in *BRCA2*. As part of the cellular response to DNA damage, the *BRCA2/RAD51* complex colocalizes to damage-induced foci, where double-strand break repair is thought to take place. It is hypothesized that *BRCA2* plays a regulatory role with respect to *RAD51* and prevents *RAD51* from binding DNA and forming nucleoprotein filaments under normal circumstances (14). However, when DNA damage occurs, there is a change in the *BRCA2/RAD51* complex (perhaps phosphorylation of either protein) resulting in the assembly of the recombination complex at the damage-induced foci for DNA repair, allowing *RAD51* to bind to single-strand DNA and participate in double-strand break repair (15). *BRCA2* is required for efficient *RAD51* delivery to DNA damage sites for homologous recombination at single-stranded/double-stranded DNA junctions (15, 16). Mutations in either *RAD51* or *BRCA2* lead to severe defects in DNA repair and potentially to chromosomal rearrangements.

This evidence suggests that *BRCA1* and *BRCA2* are involved in supercomplexes of proteins involved in networks responsible for tumor suppression (11). Therefore, we hypothesize that variation in the genes that encode *BRCA1/2* interactors modulate *BRCA1/2*

penetrance as follows: *RAD51* in *BRCA2* mutation carriers via errors in HR/*RAD51* localization; *TOPBP1* or *BRIP1* in *BRCA1* or *BRCA2* mutation carriers via errors in DNA replication-associated DNA repair; *MRN* and *CTIP* in *BRCA1* mutation carriers via errors in G<sub>2</sub>-phase checkpoint and *CHK1* activation; or *BARD1* in *BRCA1* or *BRCA2* mutation carriers by any of these mechanisms.

## Materials and Methods

**Participants and data collection.** Seventeen MAGIC centers contributed to this study: Baylor-Charles A. Sammons Cancer Center, Beth Israel Deaconess Medical Center, City of Hope National Medical Center, Creighton University, Dana-Farber Cancer Institute, NorthShore University HealthSystem, Fox Chase Cancer Center, Georgetown University, Jonsson Comprehensive Cancer Center at University of California-Los Angeles, Mayo Clinic College of Medicine, University of Chicago, University of California-Irvine, University of Pennsylvania, University of Texas Southwestern, University of Vienna, Women's College Hospital, and Kathleen Cunningham Consortium for Research into Familial Breast Cancer (17).

The protocol for this observational cohort was the same in each center. All participants were identified via high-risk programs for clinical and research purposes. Participants were referred by clinicians or self-referred because they were perceived to be at risk for hereditary breast and/or ovarian cancer. Genetic counseling and testing was done under clinical and/or research protocols specific to the institutional review board guidelines of each center. All centers identified women who had tested positive for *BRCA1/2* mutations by commercial laboratory testing or, more rarely, research testing without clinical disclosure of test results.

The participating centers provided eligibility information to the University of Pennsylvania coordinating center, which in turn determined eligibility for all participants. Eligible participants included women ages >18 years, with documented disease-associated mutations in *BRCA1* or *BRCA2*, who had never been diagnosed with cancer at any site before center ascertainment or were diagnosed with breast cancer only within 5 years or ovarian cancer within 3 years of their clinic ascertainment to minimize the potential for survival bias. As only a small proportion of our cohort (<5%) includes minority groups, we included only participants who were White, including Hispanic, non-Hispanic, and Jewish. Selection was made without respect to RRSO or exposures, and no exclusions were applied based on any risk factors, surgeries, or cancer occurrences. *BRCA1/2* mutation status of all subjects was confirmed by direct mutation testing and subjects provided full informed consent for this study under protocols approved by the human subjects

review boards at each institution. Some participants were simultaneously consented for both research and clinical BRCA1/2 testing, whereas others were consented separately for clinical testing and for research participation. Women with BRCA1/2 variants of unknown clinical significance were excluded. Mutations were included in the analysis if they were pathogenic according to generally recognized criteria, including (a) mutations generating a premature termination codon (except truncating variants in exon 27 of BRCA2) as a result of a nonsense substitution, a frameshift due to small deletion or insertion, aberrant splicing, or large genomic rearrangement; (b) mutations resulting in loss of expression due to deletion of promoter and transcription start site; (c) large in-frame deletions spanning one or more exons caused by aberrant splicing or large genomic rearrangement; and (d) missense mutations classified as pathogenic using the algorithms of Goldgar and colleagues (18) and Chenevix-Trench and colleagues (19).

Data were obtained on all eligible participants using medical records, telephone interviews, and/or self-administered questionnaires and included information on reproductive and exposure history, including hormone use and smoking. Vital status, cancer diagnoses, and prophylactic surgery data were verified by review of medical records, operative notes, and/or pathology reports. Follow-up from the time of ascertainment was conducted within each center on a periodic basis. This follow-up was active but did not occur at equivalent intervals for all individuals in this multicenter observational study. Follow-up was random with respect to RRSO use, cancer occurrence, or death. In addition, because this was not a randomized clinical trial of RRSO, both the case and the control groups underwent a variety of cancer surveillance programs that were not controlled for in this study. However, all occurrences of RRSO were verified by medical records if available, and these were carefully distinguished from ovarian surgeries that may have occurred in conjunction with an ovarian cancer diagnosis. Any oophorectomy that was done for therapeutic/symptomatic reasons and determined to be ovarian cancer or was done within 1 year of an ovarian cancer diagnosis was not considered "prophylactic" but was determined to be related to ovarian cancer diagnosis and treatment.

**Genotype and haplotype data.** We chose single nucleotide polymorphisms (SNP) to tag haplotypes, as well as putative functional SNPs, in nine genes that interact with BRCA1 and/or BRCA2 (Fig. 1; Supplementary Tables S1 and S2): ATM, BRIP1 (BRIP1/FANCJ), BARD1, CTIP, MRE11, NBS1, RAD50, RAD51, and TOPBP1. Haplotype tag SNPs at each locus were selected using Tagging Wizard in SNPBrowser from publicly available HapMap data (release 16) if they had haplotype R<sup>2</sup> > 95% and minor allele frequencies of ≥5%. We also excluded SNPs that had not been validated for the TaqMan platform based on SNPBrowser data. We identified 127 haplotype tag SNPs that met these criteria. In addition, we identified 51 putative functional nonsynonymous SNPs that had been reported in HapMap

(release 16) or in the literature. No minor allele frequency restrictions were placed on these nonsynonymous SNPs. To generate pools for genotype analysis, seven haplotype tag SNPs were excluded because the sequence surrounding the SNP was incompatible with the primers or probes used for SNPlex or would interfere with another SNP being queried. One nonsynonymous SNP was excluded because one or more additional SNPs was found to be in close proximity to the SNP of interest and therefore could not be interrogated by SNPlex. After completion of laboratory analysis, SNPs were also excluded from subsequent consideration if they had assay failure rates >20% and minor allele frequency <1% or if they showed statistically significant deviations from Hardy-Weinberg equilibrium in unrelated noncancer Caucasian women were excluded with  $P < 0.005$  (Supplementary Table S1). At the conclusion of this process, 56 SNPs in BRCA1 carriers and 51 SNPs in BRCA2 carriers were included in the analyses presented here.

Genomic DNA samples were extracted from peripheral blood at each center and shipped to the Penn data coordinating center. Samples were genotyped using the SNPlex System genotyping kit (Applied Biosystems) using the standard protocol. Briefly, 40 ng DNA extracted from peripheral blood was fragmented using heat. Samples then underwent the Oligo Ligation Assay where allele-specific oligos, each containing a unique identifying code (ZIP code), were ligated to locus-specific oligos to generate single-stranded products. The products were cleaned using exonuclease to remove all unligated products. Cleaned Oligo Ligation Assay product then underwent PCR. PCR products were then bound to a plate coated with streptavidin and underwent several washes where reporters unique to each genotype (ZIP chutes) were hybridized to the products at the ZIP code. ZIP chutes were then eluted and run on a 3130xl Capillary Sequencer. Genotypes were read using GeneMapper 4.0.

**Statistical analysis.** Analysis was undertaken using the weighted cohort approach of Antoniou and colleagues (20). The weighted approach was implemented to address the issue that study carriers may be ascertained from multiple-case families selected for genetic testing. In addition, because the presence of disease may influence the likelihood of testing, affected carriers may be overrepresented in our cohort. The approach provides reasonably unbiased risk estimates (20). Five-year interval weights were applied based on published ovarian cancer relative risks for BRCA1 and BRCA2 mutation carriers separately (21).

The primary event of interest was diagnosis of ovarian cancer. Observations were censored at the earliest of the following events: RRSO, death, or having reached the end of follow-up without an ovarian cancer or other censoring event. Time to event was computed from age at birth to age at first ovarian cancer diagnosis or age at censoring. Analyses were adjusted for ethnicity (Jewish, Hispanic, or non-Hispanic non-Jewish White) and birth cohort (decade of birth). Breast cancer diagnosis was included in the

**Table 1.** Descriptive characteristics of study participants

Variable	BRCA1			BRCA2		
	Ovarian cancer (n = 179)	No ovarian cancer (n = 1,396)	P	Ovarian cancer (n = 47)	No ovarian cancer (n = 809)	P
Mean (range) birth year	1946 (1,921-1,967)	1958 (1,899-1,986)	<0.001	1941 (1,917-1,960)	1955 (1,911-1,985)	<0.001
Jewish (%)	36 (20)	223 (16)	0.164	11 (23)	108 (13)	0.078
Ascertainment year (range)	1998 (1,978-2,006)	1998 (1,973-2,006)	0.724	2000 (1,978-2,005)	1999 (1,978-2,006)	0.481
RRSO (%)	8 (4)	655 (47)	<0.001	1 (2)	318 (39)	<0.001
Alive at end of follow-up (%)	112 (63)	1,302 (94)	<0.001	33 (70)	749 (93)	<0.001
Also have a BRCA2 deleterious mutation (%)	1 (1)	2 (0.1)	—	NA	NA	NA
Also have a BRCA1 deleterious mutation (%)	NA	NA	NA	1 (2)	2 (0.2)	—
Breast cancer diagnosis (%)	51 (28)	648 (46)	<0.001	18 (38)	418 (52)	0.098
Mean (range) age at breast cancer diagnosis	45.6 (23.6-73.2)	40.0 (22.2-74.0)	<0.001	52.3 (29.5-70.2)	44.1 (23.6-77.3)	<0.001
Mean (range) age at ovarian/primary peritoneal cancer diagnosis	50.5 (23.6-81.0)	NA	—	57.6 (41.5-77.7)	NA	—
Age at censoring (range)	50.5 (23.6-81.0)	42.4 (15-100)	0.00	57.6 (41.5-77.7)	47.2(19.7-94.0)	0.00

**Table 2.** Haplotype analysis: *BRCA1*

Locus	SNP								Frequency	Haplotype	HR (95% CI)	<i>P</i> ( <i>P</i> <sub>corr</sub> )
<i>ATM</i>	7	5	1	2	6							0.038 (0.058)
	T	T	G	A	C				0.425	Reference	1.00	
	T	C	G	G	T				0.003	Rare	0.01 (0.00-0.35)	
	A	C	G	G	T				0.411	A	0.93 (0.70-1.24)	
	A	T	G	G	C				0.011	B	1.24 (0.49-3.10)	
<i>BRIP1</i>	T	T	A	A	C				0.149	C	0.90 (0.62-1.31)	0.003 (0.012)
	4	21	33	6	15	2	26	7				
	T	A	C	C	A	G	G	C	0.206	Reference	1.00	
	C	G	C	C	G	G	G	T	0.008	Rare	0.60 (0.26-1.35)	
	T	A	C	T	A	A	G	T	0.003	Rare		
	T	G	C	T	G	G	A	T	0.006	Rare		
	C	G	C	T	G	G	G	T	0.003	Rare		
	C	G	C	T	A	A	A	T	0.005	Rare		
	T	G	C	T	G	G	G	C	0.005	Rare		
	C	G	C	C	A	A	G	T	0.010	A	0.44 (0.06-3.15)	
	C	G	C	C	A	G	A	T	0.061	B	0.71 (0.34-1.49)	
	C	G	C	C	A	G	G	C	0.097	C	0.97 (0.54-1.75)	
	C	G	C	C	A	G	G	T	0.167	D	0.80 (0.47-1.36)	
	C	G	C	T	A	A	G	C	0.018	F	1.94 (0.45-8.33)	
	C	G	C	T	A	A	G	T	0.116	F	0.77 (0.42-1.43)	
	C	G	C	T	A	G	G	C	0.033	G	0.78 (0.39-1.57)	
	C	G	C	T	A	G	G	T	0.030	H	1.07 (0.44-2.65)	
	C	G	G	T	A	A	G	T	0.034	I	0.73 (0.42-1.27)	
	T	A	C	C	A	G	A	T	0.052	J	0.62 (0.30-1.26)	
	T	A	C	C	A	G	G	T	0.059	K	0.95 (0.45-2.04)	
T	G	C	T	G	G	G	T	0.061	L	0.86 (0.51-1.47)		
<i>BARD1</i>	7	18	12	15	6	17	2	8				0.042 (0.058)
	T	C	T	G	C	A	T	T	0.268	Reference	1.00	
	T	T	A	G	C	G	T	T	0.008	Rare	0.88 (0.50-1.55)	
	C	T	T	A	C	G	T	C	0.009	Rare		
	T	C	A	G	C	A	C	T	0.009	Rare		
	T	C	A	G	C	A	T	T	0.006	Rare		
	T	T	T	A	C	G	T	C	0.008	Rare		
	C	T	A	A	C	A	T	C	0.038	A	0.95 (0.48-1.88)	
	C	T	A	G	C	A	T	T	0.035	B	1.01 (0.50-2.06)	
	T	C	T	G	C	A	C	C	0.019	C	1.27 (0.50-3.19)	
	T	T	A	A	T	A	T	T	0.048	D	0.83 (0.38-1.82)	
	T	T	A	G	C	A	C	T	0.056	E	1.40 (0.80-2.43)	
	T	T	A	G	C	A	T	T	0.152	F	1.10 (0.72-1.67)	
	T	T	A	G	C	G	C	C	0.204	G	0.93 (0.65-1.32)	
	T	T	T	A	T	A	T	T	0.062	H	1.16 (0.63-2.14)	
T	T	T	G	C	A	T	T	0.052	I	0.71 (0.37-1.35)		
<i>CTIP</i>	7	8	5	3	6	4	2				0.046 (0.059)	
	A	A	G	A	G	C	A		0.653	Reference		1.00
	A	A	G	G	A	C	A		0.006	Rare		0.92 (0.32-2.64)
	A	A	A	G	G	C	A		0.004	Rare		
	A	A	A	G	A	C	A		0.107	A		1.17 (0.79-1.71)
	G	C	A	A	A	C	A		0.018	B		1.42 (0.74-2.72)
	G	C	A	G	A	C	G		0.013	C		1.81 (0.79-4.15)
G	C	A	G	A	T	G		0.193	D	1.20 (0.93-1.55)		

(Continued on the following page)

Cox model as a censoring event. All analyses were undertaken in BRCA1 and BRCA2 mutation carriers separately. Finally, we present both the uncorrected *P* values and the *P* values corrected for multiple hypothesis testing (denoted *P*<sub>corr</sub>) to adjust the overall association between nine candidate genes in BRCA1 and BRCA2 mutation carriers separately for a total of 18 hypothesis tests. The Benjamini-Hochberg method was used to generate the *P* values

corrected for multiple testing (22). All survival analyses were conducted in SAS version 9.1 (SAS Institute).

To investigate haplotype associations, the EM algorithm (23, 24) was used to estimate haplotype frequencies as implemented in R version 2.1.1 subroutine haplo.em (25). To assess the association between haplotypes and survival outcome, we created a user-defined model matrix to estimate

**Table 2.** Haplotype analysis: *BRCA1* (Con't)

Locus	SNP						Frequency	Haplotype	HR (95% CI)	<i>P</i> ( <i>P</i> <sub>corr</sub> )	
<i>MRE11</i>	1	3	5	6	7	14	18			<0.0001 (0.007)	
	C	A	A	G	C	A	A	0.314	Reference		1.00
	T	A	A	G	C	A	A	0.004	Rare		0.47 (0.10-2.10)
	T	G	A	G	C	A	A	0.003	Rare		
	C	A	A	G	C	A	G	0.031	A		0.93 (0.43-2.00)
	C	A	G	A	T	G	G	0.049	B		0.63 (0.28-1.44)
	T	A	A	G	T	A	G	0.021	C		1.63 (0.71-3.73)
	T	A	G	A	T	G	G	0.187	D		0.92 (0.57-1.47)
	T	A	G	G	T	G	G	0.060	E		1.25 (0.73-2.15)
	T	G	A	G	T	A	A	0.015	F		1.09 (0.43-2.76)
	T	G	A	G	T	A	G	0.149	G		0.55 (0.34-0.91)
	T	G	G	A	T	G	G	0.038	H		1.00 (0.50-2.02)
	T	G	G	G	T	A	G	0.116	I		0.70 (0.40-1.23)
<i>NBS1</i>	2	4	7	8	11	12				0.008 (0.024)	
	C	G	C	T	G	G		0.492	Reference		1.00
	C	G	C	C	G	G		0.005	Rare		1.50 (0.50-4.47)
	C	A	C	T	G	G		0.132	A		0.93 (0.60-1.43)
	C	G	C	T	G	C		0.018	B		2.17 (0.98-4.81)
	C	G	T	T	A	G		0.026	C		1.33 (0.68-2.60)
	G	A	C	T	G	G		0.024	D		1.20 (0.52-2.76)
	G	G	C	C	G	C		0.071	E		1.90 (0.96-3.74)
	G	G	C	T	G	C		0.193	F		1.20 (0.82-1.75)
	G	G	C	T	G	G		0.031	G		1.06 (0.35-3.25)
<i>RAD50</i>	2	3	4	5						0.022 (0.044)	
	G	G	C	C				0.391	Reference		1.00
	G	G	T	C				0.008	Rare		1.04 (0.42-2.60)
	G	T	C	C				0.293	A		0.94 (0.67-1.32)
	G	T	T	C				0.069	B		0.74 (0.37-1.51)
	G	T	T	T				0.020	C		1.59 (0.70-3.63)
	T	T	C	C				0.084	D		0.87 (0.48-1.57)
	T	T	T	C				0.063	E		0.53 (0.27-1.04)
T	T	T	T				0.067	F	0.85 (0.42-1.71)		
<i>RAD51</i>	2	7	5	1	10					0.179 (0.205)	
	A	C	T	A	T			0.416	Reference		1.00
	C	C	T	A	T			0.006	Rare		1.63 (0.61-4.33)
	C	C	T	G	C			0.004	Rare		
	A	C	C	A	C			0.030	A		1.10 (0.48-2.47)
	A	C	C	A	T			0.044	B		0.96 (0.39-2.36)
	C	C	C	G	C			0.321	C		1.08 (0.79-1.48)
C	T	C	G	C			0.177	D	0.87 (0.60-1.27)		
<i>TOPBP1</i>	13	10	3	5	1	6				0.035 (0.058)	
	T	C	G	C	C	G		0.533	Reference		1.00
									Rare		3.68 (0.74-18.24)
	C	A	G	C	A	A		0.136	A		1.10 (0.76-1.60)
	C	A	G	C	C	G		0.029	B		1.06 (0.42-2.68)
	C	C	G	C	A	G		0.103	C		1.37 (0.89-2.10)
T	C	A	T	C	G		0.193	D	0.96 (0.68-1.35)		

haplotype associations. First, haplo.em was used to estimate haplotype frequencies under the null hypothesis of no association (in the pool of all data). This approach enumerated all possible haplotype pairs per subject along with the posterior probabilities of each haplotype pair, conditional on the genotype data. The posterior probabilities were then used to average the rows of the model matrix per subject and the resulting matrix was used in a Cox regression model. Global tests for association (to test the association between all haplotypes together with disease status) as well as haplotype-specific tests (to test the association between each haplotype and disease status) were conducted. Cox regression for haplotype analyses were conducted as implemented in SAS Genetics. Phase ambiguity was quantified by estimating the percentage of uncertainty in the imputed

diplotypes. The majority of haplotypes had a maximum posterior probability of >80%; hence, we felt comfortable proceeding with the haplotype association method outlined above rather than assigning the most likely haplotype pair to each subject.

## Results

**Sample set description.** We identified a cohort of 1,575 *BRCA1* and 856 *BRCA2* female mutation carriers. Of *BRCA1* mutation carriers, 179 (11%) had ovarian cancer and 1,396 were censored. Among *BRCA2* mutation carriers, 47 (5.5%) had ovarian cancer and 809 were censored. The characteristics of the participants are

**Table 3.** Haplotype analysis: *BRCA2*

Locus	SNP								Frequency	Haplotype	HR (95% CI)	<i>P</i> ( <i>P</i> <sub>corr</sub> )
<i>ATM</i>	5	4	1	2	6							0.022 (0.044)
	T	T	G	A	C				0.435	Reference	1.00	
	T	T	G	G	C				0.009	Rare	10.93 (4.43-26.96)	
	C	T	G	G	T				0.415	A	1.09 (0.59-2.01)	
	T	C	A	A	C				0.032	B	1.09 (0.11-10.87)	
	T	T	A	A	C				0.107	C	1.28 (0.53-3.11)	
<i>BRIP1</i>	21	6	25	2	18	29	14	27				0.001 (0.011)
	G	C	A	G	G	G	C	G	0.305	Reference	1.00	
	G	C	A	A	T	A	T	A	0.009	Rare	0.49 (0.11-2.17)	
	A	C	A	G	G	G	C	A	0.004	Rare		
	G	T	G	A	T	A	C	G	0.009	Rare		
	G	C	A	G	G	A	T	A	0.005	Rare		
	A	T	G	A	T	A	T	A	0.005	Rare		
	G	T	A	G	T	G	C	G	0.004	Rare		
	A	C	A	G	T	A	T	A	0.009	Rare		
	A	C	A	G	G	G	C	G	0.273	A	1.17 (0.52-2.63)	
	G	C	A	G	G	G	C	A	0.020	B	6.59 (1.10-39.65)	
	G	C	A	G	T	A	T	A	0.014	C	0.49 (0.03-8.98)	
	G	T	A	G	G	G	C	G	0.023	D	6.83 (0.43-107.31)	
	G	T	A	G	T	A	T	A	0.015	E	2.22 (0.24-20.50)	
	G	T	A	G	T	G	C	A	0.037	F	2.78 (0.81-9.52)	
G	T	G	A	G	G	C	G	0.024	G	7.28 (1.67-31.82)		
G	T	G	A	T	A	T	A	0.159	H	1.16 (0.46-2.96)		
G	T	G	G	G	G	C	G	0.058	I	0.54 (0.08-3.59)		
<i>BARD1</i>	7	18	13	6	10	17	2	8				0.003 (0.012)
	T	C	T	C	G	A	T	T	0.271	Reference	1.00	
	T	T	C	C	G	A	T	C	0.004	Rare	4.62 (1.31-16.31)	
	C	T	C	C	G	A	T	T	0.008	Rare		
	T	T	T	C	G	G	C	T	0.004	Rare		
	T	T	T	C	G	G	T	T	0.008	Rare		
	C	T	C	C	G	A	T	C	0.043	A	2.16 (0.75-6.23)	
	C	T	T	C	A	A	T	T	0.019	B	0.78 (0.11-5.52)	
	C	T	T	C	G	G	T	C	0.011	C	0.68 (0.06-7.28)	
	T	C	C	C	G	A	C	T	0.020	D	0.68 (0.09-4.96)	
	T	C	T	C	G	A	C	C	0.019	E	2.92 (0.48-17.83)	
	T	T	C	C	G	A	C	T	0.049	F	2.33 (0.68-8.02)	
	T	T	C	C	G	A	T	T	0.017	G	0.55 (0.08-4.01)	
	T	T	T	C	G	A	T	T	0.170	H	1.88 (0.75-4.72)	
	T	T	T	C	G	G	C	C	0.192	I	1.41 (0.49-4.10)	
	T	T	T	C	G	G	T	C	0.018	J	0.30 (0.02-5.59)	
	T	T	T	T	A	A	T	T	0.062	K	0.59 (0.22-1.57)	
T	T	T	T	G	A	T	T	0.064	L	0.74 (0.16-3.42)		
<i>CTIP</i>	7	8	5	3	6	4	2				0.183 (0.205)	
	A	A	G	A	G	C	A		0.647	Reference		1.00
	A	A	G	G	A	C	A		0.007	Rare		2.82 (1.06-7.48)
	A	C	A	G	A	T	G		0.004	Rare		
	G	C	A	G	A	C	G		0.009	Rare		
	A	A	A	G	A	C	A		0.120	A		1.15 (0.45-2.92)
	G	C	A	A	A	C	A		0.015	B		2.18 (0.30-15.98)
G	C	A	G	A	T	G		0.191	C	1.42 (0.77-2.60)		

(Continued on the following page)

described in Table 1. Ovarian cancer cases were significantly more likely to be members of an older birth cohort than censored controls for both *BRCA1* and *BRCA2* ( $P < 0.0001$ ). Data for oral contraceptive use were available for 1,391 (88%) of participants with a *BRCA1* mutation. Of those who were diagnosed with an ovarian cancer, 98 (62%) had ever used oral contraceptive and

1,021 (83%) of nonovarian cancer cases ever used oral contraceptive. Significantly more women without an ovarian cancer diagnosis used oral contraceptive ( $P < 0.001$ ). For *BRCA2* carriers, data for oral contraceptive use were available for 777 (91%). Of those who were diagnosed with an ovarian cancer, 25 (57%) ever used oral contraceptive and 592 (81%) of nonovarian cancer cases

**Table 3.** Haplotype analysis: *BRCA2* (Con't)

Locus	SNP						Frequency	Haplotype	HR (95% CI)	<i>P</i> ( <i>P</i> <sub>corr</sub> )	
<i>MRE11</i>	1	3	6	8	13	14	19			0.003 (0.012)	
	C	A	G	T	C	A	G	0.318	Reference		1.00
	T	G	G	C	T	A	G	0.008	Rare		5.13 (1.24-21.24)
	T	A	G	T	C	A	A	0.004	Rare		
	C	A	A	C	T	G	A	0.047	A		2.09 (0.40-10.76)
	C	A	G	T	C	A	A	0.020	B		0.00 (0.00-0.30)
	T	A	A	C	T	G	A	0.184	C		2.33 (1.39-3.91)
	T	A	A	C	T	G	G	0.010	D		0.00 (0.00-23.37)
	T	A	G	C	T	G	A	0.047	E		1.09 (0.23-5.23)
	T	A	G	C	T	G	G	0.012	F		1.28 (0.18-9.06)
	T	A	G	T	C	A	G	0.029	G		1.49 (0.29-7.58)
	T	G	A	C	T	G	A	0.023	H		2.19 (0.18-26.88)
	T	G	G	C	C	A	G	0.012	I		1.47 (0.14-15.63)
	T	G	G	C	T	A	A	0.077	J		0.67 (0.16-2.92)
T	G	G	T	C	A	A	0.015	K	0.00 (0.00-0.0)		
T	G	G	T	C	A	G	0.171	L	1.12 (0.27-4.69)		
<i>NBS1</i>	1	3	4							0.040 (0.058)	
	A	A	G					0.480	Reference		1.00
	G	A	A					0.008	Rare		3.15 (0.66-15.04)
	A	C	G					0.005	Rare		
	A	A	A					0.118	A		0.73 (0.18-3.05)
	G	A	G					0.011	B		0.00 (0.00)
	G	C	A					0.07	C		1.51 (0.10-22.46)
	G	C	G					0.361	D		0.93 (0.49-1.75)
<i>RAD50</i>	2	3	4	5						0.342 (0.342)	
	G	G	C	C				0.401	Reference		1.00
	G	G	T	C				0.005	Rare		0.43 (0.03-5.98)
	G	T	C	C				0.300	A		1.19 (0.54-2.59)
	G	T	T	C				0.068	B		1.38 (0.31-6.06)
	G	T	T	T				0.018	C		0.83 (0.16-4.42)
	T	T	C	C				0.080	D		0.87 (0.23-3.35)
	T	T	T	C				0.048	E		0.88 (0.10-7.76)
T	T	T	T				0.075	F	0.69 (0.19-2.48)		
<i>RAD51</i>	8	2	1	10						0.010 (0.026)	
	C	A	A	T				0.445	Reference		1.00
	T	C	A	T				0.006	Rare		4.49 (0.54-37.69)
	C	A	A	C				0.012	A		1.16 (0.11-12.69)
	C	C	G	C				0.418	B		1.45 (0.66-3.17)
T	C	G	C				0.119	C	3.53 (1.77-7.05)		
<i>TOPBP1</i>	10	8	4	3	5	6				0.241 (0.255)	
	C	G	G	G	C	G		0.334	Reference		1.00
									Rare		0.03 (0.00-1.44)
	A	A	A	G	C	A		0.122	A		0.60 (0.24-1.48)
	A	G	A	G	C	G		0.021	B		2.77 (0.59-12.96)
	C	G	A	A	T	G		0.213	C		0.99 (0.50-1.98)
C	G	A	G	C	G		0.308	D	0.83 (0.39-1.73)		

ever used oral contraceptive. The difference in oral contraceptive use between the two groups was significant ( $P < 0.001$ ) in *BRCA2* carriers. Censored controls were more likely to have undergone RRSO, to have had a breast cancer diagnosis, and to be alive at the end of follow-up.

**BRCA1.** In *BRCA1* mutation carriers, no statistically significant associations were observed between haplotypes (Table 2) at *ATM*, *BARD1*, *CTIP*, *RAD51*, or *TOPBP1*. At *ATM*, a significant relationship among rare haplotypes was observed, but no overall significance across all haplotypes was observed.

We observed a significant multiple testing corrected global  $P$  ( $P_{\text{corr}}$ ) of 0.012 for haplotypes at *BRIP1*, but no individual

haplotype was significantly associated with risk (Table 2). It is possible that the combination of multiple haplotypes may be associated with risk (because several haplotype associations are in the same direction), but we did not attempt to combine haplotypes based on observed hazard ratio (HR) estimates and did not have any a priori justification for combining haplotypes. Therefore, we cannot conclude with a high degree of confidence that there is a relationship between *BRIP1* haplotypes and ovarian cancer risk in *BRCA1* mutation carriers.

At *MRE11*, we observed a significant association of haplotypes ( $P_{\text{corr}} = 0.007$ ). Haplotype G, with a frequency of 14.9% and containing the variant G allele at SNP3, was inversely and significantly

associated with risk [HR, 0.55; 95% confidence interval (95% CI), 0.34-0.91].

At *NBS1*, we observed a significant association of haplotypes ( $P_{\text{corr}} = 0.024$ ). Although some haplotypes containing the individually significant SNPs had HR associations in the same direction as these individual SNP associations, no single haplotype was significantly associated with risk.

At *RAD50*, we observed a significant global association of haplotypes ( $P_{\text{corr}} = 0.044$ ), but no individual haplotype was significantly associated with risk. These results do not support the hypothesis that a relationship between *RAD50* and ovarian cancer risk in *BRCA1* mutation carriers exists.

Whereas the primary analysis undertaken here involved haplotype-based associations, associations involving single SNPs that comprised these haplotypes are shown in Supplementary Table S3.

**BRCA2.** In *BRCA2* mutation carriers, no haplotype associations were observed for *CTIP*, *NBS1*, *RAD50*, or *TOPBP1* (Table 3). In *ATM*, we observed a significant association with haplotypes ( $P_{\text{corr}} = 0.044$ ; Table 3). The "rare" TTGGC haplotype at *ATM* (frequency = 0.9%; Table 3), which represents a difference in SNPs 2 (rs664982) and 6 (rs664143), compared with the reference haplotype, was significantly associated with risk (HR, 10.93; 95% CI, 4.43-26.96).

At *BRIP1*, we observed a significant association of haplotypes ( $P_{\text{corr}} = 0.011$ ; Table 3). The B and G haplotypes (frequencies of 2.0% and 2.4%, respectively; Table 3) were significantly associated with risk (HR, 6.59; 95% CI, 1.10-39.65 and HR, 7.28; 95% CI, 1.67-31.82, respectively). Haplotype B differs from the reference haplotype in SNP 27 (rs4988340) only. Haplotype G differs from the reference haplotype in SNPs 2 (rs12453935), 6 (rs169456280), and 25 (rs10515211).

At *BARD1*, we observed a significant association of haplotypes ( $P_{\text{corr}} = 0.012$ ; Table 3). The "rare" haplotypes at *BARD1* were significantly associated with risk (HR, 4.62; 95% CI, 1.31-16.31;  $P_{\text{trend}} = 0.012$ ; Table 3). One of the rare haplotypes (TTTCGGCT) differs from the reference haplotype by only SNP 2 (rs6712055). These results support the hypothesis that a relationship of rare *BARD1* haplotypes and ovarian cancer risk in *BRCA2* mutation carriers exists.

At *MRE11*, we observed a significant association of haplotypes ( $P_{\text{corr}} = 0.012$ ; Table 3). The "rare" haplotypes at *MRE11* were significantly associated with risk (HR, 5.13; 95% CI, 1.24-21.24). In addition, the C haplotype in *MRE11* with a frequency of 18.4% was significantly associated with risk (HR, 2.33; 95% CI, 1.39-3.91). Haplotype C differed for all SNPs, except SNP 3 (rs6483327), compared with the reference haplotype.

Finally, at *RAD51*, we observed a significant association of haplotypes ( $P_{\text{corr}} = 0.026$ ). Haplotype C at *RAD51* (frequency = 11.9%; Table 3) was significantly associated with risk (HR, 3.53; 95% CI, 1.77-7.05). Haplotype C differs from the reference haplotype at all individual SNPs. Consistent with what has been observed in *BRCA2*-associated breast cancer (26-28), a relationship of rare *RAD51* haplotypes and ovarian cancer risk in *BRCA2* mutation carriers may exist. Given that rs1801320 (135G>C) in *RAD51* has been reported previously as a modifier of breast cancer risk in *BRCA2* mutation carriers, we also evaluated this SNP as a candidate modifier of ovarian cancer risk. This SNP was not included as a haplotype tag SNPs for haplotype analysis. The HR (95% CI) associated with carriage of the C allele, previously associated with breast cancer risk modification, was 0.40 (0.05-3.40). However, the

variant was relatively rare, occurring in only 5% of 192 *BRCA2* mutation carriers, so that the power to detect associations with this sample size was small. This SNP was in strong linkage disequilibrium ( $D' > 0.95$ ) with other SNPs at this locus for which haplotype associations were observed, including rs11070291, rs2619680, rs957603, and rs2619681. Therefore, it is likely that we have detected the same association in ovarian cancer as has been reported previously at this locus for breast cancer.

Whereas the primary analysis undertaken here involved haplotype-based associations, associations involving single SNPs that comprised these haplotypes are shown in Supplementary Table S4.

**Nonsynonymous SNP analysis.** In addition to haplotype-based analyses, we evaluated 28 candidate nonsynonymous SNPs in the nine loci studied here. No polymorphic variation was detected in several SNPs reported in HapMap data and genotyped here, including K312N, R658C, N470S, Q564H, and N295S in *BARD1*; Q540L, C832Y, L195P, F531V, P47A, or V193I in *BRIP1*; D488Y in *CTIP*; V31A or M670V in *MRE11*; T497A or P672L in *NBS1*; T191I in *RAD50*; L109V or K313Q (K216Q) in *RAD51*; or H1140P in *TOPBP1*. Among SNPs that showed polymorphic variation, we found no association with ovarian cancer risk for D1853N (D505N) or F858L in *ATM*; C557S or R378S in *BARD1*; or K370Q, S730L, or N955S in *TOPBP1*. The only SNP for which a significant trend was observed was Q185E (rs1805794) in *NBS1* for *BRCA1* mutation carriers (HR, 1.40; 95% CI, 0.92-2.14 for the QE genotype and HR, 1.91; 95% CI, 1.02-3.56 for the EE genotype relative to the QQ genotype;  $P_{\text{linear trend}} = 0.026$ ).

## Discussion

We identified several biologically plausible associations in genes that are involved in DNA damage response, interact with *BRCA1* and/or *BRCA2* (Fig. 1), and may act in concert with a mutated *BRCA1/2* to modify cancer risk. Rare haplotypes at *ATM* were associated with ovarian cancer risk in *BRCA2* mutation carriers. In response to the formation of double-strand breaks, ATM kinase phosphorylates the BRCA2 protein, leading to activation of a S-phase checkpoint (29, 30). Thus, the regulation of BRCA2 by ATM suggests a plausible mechanism by which rare *ATM* haplotypes may influence *BRCA2*-associated ovarian cancer risk.

*BARD1* and *BRIP1* associate with *BRCA1* as cells progress through the S phase of the cell cycle (31, 32). Although *BRIP1* probably does not associate with *BRCA2*, *BARD1* is a stoichiometric partner of *BRCA1* and remains associated with *BRCA1* throughout the cell cycle. *BARD1* also interacts with *BRCA2* in a substoichiometric manner. This observation provides a plausible explanation for the observation in our data that *BRIP1* is associated with ovarian cancer in *BRCA1* and *BRCA2* mutation carriers and that *BARD1* is associated with ovarian cancer in *BRCA2* mutation carriers. *BRIP1* is a DNA helicase that interacts with the COOH-terminal BRCT repeat of *BRCA1*. *BRIP1* is associated with GM1/2 checkpoint and CHK1 activation as well as regulation of entry into the S phase of the cell cycle and maintenance of genomic stability (32). Thus, if the complex involving *BRCA1*, *BRCA2*, *BARD1*, and *BRIP1* is involved in tumor suppression, mutations in the genes that encode these proteins should be associated with altered cancer risk. Our data indicating that variants in *BRIP1* and *BARD1* modify *BRCA1/2*-associated ovarian cancer support this hypothesis.

We also identified associations of *MRE11*, *RAD50*, and *NBS1* (MRN) in *BRCA1* mutation carriers and *MRE11* in *BRCA2* mutations carriers. The MRN proteins interact directly with one

another and interact with BRCA1 in a DNA damage-inducible manner (11). NBS1 protein and the activated form of BRCA2 colocalize in subnuclear foci in response to mitomycin C-induced DNA damage and interact in the cellular response to DNA crosslink formation (33). The MRN complex therefore interacts with BRCA2 and the Fanconi anemia pathway in S-phase checkpoint response (29). These observations are consistent with our findings that *MRE11* and *NBS1* are associated with ovarian cancer risk in *BRCA1* mutation carriers, and *MRE11* was associated with ovarian cancer in *BRCA2* mutation carriers. Polymorphisms in *NBS1* have been studied for an association with early-onset breast cancer with varying results (34–41), but studies of *MRE11* and *RAD50* as ovarian cancer susceptibility in *BRCA1/2* mutation carriers have not been published.

We also report that *RAD51* is associated with altered ovarian cancer risk in *BRCA2* mutation carriers. This result is consistent with previous validated studies of *RAD51* as a modifier of BRCA2-associated breast cancer risk (26, 42, 43). *RAD51* interacts with BRCA2 (Fig. 1; ref. 44). Levy-Lahad and colleagues (43) and Wang and colleagues (26) reported that a 135G→C substitution in the 5' untranslated region of the *RAD51* gene was associated with increased breast cancer risk in *BRCA2* mutation carriers. A large consortium study has validated the relationship of *RAD51* genotypes with breast cancer risk (42). Whereas studies did not detect an association of this variant in *BRCA1* mutation carriers, one study has reported an inverse association of this SNP with breast cancer risk (45). In addition, the study of Antoniou and colleagues (28) reported a potential functional link between the 135G→C variant and *RAD51* protein expression. Our findings, along with the previous validation of *RAD51* in *BRCA2*-associated breast cancer, represent biologically plausible associations that have been validated in multiple studies as a *BRCA2*-associated cancer risk modifier.

To date, few ovarian cancer risk modifiers have been identified. Use of oral contraceptives may reduce ovarian cancer risk in *BRCA1/2* mutation carriers (46). Candidate modifier genes include the PROGINs progesterone receptor allele and oral contraceptive use (8) and rare *HRAS1* alleles (7). However, neither of these associations has been validated. Our study expands the possible ovarian cancer risk modifier genes by reporting that several genes that encode *BRCA1/2*-interacting proteins explain interindividual variability in ovarian cancer risk in *BRCA1/2* mutation carriers.

Strengths of this study include a large cohort of *BRCA1/2* mutation carriers and a focus on biologically plausible associations involving genes that encode proteins that interact with *BRCA1/2*. Despite the relatively large sample size used here, power was still low to detect some small associations involving rare haplotypes. Several significant results may be driven by "rare" haplotypes. This is of concern because the SNP selection strategy was not designed to fully capture rare haplotypes, and combined with the low power of the study to evaluate rare events, these associations may represent false-positive findings. Finally, we did not have the power to study interactions or higher-order associations among genes or with exposures. Therefore, additional large-scale studies should be undertaken to confirm the results reported here.

Despite the biological plausibility of our results, we cannot make strong inferences about the mechanism of these associations. The SNPs selected here are, for the most part, not functionally relevant, and we do not have information about the causative alleles that may be in linkage disequilibrium with the haplotype or SNP associations identified here. Therefore, the inferences made here

allow us to test the hypothesis that genomic variation in our candidate genes represents potential modifiers of ovarian cancer risk in *BRCA1/2* mutation carriers. Additional studies are required to evaluate the biological mechanism of these associations.

Approximately 10% of ovarian cancer can be explained by *BRCA1/2* mutations, and the majority of breast and ovarian cancer families are attributable to *BRCA1/2*. Over 100,000 patients are currently tested for *BRCA1/2* mutations each year. Thus, a substantial proportion of women at risk for ovarian cancer because of a *BRCA1/2* mutation could benefit from improved knowledge of factors that influence risk. Because there are no effective screening strategies and ovarian cancer prevention revolves around the use of RRSO, reliable models of individualized ovarian cancer risk assessment must be developed. Our limited understanding of factors that modify these risks in *BRCA1/2* mutation carriers hampers our clinical decision-making ability, including decisions about the appropriate type and timing of preventive interventions. The long-term goal of this research is to inform ovarian cancer risk prediction estimates that can be used to focus the timing and method of ovarian cancer risk reduction. This type of information is currently unavailable to the field of hereditary ovarian cancer. Although our current results are insufficient to guide clinical practice, they may represent a first step in helping to improve our understanding of ovarian cancer risk and prevention in *BRCA1/2* mutation carriers. In addition, this research could motivate additional studies that can elucidate mechanisms of *BRCA1/2*-associated ovarian carcinogenesis.

## Disclosure of Potential Conflicts of Interest

The authors disclosed no potential conflicts of interest.

## Acknowledgments

Received 2/19/09; revised 4/27/09; accepted 4/27/09; published OnlineFirst 7/7/09.

**Grant support:** Revenue from Nebraska cigarette taxes awarded to Creighton University by the Nebraska Department of Health and Human Services. NIH grants 5U01 CA86389 (H.T. Lynch), R01-CA083855 and R01-CA74415 (S.L. Neuhausen), P30-CA51008-19 (C. Isaacs), and R01-CA102776 and R01-CA083855 (T.R. Rebbeck) and NIH General Clinical Research Center grant M01 RR00043 awarded to the City of Hope National Medical Center (J.N. Weitzel). The Kathleen Cunningham Consortium for Research into Familial Breast Cancer is supported by grants from the National Breast Cancer Foundation, the National Health and Medical Research Council, the Queensland Cancer Fund, the Cancer Councils of New South Wales, Victoria, Tasmania, and South Australia, and the Cancer Foundation of Western Australia.

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We thank Heather Thorne, Eveline Niedermayr, all the Kathleen Cunningham Consortium for Research into Familial Breast Cancer research nurses and staff, the heads and staff of the Family Cancer Clinics, and the Clinical Follow-up Study (funded by National Health and Medical Research Council grants 145684, 288704, and 454508) for contributions to this resource and the many families who contributed to the Kathleen Cunningham Consortium for Research into Familial Breast Cancer.

The MAGIC Consortium includes the following centers and individuals: Baylor-Charles A. Sammons Cancer Center (Joanne L. Blum, M.D., Ph.D.; Becky Althaus, R.N., C.G.C.; Gaby Ethington), Baylor College of Medicine (Claire Noll; Sharon Plon, M.D., Ph.D.), Beth Israel Deaconess Medical Center (Nadine Tung, M.D.), City of Hope National Medical Center (Sharon Sand; Jeffrey N. Weitzel, M.D.), Creighton University (Carrie Snyder, B.A.; Henry T. Lynch, M.D.; Patrice Watson, Ph.D.), Dana-Farber Cancer Institute (Kathryn Stoeckert; Judy E. Garber, M.D., M.P.H.), Duke University (Sydney Crankshaw; Joellen Schildkraut, Ph.D.), Evanston Northwestern Healthcare Center for Medical Genetics (Suzanne M. O'Neill, Ph.D.; Christina Selkirk; Wendy S. Rubinstein, M.D., Ph.D.), Fox Chase Cancer Center (Mary B. Daly, M.D., Ph.D.; Andrew Godwin, Ph.D.), Queensland Institute of Medical Research (Georgia Chenevix-Trench), Georgetown University (Claudine Isaacs, M.D.), Jonsson Comprehensive Cancer Center at the University of California-Los Angeles (Joyce Seldon; Patricia A. Ganz, M.D.), Mayo Clinic College of Medicine (Linda Wadum; Fergus Couch, Ph.D.), University of Chicago (Shelly Cummings; Olufunmilayo Olopade, M.D.), University of California-Irvine (Susan L. Neuhausen, Ph.D.; Linda Steele), University of Pennsylvania Health System (Susan Domchek, M.D.; Katherine Nathanson, M.D.; Tara Friebe, M.P.H.; Timothy Rebbeck, Ph.D.), University of Texas Southwestern (Gail Tomlinson, M.D.), University of Vienna (Christian Singer, M.D.), and Women's College Hospital (Steven A. Narod, M.D.).

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