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Common variants of the *BRCA1* wild-type allele modify the risk of breast cancer in *BRCA1* mutation carriers

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INTRODUCTION

Germline mutations in *BRCA1* drastically increase breast cancer risk. They mainly consist of truncating mutations leading to loss of function of the mutant allele. In a meta-analysis of families with *BRCA1* mutations detected through population-based studies, the risk of breast cancer by the age of 70 among *BRCA1* mutation carriers was estimated to be 65% (1,2). This is lower than estimates based on families with multiple affected individuals (3). Furthermore, factors such as the age at diagnosis and the type of cancer in the index patient that led to the family ascertainment have been associated with differences in breast cancer risk among *BRCA1* mutation carriers (1,2,4,5). The extent of breast cancer risk variability among mutation carriers in terms of a polygenic-modifying variance was estimated using segregation-analysis models (2,6). These observations led to the hypothesis that breast cancer risk among mutation carriers is modified by other genetic or environmental factors. In order to facilitate the analysis of genetic modifiers of risk for *BRCA1* and *BRCA2* mutation carriers across several studies, CIMBA (Consortium of Investigators of Modifiers of *BRCA1/2*) was established in 2005 (7), and since then multiple loci that modify risk among *BRCA1/2* mutation carriers have been identified by this consortium (8–17).

The wild-type copy of *BRCA1* is an interesting candidate for cancer risk modification given that the activity of the protein produced by the intact allele may influence cancer penetrance in individuals who have one inactivated *BRCA1* copy, as it is the case for *BRCA1* mutation carriers. Indeed, the BRCA1 protein plays a central role in DNA repair: it is not only essential for the repair of double-strand breaks by homologous recombination, but it is also required to signal the presence of these severe lesions to the cell (18). Yet, both environmental factors and normal biological activities constantly damage DNA through the course of an individual's lifetime. When normal repair processes fail and apoptosis does not occur, irreparable DNA damage in oncogenes and tumor suppressor genes may occur, ultimately leading to unregulated cell division and in turn to the formation of a tumor. Evidence that the amount and/or quality of the BRCA1 protein produced in cells might be important for keeping DNA integrity comes from several studies that have investigated cellular response to DNA damage in *BRCA1* mutation carriers. High frequencies of micronuclei induction and chromosomal aberrations after exposure to mutagens have been reported (19–24), although not consistently (25–27). Furthermore, the observation that mRNA profiles are altered in normal breast epithelial cells heterozygous for mutations in *BRCA1*, including those of critical genes involved in *BRCA* signaling pathways, suggests that even a small alteration in the levels of BRCA1 may result in differential gene expression. It also indicates that *BRCA1* haploinsufficiency is likely to be a driving mechanism leading to tumorigenesis in carriers (28) and, in turn, supports the hypothesis that individual *BRCA1* variations may affect cancer risks in this population.

BRCA1 single nucleotide polymorphisms (SNPs) could exert their effect through two non-exclusive ways: missense polymorphisms could slightly modify BRCA1 protein function or stability, or SNPs could alter *BRCA1* expression by acting on transcription, splicing or translation. It is not known as yet whether the few reported frequent *BRCA1* missense polymorphisms alter BRCA1 function or stability. This is certainly not surprising given the technical challenges presented by the precise assessment of subtle changes in protein efficiency or stability, even more so in the case of BRCA1 because of its many described functions, large size and the poor quality of the antibodies directed against this protein. On the other hand, it is technically straightforward to accurately measure transcript levels in order to monitor gene expression and numerous such studies on *BRCA1* have been published. In this regard, allelic imbalance of *BRCA1* expression has been repeatedly reported, fueling the hypothesis that SNPs could influence *BRCA1* transcription efficiency even if the mechanism(s) leading to the observed allelic imbalance is for the most part unknown (29–31).

Conflicting results concerning the associations between polymorphisms in *BRCA1* and breast cancer risk in the general population have been reported (29,31–35). While three studies on Caucasian populations could not demonstrate any association (32,34,35), a more recent study of *BRCA1* promoter polymorphisms identified four variants altering promoter activity which could affect susceptibility to breast cancer in the Chinese population (33). Furthermore, *BRCA1* allelic imbalance has been shown to be associated with enhanced susceptibility to breast and/or ovarian cancer (29,31).

In 2003, we tested the hypothesis that polymorphisms in the wild-type copy of the *BRCA1* gene could modify the risk of breast cancer among women with *BRCA1* mutations, but the limited number of *BRCA1* carriers that we were then able to genotype, and the less advanced state of knowledge of the patterns of human common genetic variation, prevented us from providing a convincing result (36).

With the establishment of CIMBA, we readdressed this question by determining common *BRCA1* haplotypes and studying their effect in 9874 women with germline *BRCA1* mutations. We also assessed the functional significance of the major *BRCA1* promoter haplotypes.

RESULTS

In the present study, we genotyped SNPs tagging the *BRCA1* region in *BRCA1* mutation carriers and whenever possible reconstructed haplotypes in order to test the hypothesis that the wild-type copy of *BRCA1*, in these carriers, might modify cancer risk. As a second step, we studied the possibility that such an effect could be exerted through differential transcription efficiency.

Analysis of the association between wild-type *BRCA1* genotypes and breast cancer risk in *BRCA1* mutation carriers

While the *BRCA1* gene is large (>80 kb), only one block of linkage disequilibrium (LD) exists across the entire locus (Fig. 1), resulting in the occurrence of two major haplogroups tagged by the rs16942 SNP (see Materials and Methods). This tagging SNP was genotyped in 9874 *BRCA1* mutation carriers available for these analyses when combining samples from 32 participating CIMBA centers (Table 1). Allele frequencies of rs16942 in our analysis were, on average, similar to those observed for other white populations (minor allele frequency ~33%).

The results of association tests between rs16942 of the wild-type *BRCA1* allele and breast cancer risk among *BRCA1* mutation carriers are shown in Table 2. The analysis restricted to women homozygous for rs16942, in whom the phase of rs16942 with respect to their mutation was unambiguous ($n = 5652$), showed an inverse association between the C (minor) allele and breast cancer risk [hazard ratio (HR) 0.85, 95% confidence interval (CI) 0.74–0.96 $P = 0.01$]. Using familial information, we were able to phase an additional 1396 subjects ($n = 7048$), further refining the risk estimate to 0.86, 95% CI 0.77–0.95, $P = 0.003$. In these analyses, no heterogeneity was observed among centers ($P = 0.94$) (Fig. 2). HRs in analyses that excluded breast cancer cases diagnosed more than 1 year prior to the interview were similar to the overall results (HR 0.84, 95% CI 0.75–0.93). No differences in breast cancer risk between *BRCA1* carriers of Class 1 (loss of function) or Class 2 (likely to generate potentially stable mutant protein) mutations were observed (Table 2).

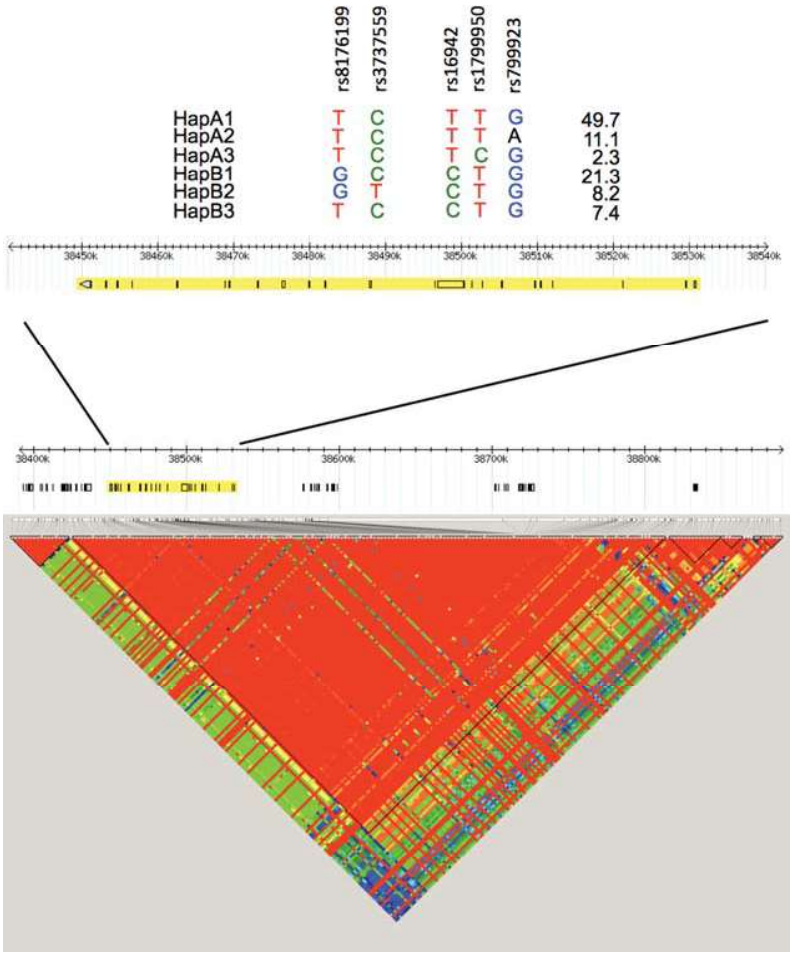


Figure 1. Haplotypes and LD structure of the *BRCA1* gene. Region surrounding *BRCA1* on chromosome 17 using HapMap Data Release 27 Phase II and III data, February 2009 build (accessed on 2 September 2010), on NCBI B36 assembly and dbSNP b126. SNPs used in haplotype analyses are shown, relative to their position in the *BRCA1* gene.

For some centers, additional SNPs that tag the six frequent haplotypes of the haplogroups A and B were genotyped. *BRCA1* wild-type haplotypes could be inferred for a total of 1033 breast cancer cases and 1049 unaffected controls (Table 3, Fig. 1). Haplotype A2 showed an inverse association with breast cancer risk when compared with the referent haplotype A1 (HR 0.67, 95% CI 0.51–0.90, $P = 0.007$). Actually, all other haplotypes (A3, B1, B2 and B3) also had inverse, though weaker, associations with breast cancer risk.

Functional studies

More than 120 common SNPs (allele frequency >5%) have been detected across the *BRCA1* LD block. Ten are located in the *BRCA1* coding sequence, of which 7 are non-synonymous. The minor alleles of these seven missense SNPs are carried on the haplotype A2 (Asp693Asn—rs4986850), haplotype A3 (Gln356Arg—rs1799950 and Ser1040Asn—rs4986852) or haplogroup B (Pro871Leu—rs799917, Glu1038Gly—rs16941, Lys1183Arg—rs16942 and Ser1613Gly—rs1799966). These missense variants could potentially alter the function or the stability of the BRCA1 protein. However, none of them is located in a recognized functional domain of BRCA1 and they are predicted to be neutral by commonly used algorithms for assessing the functional effects of missense variants such as Align-GVGD and SIFT, mainly based on phylogenetic information and biochemical differences between the reference and variant amino acid.

On the other hand, differential allelic expression for *BRCA1* has been reported in different studies, and it was hypothesized that variants in the promoter region could be involved in the regulation of this differential expression. We therefore chose to investigate the effect of SNPs located in the *BRCA1* promoter region on transcription efficiency to try to explain the genetic effect revealed by our study.

Haplogroups A and B carry five frequent SNPs in the 2 kb region upstream *BRCA1*; the haplotype regions corresponding to the *BRCA1* promoter were named thereafter pHapA and pHapB. As an initial approach to assess the functional significance of DNA variations in the promoter region, we performed transcriptional activity analyses using a gene-reporter assay in a HeLa cell line. As illustrated in Figure 3, differential

Table 1. Number of eligible *BRCA1* mutation carriers by study group

Study	Country[a]	*BRCA1* carriers	Genotyping platform
Breast Cancer Family Registry (BCFR)	USA/Australia/Canada	592	Taqman
Copenhagen Breast Cancer Study (CBCS)	Denmark	162	Taqman
Spanish National Cancer Centre (CNIO)	Spain	156	Taqman
CONsorzio Studi ITaliani sui Tumori Ereditari Alla Mammella (CONSIT TEAM)	Italy	416	Taqman
Deutsches Krebsforschungszentrum (DKFZ)	Germany	160	Taqman
HEreditary Breast and Ovarian study Netherlands (HEBON)	Netherlands	773	iPLEX[b]
Epidemiological Study of BRCA1 and BRCA2 Mutation Carriers (EMBRACE)	UK/Eire	843	iPLEX[b]
Fox Chase Cancer Centre (FCCC)	USA	80	iPLEX[b]
German Consortium of Hereditary Breast and Ovarian Cancer (GC-HBOC)	Germany	916	Taqman
Genetic Modifiers of cancer risk in BRCA1/2 mutation carriers (GEMO)	France/USA	1100	Taqman
Georgetown University (GEORGETOWN)	USA	33	iPLEX[b]
Gynecologic Oncology Group (GOG)	USA	406	Taqman
Hospital Clinico San Carlos (HCSC)	Spain	116	Taqman
Helsinki Breast Cancer Study (HEBCS)	Finland	103	iPLEX[b]
Institut Català d'Oncologia (ICO)	Spain	113	Taqman
Iceland Landspitali - University Hospital (ILUH)	Iceland	6	iPLEX[b]
Interdisciplinary Health Research International Team Breast Cancer Susceptibility (INHERIT BRCAs)	Canada (Quebec)	73	Taqman
Istituto Oncologico Veneto—Hereditary Breast Ovarian Cancer Study (IOVHBOCS)	Italy	108	Taqman
Kathleen Cuningham Foundation Consortium for research into Familial Breast cancer (kConFab)	Australia/New Zealand	526	iPLEX[b]
Mayo Clinic (MAYO)	USA	218	iPLEX[b]
Modifier Study of Quantitative Effects on Disease (MOD SQUAD)	Czech Republic/ Belgium	271	Taqman
General Hospital Vienna (MUV)	Austria	294	iPLEX[b]
National Cancer Institute (NCI)	USA	142	Taqman
Ontario Cancer Genetics Network (OCGN)	Canada	224	Taqman
Ohio State University Clinical Cancer Center (OSU CCG)	USA	80	Taqman
Odense University Hospital (OUH)	Denmark	263	Taqman
Pisa Breast Cancer Study (PBCS)	Italy	75	iPLEX[b]
Sheba Medical Centre (SMC)—Tel Hashomer	Israel	501	Taqman
Swedish Breast Cancer Study (SWE-BRCA)	Sweden	470	iPLEX[b]
University of California Irvine (UCI)	USA	193	Taqman
UK and Gilda Radner Familial Ovarian Cancer Registries (UKGRFOCR)	UK/USA	187	Taqman
University of Pennsylvania (UPENN)	USA	274	iPLEX[b]
Total		9874	

[a]Country of the clinic at which carriers are recruited.
[b]Centralized genotyping at Queensland Institute of Medical Research.

transcriptional activity was observed between the two major haplotypes (27% decrease, P-value = 0.0011). These experiments were performed five times and the mean relative luciferase activity driven by pHapB was 20% lower than the levels driven by its counterpart pHapA, suggesting allele-specific differential promoter activity.

To further investigate the effect of genetic variants of the *BRCA1* promoter region on gene expression, we assessed the impact of these SNPs on DNA–protein binding capacity. Electrophoretic mobility shift assays (EMSAs) were performed for all identified SNPs using nuclear extracts from HeLa, MCF7 and MDA-MB-231 cell lines (Fig. 4). The EMSA assay performed on probes including polymorphism rs4793204 gave the most convincing results with clear-cut differential binding of nuclear proteins to probes carrying either the T or the C allele in all three cell lines. As illustrated in Figure 4A, probe-specific differential binding was observed for the complexes identified by solid arrows, which showed binding affinity for the rs4793204-T probe (Fig. 4, lane 2) but not for the rs4793204-C probe (Fig. 4, lane 8). Competition EMSAs performed in the presence of 50-fold molar excess of the unlabeled T probe confirmed binding specificity (Fig. 4, lane 3).

In an attempt to identify the specific transcriptional factors responsible for this differential binding, *in silico* analyses were performed to assess the potential functional impact of SNP rs4793204 on predicted transcription factor binding sites (TFBSs). These analyses revealed that rs4793204 might alter the recognition/binding motifs of Brg-1, Oct-1 and Nkx-3.1. Further EMSAs were performed in the presence of antibodies raised against these transcription factors. Assays performed in the presence of anti-BRG1 antibodies revealed a supershifted band with the probe carrying the T allele in all cell lines tested (Fig. 4, lanes 6, dotted arrow). No supershift was observed when the assays were performed with antibodies raised against Oct-1 and Nkx-3.1 (data not shown). In the case of rs799908, several DNA–protein-specific complexes were observed with the rs799908-C probe using nuclear extracts from MCF7 and MDA-MB-231 cells, but not from HeLa cells, as illustrated in Figure 4B (solid arrows), suggesting cell-specific transcription factor binding. *In silico* analyses predicted that the minor allele of rs799908 altered the binding

Table 2. Association between rs16942 genotypes on 'wild-type' allele of *BRCA1* and breast cancer risk

Phasing method (see Materials and Methods)	'T' allele on wild-type (reference) Unaffected	Affected	Person-years	'C' allele on wild-type Unaffected	Affected	Person-years	HR (95% CI)[a]	*P*-value
rs16942 homozygotes								
Overall	2065	2404	185 732	607	576	49 756	0.85 (0.74–0.96)	0.01
Family-based								
Overall	2339	2608	206 355	1142	959	87 576	0.86 (0.77–0.95)	0.003
Class 1 mutations	1636	1615	205 204	904	710	68 197	0.88 (0.79–0.99)	0.04
Class 2 mutations	564	776	68 103	166	167	13 290	0.92 (0.71–1.20)	0.55

[a]HRs and 95% CIs calculated using weighted Cox regressions with a robust sandwich estimator.

motif of transcription factors Elk-1, USF-1 and USF-2. However EMSAs performed in the presence of antibodies raised against any of these proteins did not yield supershifted bands, thus not allowing us to confirm that these transcription factors were responsible for the observed differential binding in these cell lines (data not shown).

The results we obtained for both of these SNPs suggest that the haplotype-specific differences observed in gene-reporter assays might involve differential binding of transcription factors in this promoter region. The other investigated SNPs located in the *BRCA1* promoter region, rs11655505–rs799906–rs8176071, did not show significant allele-specific differential binding, as revealed by EMSA analyses using nuclear extracts from HeLa, MCF7 and MDA-MB-231 cells (data not shown).

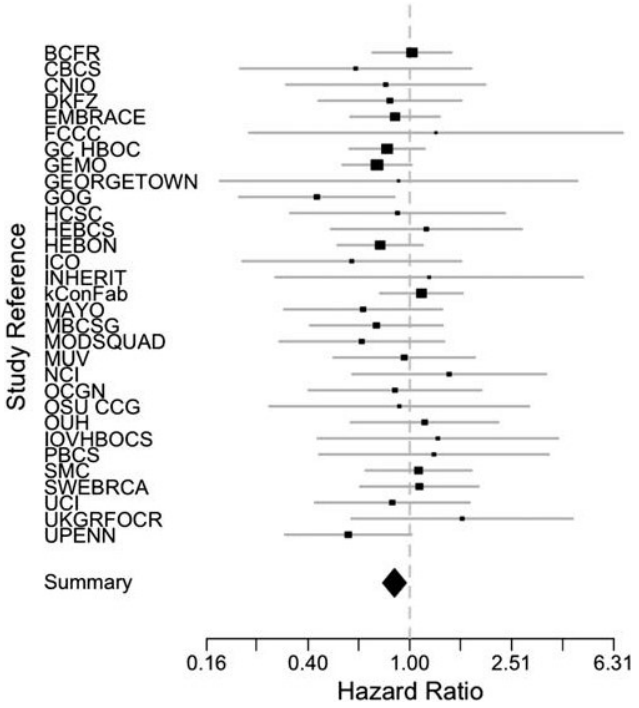


Figure 2. Study-specific HRs between rs16942 genotypes on wild-type allele of *BRCA1* and breast cancer risk. HRs were calculated in each specific study as described in Materials and Methods. The summary effect estimate for C allele carriers is 0.87 (0.78–0.97), p-heterogeneity 0.91 with a fixed effect model.

DISCUSSION

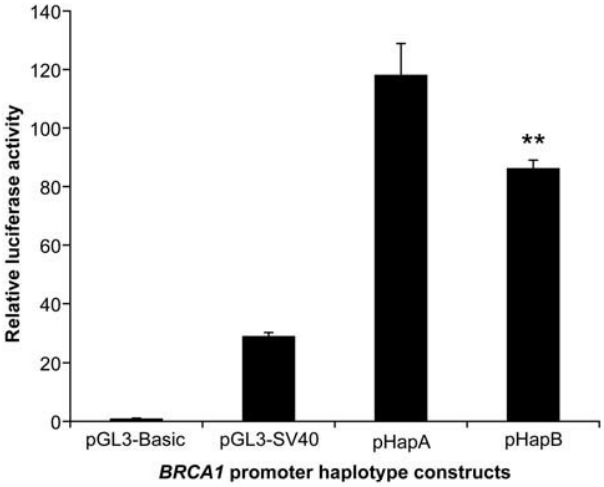
The primary objective of this study was to test the hypothesis that polymorphisms of the non-mutated (wild-type) *BRCA1* allele modify breast cancer risk among women who carry a *BRCA1* mutation. We observed an association between a *BRCA1* tag SNP, rs16942, and breast cancer risk, with women who carried the minor allele on their non-mutated *BRCA1* copy having ~14% decrease in risk (HR 0.86, 95% CI 0.77–0.95, $P = 0.003$). Perhaps not surprisingly, when phase was not taken into account, we observed weaker association between rs16942 and breast cancer risk using a per-allele test for trend ($P = 0.02$).

Characterizing the biological basis for this genetic effect is an important step towards further understanding of breast cancer susceptibility linked to *BRCA1*, and subsequent use in genetic counseling. Association studies, however, are limited in their ability to definitively identify causal variants due to correlation, or LD, between adjacent polymorphisms. This is particularly true for *BRCA1* located within a 390 kb long block of LD. A constellation of more than 120 common SNPs exists across this LD block. Ten of these common SNPs are in the *BRCA1* coding sequence, of which 7 are non-synonymous, potentially altering the function or the stability of the BRCA1 protein. However, the two most commonly used algorithms that evaluate the predicted pathogenicity of a missense variant revealed no potential effect. It should be noted that, where such an effect suspected, its assessment would prove difficult as only subtle function or stability impairment can be expected in this context. Another non-exclusive explanation for the genetic effect described here is that one or several SNPs alter(s) the level of expression of *BRCA1*, ultimately altering the amount of biologically active BRCA1. In favor of this hypothesis, previous studies have reported differential allelic expression of *BRCA1* in lymphoblastoid cell lines, in B lymphocytes and in breast tissue (29–31). This *BRCA1* differential allelic expression has been in some instances associated with breast (29) and ovarian (31) cancer susceptibility. Analysis of expression data available from the Genevar (GENe Expression VARiation) database (37) indicates that polymorphisms within the *BRCA1* locus, including rs16942, were associated with *BRCA1* expression. In lymphocytes, the most significant correlation coefficient between polymorphisms and *BRCA1*

Table 3. Association between *BRCA1* haplotypes on 'wild-type' allele of *BRCA1* and breast cancer risk using family-based phasing (see Material and Methods)

Group	Unaffected Number	Person-years	Affected Number	Person-years	HR (95% CI)[a]	*P*-value
HapA1	521	22 388	615	24 935	1.00 (Ref.)	
HapA2	116	5569	104	4306	0.67 (0.51–0.90)	0.007
HapA3	24	962	17	694	0.81 (0.41–1.61)	0.37
HapB1	224	9421	165	6673	0.77 (0.61–0.99)	0.04
HapB2	86	3686	69	2761	0.71 (0.50–1.02)	0.07
HapB3	78	3258	63	2663	0.80 (0.56–1.15)	0.23

[a]HRs and 95% CIs calculated using Cox regressions.



Haplotype constructs	rs4793204 -1890T/C	rs799908 -1508T/C	rs11655505 -969C/T	rs799906 -708A/G	rs8176071 -598(ACA)1/(ACA)2
pHapA	T	T	C	A	(ACA)1
pHapB	C	C	T	G	(ACA)2

Figure 3. Representative luciferase reporter assays of major *BRCA1* haplotype constructs in HeLa cell line. Relative luciferase activity of constructs carrying *BRCA1* promoter sequences corresponding to two haplotypes was measured following transient transfection into HeLa cells. The ratio of firefly:*Renilla* luciferase activity of each promoter construct was normalized against that of the promoter-less pGL3-Basic vector. The pGL3-SV40 vector containing the *SV40* early promoter is used as a positive control. Promoter haplotype *BRCA1*-pHapA was used as reference against which pairwise comparisons were made. Significant differences between haplotype expressions are marked with asterisks ($^{**}P = 0.0011$). The position of each SNP is given relative to the first base of the initiation codon.

expression (probe ILMN_1738027) was observed with rs16942, with a value of 0.41 ($P = 2.2\text{E}-4$). Some recent evidence that variation of expression levels is correlated with polymorphisms in the promoter region of *BRCA1* (33) prompted us further to investigate the possibility that transcription efficiency could explain the genetic effect shown in this study.

We thus tried to assess the functional significance of five common SNPs present in the 2 kb *BRCA1* promoter, using EMSA and *in vitro* transcriptional assays. Our results suggest that these polymorphisms can be involved in differential allelic expression. Indeed, EMSAs revealed that two of the five SNPs located in the promoter region showed allele-specific differential capacity of binding to nuclear proteins in HeLa cells and/or ER-positive (MCF7) and ER-negative (MDA-MB-231) breast cancer cell lines. For one of the SNPs, rs4793204, we observed in all cell lines studied a differential protein binding capacity to the BRG1 transcription factor, a subunit of the SWI/SNF chromatin remodeling complex previously shown to repress the *BRCA1* promoter reporter activity (38). One can speculate that loss or decreased binding capacity of the minor C allele, carried on haplogroup B (and associated with decreased breast cancer risk for *BRCA1* mutation carriers), to this transcription factor could lead to loss of repression resulting in an increase in the level of expression. Conversely, our gene-reporter assays show a tendency for the minor haplotype (corresponding to haplogroup B) to be expressed at lower levels than the major haplotype. However, it should be noted that only the proximal promoter region (2 kb upstream of the transcription start site) was used in these assays, as classically done and it is therefore possible that other regulatory elements or *cis*-regulatory modules present outside this region and potentially influencing the expression of this gene were missed. Indeed, regulatory elements

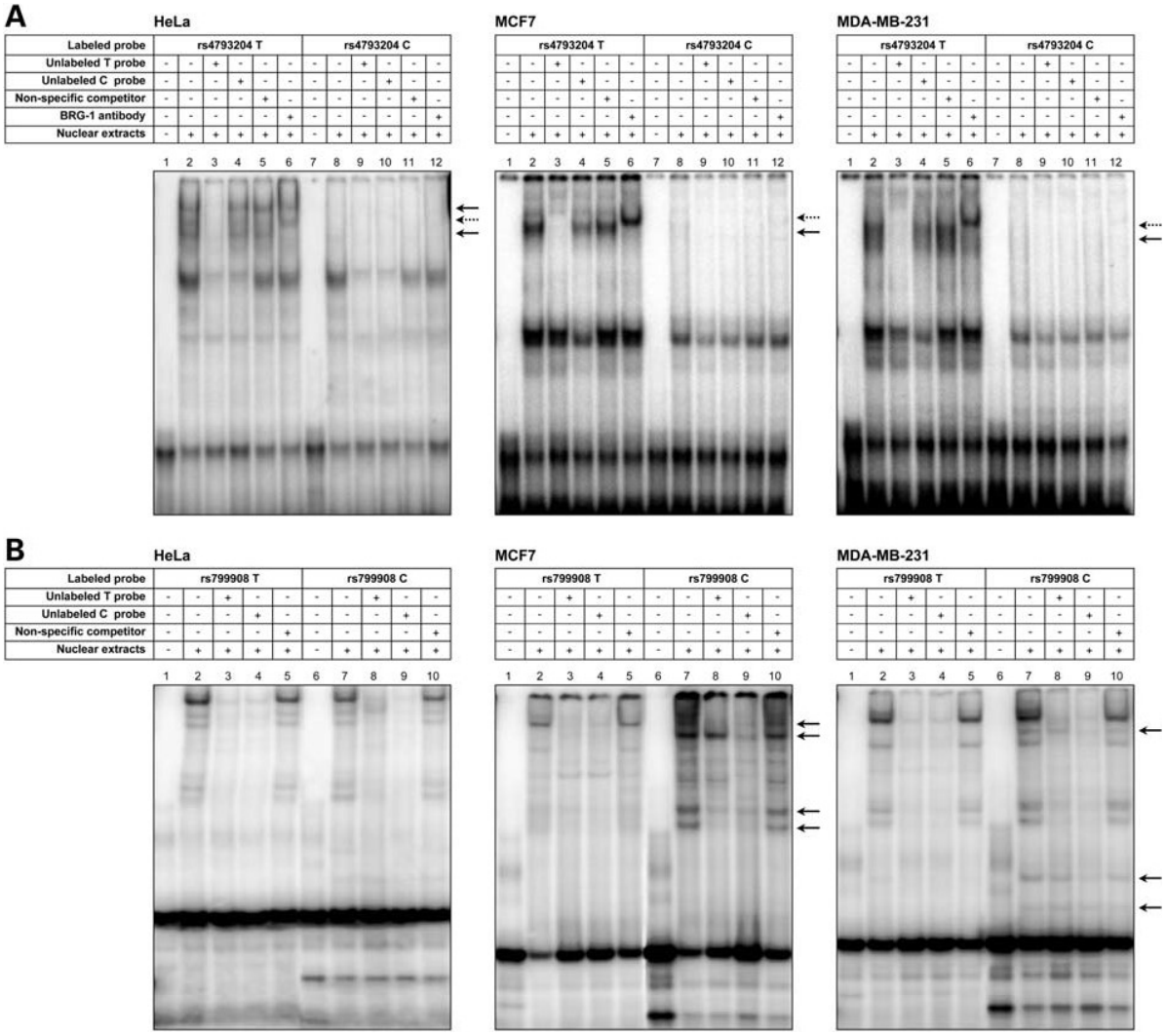


Figure 4. Representative EMSAs illustrating allelic DNA–protein interactions in the promoter region of *BRCA1*. Labeled double-stranded oligonucleotide (ds-oligo) probes containing either allele of rs4793204 (**A**) and rs799908 (**B**) were incubated with nuclear extracts from HeLa, MCF7 and MDA-MB-231 cells. (A) Lanes 1–6 represent binding to the labeled ds-oligo containing the major allele; lanes 7–12, binding to the ds-oligo containing the minor allele. Lanes 1 and 7, negative control (no extracts). Lanes 3 and 10, competition with unlabeled allelic probes (specific competitors). Lanes 4 and 9, competition with mismatched unlabeled probes. Lanes 5 and 11, competition with a non-specific probe (non-specific competitor). Lanes 6 and 12, pre-incubation with anti-BRG1 antibody. (B) Lanes 1–5 represent binding to the labeled ds-oligo containing the major allele; lanes 6–10, binding to the ds-oligo containing the minor allele. Lanes 1 and 6, negative control (no extracts). Lanes 3 and 9, competition with unlabeled allelic probes (specific competitors). Lanes 4 and 8, competition with mismatched unlabeled probes. Lanes 5 and 10, competition with a non-specific probe (non-specific competitor).

can be located in far upstream and downstream regions still within the large block of LD surrounding *BRCA1*. Furthermore, given that *BRCA1* allelic expression levels vary significantly between cell types (30), the results we obtained using luciferase reporter assays in HeLa cell line may be different in other cell types. Further studies will be needed to decipher the impact of putative regulatory SNPs in the complex transcriptional activation of the *BRCA1* gene.

We genotyped four additional SNPs in 4050–4816 of the initial 9874 *BRCA1* mutation carriers in order to define more precisely the haplotype(s) associated with modification of breast cancer risk. This analysis allowed us to define six haplotypes, three in haplogroup A and three in haplogroup B. It is interesting to note that all haplotypes carried by the wild-type allele were inversely associated with breast cancer risk when compared with the reference haplotype, haplotype A1. This implies that an allele(s) that alter(s) *BRCA1* expression may actually be carried on haplotype A1 and not on the other haplotypes. However, caution must be used when interpreting our haplotype results, due to the reduction in sample size, which led to reduced power for these analyses. Indeed, an important limitation of the power of this study is that we restricted our analyses to homozygotes and heterozygotes that we were able to phase based on family information. Hence, although a total of 9874 *BRCA1* mutation carriers were initially genotyped for rs16942, we were able to phase genotypes in 7048 individuals. For the haplotype analysis, this restriction had more serious consequence as haplotypes on the wild-type

allele of BRCA1 could only be inferred in 2082 mutation carriers.

In addition to the haplotype analysis including four SNPs in LD with rs16942, a number of other related hypotheses have been evaluated, including the associations with different mutation types. None of the reported *P*-values has been adjusted for multiple testing as it is unclear what the correct type of adjustment would be in this context. However, both the observed *P*-values for the primary single SNP analysis and secondary haplotype analysis ($P = 0.003$ and 0.007, respectively) would survive a conservative Bonferroni correction (based on five and six tests, respectively). Furthermore, the functional evidence with respect to *BRCA1* expression provides additional evidence for the association between this variant and breast cancer risk for *BRCA1* mutation carriers.

In conclusion, we have shown an inverse association between minor polymorphic variants of the wild-type allele of the *BRCA1* gene and breast cancer risk among women who carry a *BRCA1* mutation. This association was limited to women who carry the variant allele of rs16942 on their wild-type (non-mutated) allele of *BRCA1*. This association is most likely due to the influence on *BRCA1* expression of variants that are in LD with rs16942, which tags the two major haplotype groups present across the LD block of *BRCA1*. Some of these variants are likely to reside in the promoter, as we have shown here that polymorphisms located in the 2 kb promoter region of *BRCA1* appear to be involved in differential allelic expression. However, it is reasonable to suspect that the polymorphisms we have examined, both in terms of association and functional testing, are not solely responsible for the genetic effect depicted here. Identification of true causal variants will provide important insight into the mechanisms by which BRCA1 exerts its tumor suppressor role in breast cancer.

MATERIALS AND METHODS

Ethics statement and study population

Eligible study subjects were women aged ≥ 18 years who carry a pathogenic mutation in *BRCA1*. Information on study subjects was submitted from 32 studies from 20 countries (Table 1). These women participated in clinical and research studies at the host institutions under institutional review board approved protocols. Data collected included year of birth, mutation description, family membership, ethnicity, country of residence, age at last follow-up, ages at diagnosis of breast and/or ovarian cancer, and information on bilateral prophylactic mastectomy. Mutations were included in the analysis if they were pathogenic according to generally recognized criteria.

To examine whether the effects of the SNPs are different in individuals carrying different types of mutations, we classified mutations according to their functional effect. Class 1 mutations (number of carriers = 7109) were defined as loss of function mutations expected to result in a reduced transcript or protein level because of nonsense-mediated mRNA decay (NMD) and/or degradation or instability of truncated proteins (39–42), translation re-initiation but no production of stable protein (43) or the absence of expression due to deletion of transcription regulatory regions. Class 2 mutations (number of carriers = 2085) comprised mutations likely to generate potentially stable mutant proteins that might have a dominant negative action, partially preserved normal function or loss of function. Class 2 mutations are missense substitutions and truncating mutations not triggering NMD (premature stop codon occurring in the last exon). A small proportion of mutations (number of carriers = 680) could not be categorized as belonging to Class 1 or Class 2.

Selection of haplotype tagging SNPs

To select a set of SNPs efficiently capturing common variation (tagSNPs) in the genomic region of *BRCA1*, we used data available from the HapMap project on CEPH trios (Utah-USA residents with ancestry from Northern and Western Europe) (http://www.hapmap.org). The 82 kb long *BRCA1* gene is located within a 390 kb long block of LD that also comprises roughly 20 kb and 290 kb at its 5′ and 3′ ends, respectively. TagSNPs were selected using the 'Haploview 4.0' tool (http://www.broad.mit.edu/mpg/haploview/) (44). The 'Tagger' program was used to select a minimal set of tagSNPs such that all alleles to be captured (frequency >5%) were correlated at an r^2 greater than 0.8 threshold (45). This resulted in the selection of five tagSNPs: rs16942, rs179950, rs799923, rs3737559 and rs8176199. The rs16942 SNP tags the two major haplogroups (further named haplogroups A and B). The combination of rs179950 and rs799923 tags haplotype A1, rs179950 tags haplotype A3 and rs799923 tags haplotype A2. rs3737559 tags haplotype B2 and rs8176199 tags haplotype B3, with the combination of these two SNPs tagging haplotype B1 (Fig. 1).

Genotyping and phasing

SNP rs16942 and minor haplotype tagging SNPs were genotyped using the 5′ nuclease assay (TaqMan) on the ABI 7900HT Sequence Detection System (Applied Biosystems) or using the iPLEX Mass Array platform. Additional SNPs that were genotyped varied by center (see details in Supplementary Material, Table S1). All centers included at least 2% of samples in duplicate, no template controls on every plate and a random mixture of samples of affected and unaffected mutation carriers on each plate. The minimum acceptable call rate was 95%. For each study, the genotype frequencies among unrelated carriers were consistent with the expected frequencies under the assumption of Hardy–Weinberg equilibrium.

A total of 9874 *BRCA1* mutation carriers (5176 affected and 4698 unaffected) were available for these analyses. As our hypothesis was that the haplotype carried on the non-mutated *BRCA1* allele would modify breast cancer risk, we initially restricted our analyses to carriers homozygous for rs16942 (4469 T/T homozygotes, 1183 C/C homozygotes). Since both rs16942 genotype and mutation status were available from multiple family members for some of the subjects, we next used this information to infer the phase of rs16942 alleles among heterozygotes with the specific mutation in each family. Specifically, we assumed that within each family, there was little probability of recombination between

the mutation and rs16942. Therefore, if an rs16942 heterozygote and homozygote were observed within the same family, we assumed that the mutation was carried with the allele for which the family member was homozygous in that specific family. Polymorphisms rs179950, rs799923, rs3737559 and rs8176199 that define haplotypes within the major haplogroups tagged by rs16942 were genotyped in a subset of CIMBA centers (Supplementary Material, Table S1). Due to the complete LD between these SNPs, haplotypes were determined and phasing was carried out as for rs16942 described above.

In silico assessment of functional effects of missense SNPs

To predict potential functional impact of the *BRCA1* missense SNPs, we used web-based algorithms with default settings: Align-GVGD (http://agvgd.iarc.fr/) (46) and SIFT (http://sift.jcvi.org/) (47).

Statistical analyses

To evaluate the association between wild-type *BRCA1* genotype and breast cancer risk in *BRCA1* mutation carriers, their phenotype was defined by their age at diagnosis of breast cancer or their age at last follow-up. For this purpose, individuals were censored at the age of the first of the following events: breast cancer diagnosis, ovarian cancer diagnosis, bilateral prophylactic mastectomy or last observation, and only carriers censored at breast cancer diagnosis were assumed to be affected. Risk reducing salpingo-oophorectomy was not considered in the analysis as it is not expected to be associated with the underlying SNP genotype (i.e. it is not a confounder).

Studying the associations with cancer risk for *BRCA1* mutation carriers is complicated by the fact that mutation carriers in our study design are not randomly sampled with respect to their disease phenotype. Genetic testing is targeted at families with multiple affected individuals, and most genetic clinics tend to screen first young, affected family members. Therefore, the selection of mutation carriers is not random with respect to disease status or age at diagnosis. These study designs lead therefore to an oversampling of young affected mutation carriers. It has been shown in the past that under such study designs, standard cohort analysis (such as Cox regression, which assumes random sampling with respect to phenotype) yield biased estimates of the risk ratios. This can be illustrated by considering an individual affected at age t. In a standard analysis of a cohort study, the SNP genotype for the individual will be compared with those of all individuals at risk at age t. This analysis leads to consistent estimates of the HR. However, in the present design, mutation carriers are already selected on the basis of disease status (where affected individuals are over-sampled). If standard cohort analysis were applied to these data, it would lead to affected individuals at age t being compared with unaffected carriers selected on the basis of their future disease status. If the genotype is associated with the disease, the risk estimate will be biased to zero because too many affected individuals (in whom the at-risk genotype is overrepresented) are included in the comparison group. Simulation studies have shown that this effect can be quite marked.

To overcome this problem, a weighted cohort approach was previously proposed, under which affected and unaffected individuals are differentially weighted according to their age at diagnosis or last observation such that on the observed weighted age-specific *BRCA1* breast cancer incidences in the study sample agree with established breast cancer incidences in mutation carriers (47,48). This method has been shown to provide risk ratio estimates which are close to unbiased (47,48). For analyses of rs16942, we estimated the log-HRs for CC genotypes using the TT homozygotes as the baseline category. Haplotype analyses used haplogroup A1 (the most common haplotype) as the baseline category. As some of the study participants had censoring events (bilateral mastectomy or breast cancer diagnosis) prior to study inclusion interview (887 unaffected, 228 affected), we also carried out analyses restricted to patients with censoring events less than 1 year prior to their study interview in order to exclude long-term survivors. We also performed analyses to examine whether SNP associations differed by type of *BRCA1* mutations (class 1 and class 2 mutations). All analyses were stratified by study and country of residence. As sufficient detail regarding degree of family history was not available for all subjects, we were unable to take this into consideration in our analyses. In all instances, a robust variance approach was used to allow for the dependence between related carriers (49). Most statistical analyses were carried out in SAS v.9.1, with the exception of heterogeneity testing which used the rmeta package in R 2.10.1.

BRCA1 promoter polymorphisms

BRCA1 shares a well-characterized bi-directional promoter with its neighboring gene *NBR2* in a 229 bp intergenic region. As is generally done in classical promoter studies, a 2 kb region upstream of the *BRCA1* transcription start site was chosen (50,51) to assess the impact of upstream genetic variants on promoter activity. The 2067 bp of the *BRCA1* promoter region (chr17:41277361–41279427, GRCh37/hg19) was found in HapMap to contain five frequent polymorphisms that were confirmed on a population panel consisting of 40 unrelated individuals from five continental groups: rs4793204, rs799908, rs11655505, rs799906 and rs8176071. No other frequent SNPs were identified by sequencing. It should be noted that these five SNPs are in almost complete LD with rs16942 ($r^2 > 0.961$). The haplotype regions corresponding to the *BRCA1* promoter were named pHapA and pHapB.

In silico prediction of putative TFBSs

A computer-based search for putative transcription factor binding elements harbored by the *BRCA1* promoter sequence corresponding to each of the two pHaps was performed using the MatInspector software (http://www.genomatix.de/online_help/help_matinspector/matinspector_help.htm) (52). Transcription factors that putatively bind to the sense strand sequence of the *BRCA1* promoter in humans were identified, and those showing significantly altered predicted scores for any of the pHaps were selected for further analysis.

Cell culture

The human cervical carcinoma cell line HeLa was grown in EMEM (Wisent Bioproducts, St-Bruno, Québec, Canada) supplemented with 5% fetal bovine serum (FBS) and 1% penicillin–streptomycin. The human breast adenocarcinoma cell lines MCF7 and MDA-MB-231 were grown in DMEM/F12 (Wisent Bioproducts, St-Bruno, Québec, Canada) supplemented with 5% FBS, 1% penicillin–streptomycin and 10^{-9} M oestradiol (E2). All cells were grown at 37°C in a 5% CO_2 incubator.

Electrophoretic mobility shift assay (EMSA)

EMSAs were performed on the regions of the five polymorphisms found in the *BRCA1* promoter region. For each SNP, double-stranded oligonucleotide probes corresponding to the sequences surrounding the polymorphic site were ^{32}P-radiolabeled and purified using MicroSpin G-25 columns. Binding experiments were conducted using nuclear protein extracts prepared from HeLa, MCF7 and MDA-MB-231 cell lines. Briefly, nuclear proteins from each cell line were quantified with the Bradford protein assay (Bio-Rad). Nuclear extracts (10 μg) were incubated with the various radiolabeled double-stranded DNA probes (35 fmol) in a buffer containing 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 0.25 μg/μl poly deoxyinosinate–deoxycytidylate and 20% glycerol, in a total volume of 10 μl for 20 min at room temperature. For competition experiments, 50-fold molar excess of the unlabeled probe oligonucleotide, the unlabeled corresponding mutant probe or a non-specific DNA probe was added before incubation. Supershift assays were performed in the presence of 1 μl of antibodies (2 μg/μl). The variants tested and the corresponding antibodies were as follows: rs4793204: Brg-1, Oct-1, Nkx-3.1; rs799908: Elk-1, USF-1, USF-2; rs11655505: GATA-3, Oct-1; rs799906: ER; rs8176071: MEF2 (SantaCruz Biotechnology, Santa Cruz, CA, USA). Competitions and supershift experiments were carried out by pre-incubating nuclear extracts in binding buffer for 10 min at room temperature, followed by 20 min incubation at room temperature with the radiolabeled double-stranded DNA probes. Complexes were separated on a 6% non-denaturing polyacrylamide gel (acrylamide–bisacrylamide, 37.5:1) in 1× Tris-glycine-EDTA buffer (190 V at 4°C). After electrophoresis, gels were dried and subjected to autoradiographic analysis.

Promoter activity assay

Constructs. For the *BRCA1* pHaps (A and B), genomic DNA samples from known homozygous or heterozygous individuals were polymerase chain reaction-amplified to obtain the 2067 bp haplotype-specific fragments that were then subcloned into the promoterless pGL3-Basic luciferase reporter vector (Promega, Madison, WI, USA). The following primers were used for amplification (5′-GGGGCCGCTCGAGACACAGAA GTTCTCCAAGTGC-3′, 5′-GGGGCCCAAGCTTCCCGTC CAGGAAGTCTCAG-3′) and detailed information on experimental conditions is available upon request. The resulting constructs were sequenced to confirm the presence of the expected polymorphic sites, amplified and then purified using QIAfilter Plasmid Kit (Qiagen Sciences, Maryland, USA) prior to transfection.

Transient transfection and luciferase reporter assay. HeLa cells were seeded in 24-well culture dishes at a density of 7×10^4cells/well for 24 h prior to transfection. Transient transfection was performed using ExGen 500 cationic polymer transfection reagent (MBI Fermentas Inc., Ontario, Canada) according to the supplier's protocol. Briefly, HeLa cells were co-transfected with 800 ng of pGL3-promoter haplotype-specific constructs encoding a modified firefly luciferase gene and 200 ng of pRL-null vector (Promega Corporation) encoding the *Renilla* luciferase gene as an internal standard. The promoterless pGL3-basic vector and pGL3-SV40 control vector, containing the *SV40* early promoter, were used as negative and positive controls, respectively. Cells were harvested 24 h post-transfection and luciferase reporter gene activities measured with the Dual-Luciferase Reporter Assay System according to the manufacturer's instructions (Promega) in a MicroLumat Plus luminometer (EG&G Berthold). Promoter activities are expressed as a ratio of firefly luciferase to *Renilla* luciferase luminescence and are represented as the relative luciferase activity of four independent replicates (mean + standard deviation). The promoterless pGL3-basic vector was used to measure basal expression levels. Each experiment was performed five times. Pairs of haplotypes were compared by Student's unpaired *t*-test.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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