Genetic Variation in IGF2 and HTRA1 and Breast Cancer Risk among BRCA1 and BRCA2 Carriers


Abstract

Background: BRCA1 and BRCA2 mutation carriers have a lifetime breast cancer risk of 40% to 80%, suggesting the presence of risk modifiers. We previously identified significant associations in genetic variants in the insulin-like growth factor (IGF) signaling pathway. Here, we investigate additional IGF signaling genes as risk modifiers for breast cancer development in BRCA carriers.

Methods: A cohort of 1,019 BRCA1 and 500 BRCA2 mutation carriers were genotyped for 99 single-nucleotide polymorphisms (SNP) in 13 genes. Proportional hazards regression was used to model time from birth to diagnosis of breast cancer for BRCA1 and BRCA2 carriers separately. For linkage disequilibrium (LD) blocks with multiple SNPs, an additive genetic model was used. For an SNP analysis, no additivity assumptions were made.

Results: Significant associations were found between risk of breast cancer and LD blocks in IGF2 for BRCA1 and BRCA2 mutation carriers (global P values of 0.009 for BRCA1 and 0.007 for BRCA2), HTRA1 for BRCA1 carriers (global P value of 0.005), and MMP3 for BRCA2 carriers (global P = 0.0000007 for BRCA2).

Conclusions: We identified significant associations of genetic variants involved in IGF signaling. With the known interaction of BRCA1 and IGF signaling and the loss of PTEN in a majority of BRCA1 tumors, this suggests that signaling through AKT may modify breast cancer risk in BRCA1 carriers.

Impact: These results suggest potential avenues for future research targeting the IGF signaling pathway in modifying risk in BRCA1 and BRCA2 mutation carriers. Cancer Epidemiol Biomarkers Prev; 20(8); 1690–702. ©2011 AACR.

Introduction

Women who carry mutations in the BRCA1 or BRCA2 (BRCA) genes have a substantially increased risk of developing breast and ovarian cancers as compared with the general population. Estimates of the age-specific risk attributable to mutations at these loci vary depending on the ascertainment scheme. The cumulative risks of breast cancer by age 70 years have been estimated between 40% and 87% for BRCA1 and BRCA2 mutation carriers (1–5). Among BRCA mutation carriers, there is considerable variability in both age at diagnosis and incidence of breast cancer, with a higher proportion of breast cancer cases diagnosed before age 50 years for BRCA1 carriers (1–5). BRCA1 breast cancer is characterized by an increased risk of bilateral disease and a younger age at diagnosis compared with BRCA2 breast cancer (5–8).
and ovarian cancers, even among women who carry the same BRCA mutation (6–8). These observations of large variation in risk are consistent with the hypothesis that the breast cancer risk in mutation carriers is modified by other genetic and/or environmental factors. To test this hypothesis, we have focused on genetic variation in the insulin-like growth factor (IGF) signaling pathway, as it regulates both cellular proliferation and apoptosis (reviewed in refs. 9–11). We previously reported significant associations of single-nucleotide polymorphisms (SNP) in insulin receptor substrate (IRS) 1 and IGF1R in BRCA1 mutation carriers and in IGF1R and IGFBP2 in BRCA2 mutation carriers (12).

For this study, we investigated the association of variants in additional genes involved in IGF signaling. We focused on 13 genes in this pathway, which were closely related to genes with significant associations with breast cancer risk in our previous work (12). These genes included the IGF1 receptor (IGF1R) ligands IGF2 and insulin (INS); the IGF binding proteins (IGFBP) IGFBP3 (and its partners in binding IGF, IGFBALS), IGFBP4, and IGFBP6; the IGFBP proteases kallikrein–related peptidase 3 (KLK3, aka prostate-specific antigen; PSA), cathepsin D (CTSD), matrix metalloproteinases 3 and 7 (MMP3 and MMP7), HTRA serine peptidase 1 (HTRAI, aka PRSS11); the IGF1R docking protein IRS2; and its phosphorylation target phosphoinositide 3-kinase 110-kb catalytic subunit B (PIK3CB), as potential disease modifiers in deleterious mutation carriers of BRCA1 and BRCA2.

Materials and Methods

Participants

For the primary investigation, women with germline, deleterious mutations in BRCA1 or BRCA2 were identified in 14 centers in the United States, 1 center in Canada, and 1 center in Austria, including Baylor University Medical Center-Dallas, Dallas, TX, Beth Israel in Boston, City of Hope, Creighton University, Omaha, NE, Dana Farber, Boston, MA, Fox Chase Cancer Center, Philadelphia, PA, Georgetown University, Washington DC, the Mayo Clinic, Scottsdale, AZ, Medical University of Vienna, Vienna, Austria, North Shore University Health-System, Chicago, IL, University of California, Los Angeles, CA, University of California, Irvine, CA, University of Chicago, Chicago, IL, University of Pennsylvania, Philadelphia, PA, and Women’s Hospital of the University at Buffalo, Buffalo, NY. The majority of subjects were recruited from our cancer centers. The majority of subjects were recruited from our cancer centers, and 140 were selected for the University at Buffalo, Buffalo, NY. The majority of subjects were recruited from our cancer centers, and 140 were selected for the University at Buffalo, Buffalo, NY. The majority of subjects were recruited from our cancer centers, and 140 were selected for the University at Buffalo, Buffalo, NY.

SNP genotyping

The Genome Variation Server (GVS; ref. 13) at the University of Washington was used to identify SNPs in the genes and select haplotype tagging SNPs (htSNP) using the program LDSelect (14), which is integrated with the GVS. A total of 99 SNPs were selected from 13 genes to mark the common genetic variation in each gene and minimize the genotyping costs. However, because some of the genes had SNPs from the pooled HapMap race/ethnicity samples, we could not discern race and so we recalculated linkage disequilibrium (LD) blocks specifically for the European (EU) population in this article as described later.

Genotyping was done using the SNPlex assay from Applied Biosystems, Inc. (ABI), for all but 3 SNPs in IGFBP3 for which alleles were genotyped by ABI TaqMan exonuclease assays. Genotyping by SNPlex was done according to ABI’s protocols. The SNPlex assay is based on the oligonucleotide ligation assay (OLA), followed by multiplex PCR amplification using a universal primer pair, hybridization of reporter probes to amplicons, and capillary electrophoresis to rapidly identify eluted reporter probes (ABI). On the basis of the list of SNPs sent to ABI, 3 SNPlex pools were designed and ordered for genotyping. We obtained genotype data for 99 SNPs following the manufacturer’s protocol (ABI). Briefly, 120 ng genomic DNA for each sample was fragmented by denaturing at 99°C for 10 minutes. Phosphorylation of probes and allele-specific ligation between fragmented DNA and probes were done by running an OLA program on a PTC225 thermal cycler. After purification of ligation product by exonuclease digestion, PCR was carried out using universal primers with the reverse primer biotinylated. During hybridization, the captured biotinylated PCR products were hybridized with fluorescently labeled ZipChute probes, with a unique sequence corresponding to the probe sequence.

The current study uses data on a total of 1,519 adult, female non-Hispanic Caucasian mutation carriers, including 1,019 BRCA1 carriers (381 cases and 638 unaffected controls) and 500 BRCA2 carriers (219 cases and 281 unaffected controls). The BRCA mutation status of all subjects was confirmed by direct mutation testing, with full informed consent under protocols approved by the human subjects review boards at each institution. Women were eligible for entry into the study cohort if they were testing positive for a known deleterious mutation in BRCA1 or BRCA2. Women with deleterious mutations in both BRCA1 and BRCA2 were excluded. Women were excluded if they were missing information on year of birth, parity, menopausal status, and oral contraceptive use or had a diagnosis of breast or ovarian cancer more than 3 years prior to study entry. Information about breast cancer, ovarian cancer, prophylactic mastectomy, and prophylactic oophorectomy was obtained from medical records, and information on reproductive history and lifestyle habits was obtained by questionnaire.
to the unique Zip code sequences at the 5’ end of the allelic-specific probes used in OLA reactions. The Zip-Chute probes were separated by the ABI 3130xl capillary sequencer, and genotypes were called by ABI Genemapper 4.0 program. The laboratory technician was blinded as to whether samples were duplicates, cases, or controls. Each 96-well plate contained 87 unique samples, 2 positive controls, 1 negative control, and 6 ladders. Genotyping call rates ranged from 67% to 99%. SNPs that were monomorphic or with minor allele frequencies (MAF) less than 0.03 (n = 10 SNPs) or with genotyping call rates less than 80% (n = 5 SNPs) were excluded from further analysis. Because the duplicate concordance rates of the 40 duplicated samples were higher than 99.9%, we felt confident that the data were accurate even for the 10 SNPs with 82% to 89% call rates.

**Determination of LD blocks**

To define LD blocks relative to the current study population, all LD blocks were computed using the current analytic data set. Because of the relatively small proportion of non-EU subjects within the study sample, and the possibility that LD structure may differ between EU and non-EU ancestries, we restricted the analysis to only non-Hispanic Caucasians. SNPs were grouped according to their adjacent pairwise LD coefficient, \( D^2 \), which was computed between all adjacent marker pairs within each candidate gene. To account for within-family correlation, multiple outputation (15) was used to estimate \( D^2 \). In this case, a single member from each family was randomly sampled to create a single bootstrap sample, from which \( D^2 \) was computed. This process was repeated to obtain 200 bootstrap samples, yielding an empirical distribution of \( D^2 \). An LD block was defined as a set of contiguous SNPs having \( D^2 \) values exceeding 0.90 between each contiguous pair of SNPs. The boundary of an LD block would be defined by a marker pair with \( D^2 < 0.9 \). Given that individuals of Ashkenazi Jewish ancestry make up approximately 24% of the analytic sample, we further explored differential LD structure between Ashkenazi Jewish ancestry and non-Ashkenazi Jewish ancestry. No statistically significant differences were found (as defined by nonoverlapping 95% CIs for \( D^2 \) between ancestry strata). However, to guard against grouping SNPs within an ancestry that may or may not be in linkage, we used the minimum \( D^2 \) between the non-Jewish and the Jewish groups when defining the LD blocks. The LD blocks for the SNPs within each gene are shown in Supplementary Table S1 along with the MAFs.

**Statistical analysis**

For all analyses, subjects were considered at risk for breast cancer from birth until the first occurrence of breast cancer diagnosis, death, or loss to follow-up. Subjects were censored in the event that they underwent a bilateral prophylactic surgery of the breasts more than 1 year preceding the diagnosis of breast cancer. Bilateral prophylactic surgery of the breasts occurring within a year of breast cancer was considered an event to avoid potential biases resulting from informative censoring. Descriptive breast cancer rates were calculated as the observed number of breast cancers per total patient time at risk and standardized to the age distribution of the study cohort at the time of interview (16). Covariates that vary with time (ovarian cancer and prophylactic ovarian surgery) were treated as time-dependent in the calculation of rates. Thus, a subject who had a diagnosis of ovarian cancer contributed time at risk in the nonovarian cancer group prior to the diagnosis and then time at risk in the ovarian cancer group following the diagnosis. Because subjects were ascertained primarily from high-risk clinics, selection bias is likely to result in an overrepresentation of cases in the analysis sample relative to the general population. To account for potential bias in cumulative risk estimates due to nonrandom sampling from the general population, Kaplan–Meier estimates of the cumulative probability of breast cancer diagnosis were computed using age-specific sampling weights for cases and controls as obtained from Antoniou and colleagues (17).

Cox proportional hazards regression was used to estimate the association between time from birth to diagnosis of breast cancer and haplotypes in genes within the IGF pathway. In this model, the hazard or instantaneous rate of breast cancer diagnosis is modeled as a function of the predictor covariates. The relative risk or HR is then interpreted for each covariate as the proportionate change in the instantaneous rate of diagnosis for 2 individuals differing by a single unit of that covariate. When analyzing LD blocks with multiple SNPs, an additive haplotype effect was assumed where the haplotype composed of the major allele for each individual SNP within the LD block was used as the referent group for comparisons (i.e., 0000). However, when an LD block consisted of an SNP, a general genetic model making no additivity assumption was used. To account for phase uncertainty in haplotype analysis, we used a 2-step approximation to the semiparametric maximum likelihood estimator of Lin and Zeng (18). This method used the expectation–maximization algorithm to compute posterior estimates of the probability of all potential haplotypes for a subject given their known genotype, and these probabilities were used to weight the individual’s contribution to the partial likelihood. A similar approach has previously been applied to logistic regression models for analyzing case–control data and shown to provide robust inference for relatively common haplotypes with little phase ambiguity (19). To account for hierarchical clustering at the individual level (multiple records per individual were analyzed according to the number of potential diplotypes consistent with the individual’s genotype) and the family level (matched controls were often selected from the family of a case), the sandwich estimator of Lin and Wei (20) was used in combination with multiple outputation (15) to obtain robust inference about haplotype associations. All estimates were adjusted for birth cohort.
ancestry, age at first pregnancy, and region of center [North American (US) vs. EU]. Ashkenazi Jewish individuals were considered a separate group because the carriers had only 1 of 3 founder mutations. Age at first pregnancy, prophylactic oophorectomy, and ovarian cancer status were treated as time-dependent covariates in the analysis. To account for potential selection bias through recruitment from high-risk clinics, all regression models employed age-specific sampling probability weights for cases and controls as obtained from Antoniou and colleagues (17). For LD blocks exhibiting significant associations with the time to breast cancer diagnosis, secondary analyses of individual SNPs making up the LD block were conducted. In addition, we considered sensitivity analyses to assess the assumption of additivity for haplotype models and found no quantifiable differences.

In total, the current analysis involved testing of 83 SNPs in 41 LD blocks across the 13 genes, which is likely to result in an inflation of the family-wise type I error rate for the study if unadjusted critical values are used for assessing LD block significance. Noting that this analysis represents a first stage in identifying variants in the IGF signaling pathway that are associated with time to breast cancer diagnosis, we sought to control the family-wise type error rate at 10% to minimize the type II error rate, limiting the possibility of ruling out potentially important LD blocks from future investigation. Simulation was used to estimate the family-wise type I error rate, assuming a correlation of 0.75 across tests. On the basis of 100,000 simulations, it was estimated that an adjusted \( P \) value of 0.010 on any individual LD block test would result in a family-wise type I error rate of 10% for the study. Thus, an adjusted \( P \) value less than 0.010 was interpreted as a significant association.

**Results**

The characteristics of study participants, study sites, and the observed incidence rate (per 1,000 women per year) of breast cancer diagnosis stratified by \( BRCA \) status were previously described (12). Briefly, the age-standardized incidence rate of breast cancer diagnosis was estimated to be 26.94 per 1,000 per year in \( BRCA1 \) carriers (95% CI: 19.79, 34.10) compared with 25.03 per 1,000 per year in \( BRCA2 \) carriers (95% CI: 18.71–31.36). The characteristics of the study participants, limited to non-Hispanic Caucasians, are shown in Table 1. The majority of study subjects in both strata were of non-Jewish ancestry (76%). Of the study subjects, 9% underwent bilateral prophylactic mastectomy and 41% underwent prophylactic bilateral salpingo-oophorectomy. Median age at diagnosis was estimated to be 57.0 years (95% CI: 54.1–62.2) among \( BRCA1 \) carriers and was 70.5 years (95% CI: 67.7–INF) among \( BRCA2 \) carriers. The results are presented grouped by genes in components of the IGF signaling pathway. As described in Materials and Methods, only \( P \) values of less than 0.010 for individual LD blocks were considered significant.

**IGFIR ligands: IGF2 and INS**

In Figure 1, we present the estimated HR for time to diagnosis by LD block and \( BRCA \) status after adjustment for covariates (described in Materials and Methods). For both \( BRCA1 \) and \( BRCA2 \) mutation carriers, significant associations of LD blocks in \( IGF2 \) were observed. Among \( BRCA1 \) carriers, for \( IGF2 \) LD block 3 (defined by SNP

| Table 1. Characteristics of study participants by \( BRCA \) gene |
|------------------|------------------|------------------|------------------|
| Characteristic   | \( BRCA1 \) carriers | \( BRCA2 \) carriers |
| Total, n (%)     | Total, n (%)     | Cases, n (%)     | Cases, n (%)     |
| Ethnicity        |                  |                  |                  |
| Caucasian (non-Jewish, non-Hispanic) | 774 (76) | 283 (74) | 381 (76) | 176 (80) |
| Caucasian (Jewish, non-Hispanic) | 245 (24) | 98 (26) | 119 (24) | 43 (20) |
| Ovarian cancer   |                  |                  |                  |
| Yes              | 111 (11) | 26 (7) | 30 (6) | 5 (2) |
| No               | 908 (89) | 407 (83) | 513 (94) | 233 (98) |
| Bilateral BPO    |                  |                  |                  |
| Yes, before breast cancer diagnosis | 270 | 42 | 103 | 17 |
| Yes, after breast cancer diagnosis | 154 | 154 | 94 | 94 |
| No BPO           | 594 | 184 | 303 | 108 |
| Prophylactic bilateral mastectomy |                  |                  |                  |
| Yes              | 99 | 37 |
| No               | 920 | 463 |

Abbreviation: BPO, prophylactic oophorectomy.
rs1065687), women with at least 1 variant allele were estimated to experience a 76% higher relative risk of diagnosis than women with no variant allele (per-allele HR 1.76; 95% CI: 1.15–2.70; \( P = 0.009 \)). Among BRCA2 carriers, in LD block 5, the global \( P \) value was significant for LD block 4 (\( P = 0.003 \)) and LD block 5 (\( P = 0.0007 \)). In LD block 4, women with haplotype 10 had a more than 2-fold increased risk of breast cancer (HR = 2.20; 95% CI: 1.4–3.5; \( P = 0.0008 \)). In LD block 5, women with haplotypes 0001 (HR = 0.48; 95% CI: 0.30–0.76; \( P = 0.0017 \)), 0100 (HR = 0.40; 95% CI: 0.26–0.61; \( P = 0.00002 \)), and 0101 (HR = 0.39; 95% CI: 0.20–0.79; \( P = 0.009 \)) were estimated to experience a significantly decreased risk of diagnosis when compared with the reference haplotype. Given the significant HRs for the haplotypes in IGF2 LD blocks 4 and 5, we investigated the HRs for the 6 individual SNPs (rs3213232, rs3213229, rs3213223, rs3213221, and rs3213216) within the LD blocks to examine whether the observed haplotype effect could be attributed to any one SNP. Of the 6 SNPs, there was only an effect of carrying 1 copy of the variant allele of rs3213221 (HR = 0.60; 95% CI: 0.40–0.92;
There was no significant association of INS and breast cancer risk among BRCA1 or BRCA2 carriers after adjusting for multiple testing.

**IGFBPs: IGFALS (in the complex with IGFBP3), IGFBP3, IGFBP4, and IGFBP6**

Estimated HRs for the haplotypes of the IGFBPs are shown in Figure 2. No significant global tests of associations were observed for any haplotypes of the 3 IGFBP genes. The SNP in IGFALS was also not associated with risk (see Supplementary Table S1).

**IGFBP proteases: HTRA1, MMP3, MMP7, KLK3, and CTSD**

Estimated HRs for the haplotypes of the IGFBP proteases are shown in Figures 3 and 4. A highly significant

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**Figure 2.** Estimated HRs associated with haplotype presence for IGFBS (A), IGFBP3 (B), and IGFBP6 (C). Linkage blocks (D) were defined as pairwise \( D^2 \geq 0.90 \). Estimates were stratified by BRCA1/2 mutation status (BRCA1 in left column and BRCA2 in right column) and adjusted for birth cohort and ancestry as well as first pregnancy, prophylactic oophorectomy, and diagnosis of ovarian cancer as time-dependent covariates.
Figure 3. Estimated HRs associated with haplotype presence for HTRA1 (A), KLK3 (B), and CTSD (C). Linkage blocks (D) were defined as pairwise $D' \geq 0.90$. Estimates were stratified by BRCA1/2 mutation status (BRCA1 in left column and BRCA2 in right column) and adjusted for birth cohort and ancestry as well as first pregnancy, prophylactic oophorectomy, and diagnosis of ovarian cancer as time-dependent covariates.
Association in HTRA1 LD block 3, composed of 3 SNPs, was observed in BRCA1 carriers (global $P = 0.005$) with no association in BRCA2 carriers (global $P = 0.147$). Within LD block 3, the rare haplotypes grouped together were significantly associated with a 2-fold risk to develop breast cancer (HR = 2.12; 95% CI: 1.30–3.46; $P = 0.0028$). When looking at individual SNPs within HTRA1, no SNP within LD block 3 was associated with risk. There were no significant associations of LD blocks in HTRA1 and risk among BRCA2 carriers.

**Figure 4.** Estimated HRs associated with haplotype presence for MMP3 (A) and MMP7 (B). Linkage blocks (C) were defined as pairwise $D' > 0.90$. Estimates were stratified by BRCA1/2 mutation status (BRCA1 in left column and BRCA2 in right column) and adjusted for birth cohort and ethnicity as well as first pregnancy, prophylactic oophorectomy, and diagnosis of ovarian cancer as time-dependent covariates.
among BRCA2 carriers, carrying the variant allele in rs12571363 in LD block 1 conferred a 2-fold increased risk (per allele HR = 2.03; 95% CI: 1.29–3.21; P = 0.002). Haplotypes in KLK3 and CTSD were not associated with any modification of risk for developing breast cancer. For MMP3 in BRCA2 carriers, a highly significant association (global P = 0.0000003) in LD block 3 was observed because of a combination of rare haplotypes associated with a 5.1-fold higher risk (HR: 5.08; CI: 2.67–9.67; P = 0.0000007; Fig. 4A). None of the 3 SNPs constituting this haplotype, rs476762, rs679620, or rs3025058, could explain the association. For MMP7, after adjusting for multiple testing, there were no significant associations of any LD blocks with risk in BRCA1 or BRCA2 carriers.

**IGF1R downstream docking protein and phosphorylation target: IRS2 and PIK3CB**

There was no significant association of LD blocks in either IRS2 or PIK3CB and breast cancer risk (Fig. 5).

**Discussion**

The IGF pathway signals cells to grow, differentiate, and survive through activation of the phosphoinositol-3-kinase/protein kinase AKT (PI3K/AKT) pathway and the RAS/mitogen-activated protein kinase (RAS/MAPK) pathway. The role of the IGF pathway in breast cancer was reviewed, and a figure of the IGF system and its downstream effectors was presented by Hamelers and Steenbergh (21). This study is a continuation of our

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**Figure 5.** Estimated HRs associated with haplotype presence for IRS2 (A) and PIK3CB (B). Linkage blocks (C) were defined as pairwise \( D > 0.90 \). Estimates were stratified by BRCA1/2 mutation status (BRCA1 in left column and BRCA2 in right column) and adjusted for birth cohort and ancestry as well as first pregnancy, prophylactic oophorectomy, and diagnosis of ovarian cancer as time-dependent covariates.
investigation of the role of genetic variants in IGF signaling as modifiers of breast cancer risk in women who carry deleterious mutations in **BRCA1** and **BRCA2**. We previously investigated only a small number of the genes involved in IGF signaling and reported significant HRs associated with genetic variants in **IGF1R** and **IRS1** in **BRCA1** mutation carriers and in **IGFBP2** and **IGFBP5** in **BRCA2** mutation carriers (12). In this investigation of an additional 13 genes in the pathway, there were significant associations of variants in **IGF2** in both **BRCA1** and **BRCA2** mutation carriers and **HTRA1** in **BRCA1** mutation carriers and **MMP3** in **BRCA2** carriers.

There have been a limited number of epidemiologic studies of the association of sporadic breast cancer risk and genetic variation in the genes in the IGF pathway investigated in this study. In a large study in the NCI breast and prostate cancer cohort consortium of 6,292 breast cancer cases and 8,135 unaffected controls, they reported no significant associations (P < 0.00005) of SNPs in **IGF2**, **IGFALS**, **IGFBP3**, **IGFBP4**, **IGFBP6**, and **IRS2** and breast cancer risk (22). Their results were inconsistent with our observation of significant associations with haplotypes in **IGF2**, which may reflect different SNPs and different population characteristics including ages at diagnosis and **BRCA1** and **BRCA2** carrier status. There have been several studies of the −202 A>C SNP in the **IGFBP3** promoter and breast cancer risk, with most being negative (reviewed in ref. 23). In association studies of SNPs and breast cancer risk in the Cancer Prevention Study II, 3 of 11 SNPs in **IRS2** were associated with breast cancer risk (haplotype OR = 0.76; 95% CI: 0.63–0.92; ref. 24) and the **INS** rs3842752 SNP was not associated with breast cancer risk (25). The **INS** SNP was the same, whereas none of the 11 **IRS2** SNPs were the same as investigated in our study. Two studies have reported significant associations of SNPs in **MMP7** and breast cancer survival, although with different SNPs than investigated here (26, 27). As yet, there are no reported association studies in breast cancer investigating SNPs in **HTRA1**, **PIK3CB**, or **MMP3**.

**IGF1** and **IGF2** are the primary ligands for binding to the **IGF1R**, and **IGF2** levels are higher than **IGF1** levels in cells. Interestingly, in a study of women of 43 women with localized breast cancer and 38 unaffected controls, there were significantly elevated circulating free **IGF2** and **IGF1** levels yet significantly reduced total **IGF2** levels and reduced total **IGF1** levels in the breast cancer cases as compared with controls (28). They also found that **IGFBP2** levels were significantly reduced in the cases with no differences in **IGFBP1**, 3, 4 or 6 levels and suggested that the increase in free **IGF2** levels was because there was less **IGFBP2** to bind **IGF2**. **IGFBP2** and **IGFBP5** preferentially bind **IGF2** over **IGF1**. With higher amounts of free **IGFs**, there can be more signaling activity through **IGF1R**. The significant association with variants in **IGF2** fits with our previous results in which we had observed a significant association with variants at the **IGFBP2**-**IGFBP5** locus in **BRCA2** carriers and at **IGF1R** in **BRCA1** carriers.

Proteolysis of **IGFBPs** occurs through the action of **IGFBP** proteases that include serine proteases (KLKs and **HTRA1**), **MMPs**, and **CTS** (29). With proteolysis of the **IGFBPs**, there is then more bioavailability of the **IGFs** for signaling through **IGF1R**. In **BRCA1** mutation carriers, an **HTRA1** haplotype in LD block 3 was significantly associated with breast cancer (global P = 0.0005). **HTRA1**, located at 10q25.3-q26.2, was identified in 1996 as a serine protease specific for **IGFBPs** (30). **HTRA1** is also now thought to function as a tumor suppressor gene (31), possibly through regulation of TGF-B signaling (32). None of the 4 SNPs in the LD block were significantly associated with breast cancer, suggesting that the associated haplotype only marks the causal variant in the region. Further investigation of this region is needed to understand how it may be modifying risk of developing breast cancer in **BRCA1** mutation carriers. A group of rare haplotypes (combined frequency of 2%) in **MMP3**, another **IGFBP** protease, was significantly associated with breast cancer in **BRCA2** carriers (HR = 4.33). For **MMP3**, many more carriers need to be investigated to determine the actual associated haplotype within the grouped haplotypes, as neither of the 2 SNPs in the LD block showed association.

**PIK3CB** is a 110-kDa catalytic subunit of PI3K and is activated through the **IGF1R** as follows: Binding of **IGF** induces receptor autophosphorylation of the β-subunit of **IGF1R**, resulting in activation of the tyrosine kinase activity and subsequent tyrosine phosphorylation of **IRS1**. The activated **IRS1** binds the p85 α-subunit **PI3K**, which, in turn, activates **PIK3CB** for downstream signaling. **PTEN** regulates **PI3K** signaling by dephosphorylating **PI3K**, so in the absence of **PTEN**, the pathway is activated. In cells without **PTEN**, experiments have shown that **PIK3CB** is necessary for continued **PI3K** signaling (33). **PIK3CB** may be particularly relevant to cancer in **BRCA1** carriers because a majority of **BRCA1** and other basal-like breast cancers have loss of **PTEN** expression (34). Furthermore, this suggests a possible therapeutic target in **BRCA1** carriers. Clinical trials are ongoing with agents targeting the inhibition of the **IGF1R** and mTOR, although none are targeted to **BRCA2** mutation carriers. In **BRCA1** carriers, there was a marginal association of genetic variation in **PIK3CB** and breast cancer. The intronic SNP rs9878820 was associated with breast cancer (per allele HR = 0.83, CI: 0.70–0.99, P = 0.04; homozygous recessive HR = 0.69, P = 0.04), and was more strongly associated in the larger data set including Hispanics, African Americans, and others (P = 0.008).

Although this study provides an important continued step in identifying potential genetic modifiers of risk among **BRCA1** and **BRCA2** mutation carriers, there are some limitations. First, the sample size was limited, particularly for **BRCA2** carriers, so that there may be associations that we could not detect and the point estimates for the associations we did detect may be imprecise, particularly for SNPs and haplotypes of low frequency. Second, the loci we identified in this study...
that had significant HRs for risk were not previously identified as the top candidates in the genome-wide association study (GWAS) of BRCA1 (35) or BRCA2 mutation carriers (36). However, even known associations can be missed in GWAS, as only the top hits are further investigated through replication and imputation of additional SNPs in those regions. One example is the previous finding that a promoter SNP in RAD51C (37) is a modifier of risk in BRCA2 carriers with an HR of 3.2 among the rare homozygotes, yet it was not identified in the GWAS (36). Therefore, it is possible that one or more of the associations identified here were missed in the GWAS. Third, although the IGF pathway was a priori hypothesized as a source for potential modifiers, multiple LD blocks were considered for association testing and such testing could lead to inflation of the overall type I error rate for the study. With this being said, we studied only a relatively small number of the genes in IGF signaling that we a priori deemed would potentially play a role in the time to diagnosis and considered significant only those associations that decreased beyond a multiple comparison-adjusted critical value. In addition, we view the goal of the current research as hypothesis refinement where, based upon the results from this well-defined set of genes, we will further validate the results using an independent sample. As with all observational studies, there is the potential for selection bias and unmeasured confounding. However, we have adjusted for those factors that are most likely to influence the risk of breast cancer diagnosis within this cohort, thus lowering the potential for unadjusted confounding.

We and others have investigated putative risk factors, and a number of published studies have implicated loci as modifiers of breast or ovarian cancer penetrance in women who carry germline BRCA1 or BRCA2 mutations (12, 35–42). The majority of modifiers of BRCA1 cancer risk identified to date have been genetic variants in proteins that interact with BRCA1 in DNA repair. Our results suggest that IGF signaling also modifies breast cancer penetrance in BRCA1 and BRCA2 mutation carriers. IGF signaling and BRCA1 directly interact with multiple lines of evidence, including that IGF-1 enhances BRCA1 activity (43), BRCA1 regulates IGFR1 and IRS1 activity (44, 45), and BRCA1 protein expression and localization are dependent on AKT activation (46).

In conclusion, this is the second study to investigate the role of genetic variation in IGF signaling and breast cancer risk in women carrying deleterious mutations in BRCA1 and BRCA2. In the previous study, we identified significant associations in variants in IGFR1 and IRS1 for BRCA1 carriers and in IGFR1, IGFBP2, and IGFBP5 in BRCA2 carriers. On the basis of those results, and given the known interaction of BRCA1 and IGF signaling, we investigated additional variants in genes in this pathway and identified significant breast cancer risk modifiers with haplotypes in IGFR2 for both BRCA1 and BRCA2 mutation carriers and in HTRA1 and MMP3 for BRCA1 and BRCA2 mutation carriers, respectively. The identification of causal mechanisms within these loci is needed to validate and better understand these associations. We hope that with further identification of genetic factors that modulate age at diagnosis and overall incidence of breast cancer, and better elucidation of the actual mechanisms of modifying risk, these data can be used in the future to provide more precise risk estimates to develop cancer and design targeted therapeutics for carriers.

Disclosure of Potential Conflicts of Interest

T.R. Rebbeck is the Editor-in-Chief of Cancer Epidemiology, Biomarkers & Prevention. In keeping with the AACR’s Editorial policy, the manuscript was peer reviewed and a member of the AACR’s Publications Committee rendered the decision concerning acceptability. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the State of Nebraska or the Nebraska Department of Health and Human Services.

Acknowledgments

The MAGIC Consortium includes the following centers and individuals: Baylor-Charles A. Sammons Cancer Center (J.L. Blum, MD, PhD); Estelle Brothers, RN; G. Ethington), Beckman Research Institute of the City of Hope (formerly at the University of California, Irvine, S.L. Neuhausen, PhD; L. Steele; J.N. Weitzel, MD; S. Sand), Beth Israel Deaconess Medical Center (N. Tung, MD), Creighton University (C. Snyder, BA; H.T. Lynch, MD; P. Watson, PhD), Dana Farber Cancer Institute (K. Stoeckert; J.E. Garber, MD MPH), Fox Chase Cancer Center (M.B. Daly, MD, PhD; A.K. Godwin, PhD), Georgetown University (C. Isaacs, MD), Jonsson Comprehensive Cancer Center at the University of California, Los Angeles (J. Seldan, MSGC; P.A. Ganz, MD), Mayo Clinic (F. Couch, PhD), Northshore University HealthSystem Center for Medical Genetics (C. Selkirk, S.M. Weissman, W.S. Ruhnstein, MD, PhD), University of Chicago (S. Cummings; O. Olopade, MD), University of Pennsylvania HealthSystem (S. Domchek, MD), University of Texas, San Antonio (G. Tomlinson, MD), University of Vienna (C. Singer, MD), University of Wisconsin (M.B. Daly, MD, PhD), University of Pennsylvania (M.K. Isaacs, MD), University of Washington (O. Olopade, MD), University of Wisconsin-Madison (G. Tomlinson, MD), University of Zurich (F. Couch, PhD), University of California, San Francisco (F. Couch), University of Southern California (A. Narod, MD).

Grant Support

Support was received from NIH grants SUDI CA66389 (to H.T. Lynch, R01-CA80955 and R01-CA174415 (to S.L. Neuhausen), R01-CA135691 (to D.L. Gillen), R01-CA102776 and R01-CA80855 (to T.R. Rebbeck), R01-CA153328 (to J.N. Weitzel), R01CA100223 and S01CA113916 (to A. Godwin), R01-CA128978 and R01-CA166167 (to F. Couch), and U01CA69631 (to M.B. Daly) and a grant from the Breast Cancer Research Foundation (to F. Couch). This publication was supported in part by revenue from Nebraska cigarette taxes awarded to Creighton University by the Nebraska Department of Health and Human Services. S.L. Neuhausen is partially supported by the Morris and Horowitz Families Endowment.

Received December 23, 2010; revised April 22, 2011; accepted June 16, 2011; published OnlineFirst June 27, 2011.

Cancer Epidemiology, Biomarkers & Prevention

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Genetic Variation in \textit{IGF2} and \textit{HTRA1} and Breast Cancer Risk among \textit{BRCA1} and \textit{BRCA2} Carriers

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