**Short Communication**

**BRCA1 Susceptibility Markers and Postmenopausal Breast Cancer: The Iowa Women’s Health Study**


Division of Epidemiology [J. A. T., P-L. C., A. R. F.] and Department of Medicine [R. A. K., W. S. O., C. A.], University of Minnesota; Wake Forest University School of Medicine [S. S. R.]; and Department of Health Sciences Research, Mayo Clinic Cancer Center, Mayo Clinic, Rochester, Minnesota 55905 [T. A. S.]

**Abstract**

Much research on early-onset breast cancer families has been performed and has shown that breast cancer in many of these families is linked to either **BRCA1** or **BRCA2**. Fewer studies have examined the role of genetic predisposition in postmenopausal breast cancer. A nested case-control family study of breast cancer was conducted within the Iowa Women’s Health Study, a population-based prospective study of 41,836 postmenopausal women. Proband case-control were 251 incident cases diagnosed between 1988 and 1989. Three-generation pedigrees were developed through mailed questionnaires. From this collection of pedigrees, thirteen were identified for further detailed genetic analysis. Sibling-pair linkage analyses were performed using polymorphic markers in candidate regions in these 13 families with multiple cases of breast and other cancers. Four of the DNA markers are located on chromosome 17, and two of these (D17S579 and THRA1) flank the **BRCA1** locus. Significant evidence for linkage to D17S579 was obtained in the total sample, in a model without inclusion of covariates or age at onset (P = 0.005), and in a model adjusted for five measured covariates and for variable age at onset (P = 0.008). Complete sequencing of the **BRCA1** gene in these families, including all intron/exon boundaries, failed to reveal any mutations in 24 women with breast cancer from the 13 families. These data suggest that in some families identified by postmenopausal breast cancer cases, breast cancer risk may be mediated by a gene (or genes) in the **BRCA1** region, but not **BRCA1** itself.

**Introduction**

A family history of breast cancer has been shown to increase a woman’s risk of the disease. For some women, a family history of breast cancer represents inherited susceptibility, which is estimated to be responsible for approximately 5% of all cases (1). This suggests that about 1 in 160 women will develop breast cancer due to an inherited predisposition. Risk increases as age of onset in the affected family member decreases (2–4), but risk remains elevated in women whose primary relatives were diagnosed with late-onset breast cancer (5–7). In the IWHS,3 risk to sisters of postmenopausal breast cancer cases was increased for both early-onset (relative risk = 1.41) and late-onset (relative risk = 1.81) disease after controlling for measured risk factors (7). Segregation analyses have also supported the role of a major gene in late-onset breast cancer (8, 9). Genetic factors may therefore be relevant to late-onset breast cancer development.

Much research has been published on genetic factors in early-onset disease. Recently, there has been considerable focus on the **BRCA1** locus, a site at which mutation carriers are predisposed to developing breast and ovarian cancers. Estimates of the frequency of women carrying **BRCA1** mutations range from 1 in 200 to 1 in 2000 (10), and the risk of developing breast cancer by age 70 among carriers has been estimated to be up to 80% (11). Although some of the variation in the occurrence of breast cancer associated with **BRCA1** mutations may be due to differences in the mutations [with at least 254 mutations identified to date (12)], variable penetrance suggests that other genetic and environmental factors contribute to the development of the disease. One example is HRAS1 variable number tandem repeats that have been shown to modify the risk of ovarian cancer (but not breast cancer) in **BRCA1** mutation carriers (13). Narod et al. (14) also reported on risk modifiers of **BRCA1** mutations, including reproductive factors such as age at menarche (below age 12), parity (<3), and year of birth (after 1930). These factors could not explain the total variability of expression and penetrance. Beyond the findings of a few reports, it remains uncertain what factors, modifiable or not, may delay or prevent disease onset in **BRCA1** mutation carriers.

Anthropometric variables (15) and the number of pregnancies have also been shown to affect the risk of breast cancer (15–17). We previously showed in this population of women that the increase in risk of breast cancer associated with a high WHR or low parity is more pronounced among women with a family history of breast cancer compared with those without such a family history (17), especially for breast/ovarian cancer (18). These data suggest that genetic factors may be relevant to late-onset breast cancer and that heterogeneity may be influenced by nongenetic risk factors.

Mutations in the **BRCA1** gene are estimated to account for nearly one-half of early-onset breast cancer families (19). Most of the studies examining the role of **BRCA1** have identified...
linkage and mutations in families in premenopausal cases (20, 21). There have been few linkage studies in families with late-onset disease, and those results have not provided significant evidence for linkage to \textit{BRCA1} (22). Barker et al. (23), however, reported a \textit{BRCA1} mutation identified from a population-based set of families selected without respect to age at onset. Three breast cancer cases showed strong family histories of breast cancers, with ages of onset similar to cases considered “sporadic.” The \textit{BRCA1} mutation (R841W) found in these families is thought to exert less dramatic effects on protein structure than other mutations and thus may exhibit a phenotype that has a later age at onset of breast cancer. These results suggest that when only families with early-onset disease are chosen for \textit{BRCA1} screening studies, the older onset families that may carry less severe but important mutations are missed, perhaps reaching 1% of cases in the population. Indeed, it has been suggested that many of the pedigrees studied in \textit{BRCA1} research have not been typical and that mutations with milder effects (24) that include cases with older ages of onset (25) may be more common.

Other genes implicated for increased risk of breast cancer include \textit{INT2}, coding for a fibroblast growth factor (chromosome 11q), the estrogen receptor locus (\textit{ESR}, 6q) and \textit{p53}, a tumor suppressor gene (17p). \textit{MLH1} (3p) and \textit{MSH2} (2p) may also be relevant in breast cancer because inherited mutations in \textit{MSH2} are associated with the Muir Torre syndrome (26), which has breast cancer as part of the phenotype. In an effort to determine the importance of these various genes in breast cancer, the current study evaluated evidence for linkage of 12 polymorphic markers in six breast cancer candidate regions (2p, 3p, 6q, 11q, 17p, and 17q). Families selected for analysis represent a subset of a population-based cohort. We evaluated evidence for linkage at these loci in 13 three-generation families with multiple cases of postmenopausal breast cancer. Age at onset and selected environmental covariates were included in the genetic analyses.

\textbf{Materials and Methods}

\textbf{Study Population.} The IWHS is a population-based cohort study designed to identify risk factors for postmenopausal cancer (27). In 1986, a questionnaire was mailed to approximately 100,000 female Iowa residents between the ages of 55 and 69 years with a valid driver’s license. Women reporting at baseline previous cancers other than skin cancer, a prior mastectomy, or a menstrual period within the last year were excluded. The total cohort at risk for incident breast cancer was 37,105 women. Incident breast cancer cases were identified through the Health Registry of Iowa, part of the National Cancer Institute’s Surveillance, Epidemiology, and End Results program. In 1988 and 1989, there were 265 incident breast cancers in this cohort. A pedigree development form was mailed to index cases to identify first-degree female relatives (mother, sisters, and daughters). First-degree relatives received a mailed health history questionnaire to ascertain risk factor information: (a) alcohol use; (b) body measurements; (c) history of benign breast disease; (d) education level; (e) menstrual factors; (f) oral contraceptive use; and (g) pregnancy history. To assess body fat distribution, a paper tape measure was enclosed along with detailed instructions for measuring waist and hip circumferences. These measurements were used to calculate WHR. Reliability and accuracy of self-measurements of waist and hip in the IWHS are good (28). Self-reports of cancer in first-degree relatives were confirmed by medical record review.

\textbf{Pedigree Extension and Blood Collection.} Thirteen families with at least two first-degree relatives with breast cancer were identified. Twelve of these families were ascertained through index cases (probands) with postmenopausal breast cancer. One family was identified through a study participant who did not have breast cancer but had a family history of breast cancer. A genetic counselor extended each pedigree using standard protocols. Updated information was obtained on name, address, age, vital status, and cancer status of index cases’ second-degree female and male relatives and their spouses. The phenotype for analysis was defined as breast cancer, but cancers of the endometrium, cervix, ovaries, prostate, and colon were observed in some of these families.

Potentially informative individuals were invited to provide blood samples for genetic analysis. Informative persons included the index case and her mother, sisters, and daughters. If sisters or daughters with breast cancer were deceased, their available spouses and offspring were invited to have blood drawn to permit inference of their genotype. A blood kit that contained a cover letter, consent form, instructions for sample shipment, blood collection tubes, and a protective Styrofoam container was mailed to each individual. Subjects took the blood kits to their personal physicians for venipuncture and return shipment.

\textbf{Genotyping.} Genomic DNA was isolated from whole blood for genetic analysis using standard procedures. Genotypes were determined for the following microsatellite genetic markers: (a) chromosome 17q [\textit{THRA1} (29), \textit{D17S250} (30), \textit{D17S579}, and \textit{D17S588} (31)]; (b) 17p [two markers for \textit{p53} (32)]; (c) 11q [\textit{INT2} (33)]; (d) 6q [\textit{ESR} (34)]; (e) 3p [\textit{D3S1277} and \textit{D3S161}] (35); and (f) 2p [\textit{D2S123} and \textit{D2S119}] (36).

Amplification of the microsatellite markers was performed using 10 ng of genomic DNA, 2 \textit{mM} MgCl{	exttwosuperior}, 100 \textmu M deoxyribonucleotide triphosphates, 1 \texttimes buffer (Promega), 0.5–2.0 pmol of the sense and antisense primers, 0.1 pmol of the fluoridated sense primer for each pair, and 0.5 unit of Taq polymerase (Promega) in a total volume of 5 \textmu l. The PCR cycles were 3 min at 95°C for initial denaturation, followed by 30 s each at 95°C, 55°C, and 72°C and a final cycle of 3 min at 72°C for the final extension. The amplified products were separated on a 6% denaturing polyacrylamide gel on an automated DNA sequencer (Model 4000; LI-COR, Inc, Lincoln, NE) using a fluorescence detection system (37). The bands were visually inspected and analyzed densitometrically.

\textbf{DNA Sequencing.} When preliminary results suggested evidence of linkage to markers near the \textit{BRCA1} locus, we then examined the \textit{BRCA1} gene for inherited mutations in the 24 women with breast cancer available from the 13 families, including 11 index cases and 13 first-degree relatives (Table 1). This included all exons and intron/exon boundaries. Sequencing reactions were done on PCR-amplified templates (38). Individual exons were amplified using genomic DNA with the exception of exons 10, 11, 14, 16, 18, and 27, in which multiple amplification and sequencing reactions were required. Amplification primers for \textit{BRCA1} were from Friedman et al. (39) and this report. The forward primer for each PCR reaction contains an M13 tail on the 5’ end of the primer (40). Sequencing was done using fluorescence-based DNA sequencing, and the banding pattern was visualized using a LI-COR model 4200 Automated DNA sequencer (37).

\textbf{Statistical Analysis.} Descriptive characteristics of each family member (vital status, age at death, age at onset, age at exam, and number of relatives) were determined.

We performed sibling-pair linkage analysis on these families using the SIBPAL program in the S.A.G.E. computer package (41), an implementation of the Haseman-Elston approach to detection of linkage for both qualitative and quantitative traits (42, 43). This method permits investigation of the genetic component of a trait or disease without specification of the underlying mode
of transmission. It is based on the principle that although siblings share on average 50% of their genes IBD at a given genetic locus, sibling pairs who are concordant on disease status would be expected to share more alleles IBD at the disease-predisposing locus than siblings discordant on disease status. A test for genetic linkage is thereby given by a regression of the sibling-pair squared trait difference (trait = breast cancer status) on the estimated proportion of alleles at a particular marker locus shared IBD. The statistic for testing linkage is a one-sided t test of the estimated regression coefficient divided by its SE (42, 43).

In addition to simple models including only disease status and alleles IBD, models with adjustment for age at onset of breast cancer and nine breast cancer risk factors (covariates) were also fitted: (a) model 1, no adjustment for variable age at onset or covariates; (b) model 2, adjustment for age at onset; (c) model 3, adjustment for nine selected covariates; and (d) model 4, adjustment for age at onset and five covariates identified as significant in logistic regression.

The risk factors included alcohol use (g/day), age at first pregnancy (years), BMI (kg/m²), history of benign breast disease (ever/never), education level (grade school, less than high school, high school, some college, college graduate, or graduate school), age at menarche (years), menstrual status (premenopausal/postmenopausal), number of pregnancies, use of oral contraceptives (ever/never), and WHR.

**Results**

Table 1 presents the characteristics of members from these 13 families. There were 227 relatives identified, with 21 confirmed breast cancer cases among first-degree female relatives and 1 self-reported case in a second-degree female relative. Each pedigree contained two or more breast cancer cases. The mean age at onset in index cases was 65.0 ± 2.5 years among first-degree female relatives and 13.5 ± 5.8 years in first-degree female relatives. Most cases were diagnosed after age 55 years. Nine cancers other than breast cancer were identified in first- and second-degree relatives. Most cases were diagnosed after age 55 years. Nine cancers other than breast cancer were identified in first- and second-degree relatives.

A total of 74 informative relatives (11 male and 63 female) were invited to provide blood samples. Three subjects refused to participate in venipuncture, and six individuals did not return the blood kit after three reminder contacts over a 6-month period. DNA marker genotypes were determined on the final set of 65 samples. Genotypes of nine breast cancer cases were not determined because they were deceased (n = 7) or refused to participate (n = 2).

Table 2 shows the proportion of alleles shared IBD at each of the 12 markers analyzed and the statistical evidence for linkage based on four models for three groups of sibling-pairs: (a) concordant unaffected; (b) discordant on cancer status; and (c) discordant on cancer status. Model 1, which is unadjusted for age at onset or covariates, revealed evidence for linkage to the chromosome 17 marker D17S579. Note that the proportion of alleles IBD is greatest for the relative pairs concordant on disease status at this marker locus. Because breast cancer occurs mainly in older women, and these families were selected from a study of postmenopausal females, model 2 included adjustment for variable age at onset (or age at exam). None of the results were statistically significant, although D17S579 yielded the lowest P (0.15). Next, epidemiological risk factors were added to the model in an attempt to eliminate the amount of disease concordance attributed to shared nongenetic factors. Model 3 included education, history of benign breast disease, age at menarche, menstrual status, age at first pregnancy, number of pregnancies, oral contraceptive use, BMI, and WHR (the maximum number of covariates allowed by the program) without adjustment for age at onset. D17S579 was the only locus demonstrating evidence for linkage. The fourth model included adjustment for age at onset and five covariates (the maximum number allowed by the program) that had been previously reported to be the most significant in this study population (7): (a) age at menarche; (b) menstrual status; (c) use of oral contraceptives; (d) BMI; and (e) WHR. Again, D17S579 provided the only evidence of linkage in the total sample, with a P of 0.008.

Because the D17S579 locus is near BRCA1, sequencing of the BRCA1 gene was performed to determine whether BRCA1 was responsible for the linkage results. No mutations were found in any of the samples tested. Thus, it appears unlikely that the observed linkage is a reflection of inherited truncating mutations in the BRCA1 gene.

**Discussion**

We present results from a sibling-pair analysis of postmenopausal breast cancer in 13 population-based families from Iowa. Results from multiple analyses consistently indicate evidence for linkage to D17S579, a marker flanking BRCA1. Even without adjustment for environmental covariates or age at on-
set, evidence for linkage was detected at D17S579. The only other suggestive results were obtained at other markers also close to BRCA1 [THRA1 (also flanking BRCA1) and D17S250]. No other DNA markers tested here provided evidence of linkage to breast cancer in these families.

The function of BRCA1 is unknown, but recent studies suggest that the protein may act as a transcription factor and may be involved in the cellular mechanisms of DNA repair and maintenance of genome integrity (44–47). Normal functioning BRCA1 protein inhibits breast cell growth in vitro, and transfection of wild-type BRCA1 into MCF-7 breast cancer cells inhibits tumor formation in nude mice (48). Moreover, BRCA1 levels increase during pregnancy (49). These studies suggest that the protective effect of pregnancy may be mediated by BRCA1 through inhibition of breast tissue proliferation. Narod et al. (14) provided evidence that increasing parity reduced the lifetime risk of cancer in a historical cohort study of 333 BRCA1 mutation carriers, but the variables did not account for the total penetrance variability. Thus, there are likely to be other factors that mediate expression of inherited susceptibility. Identification of modifiable risk factors that can delay or prevent onset clearly has important implications for mutation carriers and their families.

Despite the evidence for linkage of breast cancer to the BRCA1 region, direct sequencing of the gene failed to reveal evidence for truncating mutations in the gene. Therefore, one must conclude that either the BRCA1 gene is not involved, that nontruncating alterations in the BRCA1 gene outside of the coding regions (variants of uncertain significance) may be involved, or that another gene in the same chromosomal location is involved. For example, the gene for insulin-like growth factor-binding protein-4 is located between D17S579 and THRA1 (50). Given the hormonal and growth factor perturbations associated with high WHR, genetic variation in the gene for insulin-like growth factor-binding protein would be important to investigate (51). Alternatively, given the relatively small sample size and the nominal statistical significance of the linkage analysis, the results are also consistent with chance.

Limitations of these data include the small number of families available for analysis. Larger families with multiple late-onset breast cancer cases may have been helpful in providing more informative results for stratified analysis. Four of the families in this sample reported cancers other than breast cancer. In this set of families, the average age of second-degree female relatives was approximately 38 years, so these relatives were at low risk of disease and were too young to accurately identify their breast cancer susceptibility phenotype. Finally, these data were analyzed before BRCA1 had been localized, so markers selected for linkage are now known to be within the gene. The fact that no mutations were found after sequencing of the coding region of the BRCA1 gene negates this limitation. Similarly, BRCA2, another breast cancer susceptibility gene on chromosome 13, was not yet identified; this study included no markers on chromosome 13. Nonetheless, we sequenced the BRCA2 gene and detected no mutations. The gene for Cowden disease, which can be associated with elevated breast cancer risk, has recently been localized to chromosome 10q (52), another locus for which no markers were typed in this study. With recent identification of these genes and the multitude of studies examining mutations, it is now possible to more precisely examine the relationship between postmenopausal breast cancer and genes that predispose women to breast cancer.

In summary, these data provide supportive evidence for linkage of families with postmenopausal breast cancer to a marker near BRCA1, suggesting that risk of late-onset breast cancer may be mediated by BRCA1-linked genes, but not likely BRCA1 itself.

References

<table>
<thead>
<tr>
<th>Locus</th>
<th>Proportion of alleles IBD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concordant unaffected</td>
<td>Discordant</td>
</tr>
<tr>
<td>D2S119</td>
<td>0.43</td>
<td>0.46</td>
</tr>
<tr>
<td>D2S123</td>
<td>0.38</td>
<td>0.47</td>
</tr>
<tr>
<td>D3S1277</td>
<td>0.49</td>
<td>0.51</td>
</tr>
<tr>
<td>D3S1611</td>
<td>0.55</td>
<td>0.54</td>
</tr>
<tr>
<td>INT2</td>
<td>0.48</td>
<td>0.51</td>
</tr>
<tr>
<td>ESR</td>
<td>0.46</td>
<td>0.55</td>
</tr>
<tr>
<td>P531</td>
<td>0.45</td>
<td>0.43</td>
</tr>
<tr>
<td>P532</td>
<td>0.50</td>
<td>0.45</td>
</tr>
<tr>
<td>D17S579</td>
<td>0.59</td>
<td>0.37</td>
</tr>
<tr>
<td>THRA1</td>
<td>0.57</td>
<td>0.44</td>
</tr>
<tr>
<td>D17S250</td>
<td>0.51</td>
<td>0.45</td>
</tr>
<tr>
<td>D17S588</td>
<td>0.58</td>
<td>0.50</td>
</tr>
</tbody>
</table>

Model 1:  
Model 2:  
Model 3:  
Model 4:  

\(^a^\) Model 1, no adjustment for measured covariates and variable age at onset.  
\(^b^\) Model 2, adjustment for variable age at onset.  
\(^c^\) Model 3, adjustment for nine measured covariates (education, benign breast disease, age at menarche, menopausal status, age at first pregnancy, number of pregnancies, oral contraceptive use, BMI, WHR).  
\(^d^\) Model 4, adjustment for covariates identified as significant in logistic regression (age at menarche, menstrual status, oral contraceptive use, BMI, and WHR) and variable age at onset.