Evaluation of linkage of breast cancer to the putative BRCA3 locus on chromosome 13q21 in 128 multiple case families from the Breast Cancer Linkage Consortium

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The known susceptibility genes for breast cancer, including BRCA1 and BRCA2, only account for a minority of the familial aggregation of the disease. A recent study of 77 multiple case breast cancer families from Scandinavia found evidence of linkage between the disease and polymorphic markers on chromosome 13q21. We have evaluated the contribution of this candidate ''BRCA3'' locus to breast cancer susceptibility in 128 high-risk breast cancer families of Western European ancestry with no identified BRCA1 or BRCA2 mutations. No evidence of linkage was found. The estimated proportion (α) of families linked to a susceptibility locus at D13S1308, the location estimated by Kainu *et al.* [(2000) *Proc. Natl. Acad. Sci. USA* 97, 9603–9608], was 0 (upper 95% confidence limit 0.13). Adjustment for possible bias due to selection of families on the basis of linkage evidence at BRCA2 did not materially alter this result ($\alpha = 0$, upper 95% confidence limit 0.18). The proportion of linked families reported by Kainu *et al.* (0.65) is excluded with a high degree of confidence in our dataset [heterogeneity logarithm of odds (HLOD) at $\alpha = 0.65$ was -11.0]. We conclude that, if a susceptibility gene does exist at this locus, it can only account for a small proportion of non-BRCA1/2 families with multiple cases of early-onset breast cancer.

Several genes are known to predispose to breast cancer. In the context of large multiple case families, the BRCA1 and BRCA2 genes are numerically the most important, accounting for most families segregating both early-onset breast cancer and ovarian cancer. However, as many as 60% of families with site-specific female breast cancer cannot be explained by BRCA1 and BRCA2 (1, 2). Moreover, population studies have demonstrated that these genes only account for ≈15% of the overall familial risk of breast cancer (3, 4). Even after allowing for other susceptibility genes that confer increased risk in the context of familial cancer syndromes, including TP53 (Li Fraumeni), PTEN (Cowden), and ATM (ataxia telangiectasia), at least 80% of familial breast cancer risk is not explained by known genes, suggesting that other important susceptibility genes remain to be mapped.

Outside the context of these specific syndromes, known genes other than BRCA1/BRCA2 do not appear to account for a substantial proportion of high-risk breast cancer families. Linkage analysis in a set of 56 families with 3 or more cases of breast cancer yielded no evidence for a significant role of PTEN,

Abbreviation: HLOD, heterogeneity logarithm of odds.

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although an attributable fraction of up to 35% could not be ruled out in a family set of this size (5). However, direct mutation testing of the PTEN gene in a subset of these families has failed to identify any mutations, lending further support to the linkage results indicating that this locus is unlikely to account for a significant fraction of hereditary breast cancer.

To date, few additional candidate breast cancer susceptibility loci have been identified in families not attributable to any of the known genes. A potential susceptibility locus on chromosome 8p12–8p22 was identified through targeted linkage analysis of a region of frequent loss in breast tumors (6, 7). However, our analysis of a larger family series did not support the contribution of a putative gene at this locus to more than a small proportion [HLOD = 0.03, $\alpha = 0.03$, upper 95% confidence limit (CL) 0.30] of high-risk families (8).

These findings illustrate the difficulties inherent in efforts to identify additional susceptibility genes for a disease with high population prevalence. First, breast cancer is a genetically heterogeneous disease, and it is likely that there are multiple genes remaining to be identified among non-BRCA1/BRCA2 families, with any one accounting only for a small proportion of such families. Second, in moderate-size families with a mixture of cases diagnosed at early and late ages, chance familial clustering of cases may confound linkage-based approaches. Finally, penetrances of additional breast cancer susceptibility genes are likely to be lower than those associated with BRCA1 and BRCA2 (9). Thus, analysis of a large family series with stringent selection criteria is required to achieve sufficient statistical power for unambiguous localization of novel susceptibility loci and meaningful evaluation of candidate genomic regions. To surmount these obstacles, our international collaborative group [Breast Cancer Linkage Consortium (BCLC)] has accrued, and continues to accrue, a collection of families appropriate to address the problem.

Recently, Kainu *et al.* (10) reported evidence for a novel breast cancer susceptibility locus on chromosome 13q21. They studied 77 families with multiple cases of breast cancer from Finland, Sweden, and Iceland in which no germline BRCA1 or BRCA2 mutations had been identified. Families were not specifically selected for early onset disease, nor were they excluded if one or more cases of ovarian cancer were present.

Initial analysis by comparative genomic hybridization (CGH) of tumors from 23 of these families and 14 others not analyzed further by linkage identified loss of 13q21–31 as a frequent and early event. Consistent loss of 13q21 in all five tumors from one family delineated a minimal region of haplotype sharing in these individuals as the target locus for a susceptibility gene. However, no evidence was presented for specific loss of the wild-type allele in these tumors, as would be expected for the underlying genetic model (inactivation of a tumor suppressor gene).

Genetic linkage analysis using 23 microsatellite markers from this region revealed supportive evidence of linkage to breast cancer. A maximum multipoint HLOD of 3.46 was found at marker D13S1308, with an estimated 65% of families linked. This marker lies ≈25 cM distal to *BRCA2* on chromosome 13q. Simulation studies to account for the possible confounding of linkage results by the proximity of these loci indicated that the linkage was unlikely to be the result of unidentified *BRCA2* mutations in a subset of families. However, the evidence for linkage was confined to a single pair of tightly linked markers (D13S1308/D13S1296) in this region, with linkage evidence dropping off quite rapidly surrounding this peak; indeed markers flanking a 2.1-cM region surrounding this peak yielded negative two-point LOD scores at recombination fractions up to 20%.

We present results from our attempt to confirm this linkage result through analysis of our series of 128 breast cancer families. In the remainder of this article, we refer to this locus as "BRCA3," the quotation marks serving to emphasize the uncertainty regarding the existence and location of one or more such susceptibility loci.

Table 1. Summary of the families used in the 13q21 analysis

	Number of breast cancer cases in family				
Age of diagnosis	<3	3	4	5	>5
<50 years	51	48	19	5	5
<60 years	0	58	39	14	17
All cases:	0	26	36	25	41
Cases sampled/ genotyped	26	68	20	9	5

Entries are the number of families with the specified number of breast cancer cases of the indicated diagnostic criteria and sample availability.

Methods

Families. Families were ascertained from cancer genetics or oncology centers in Europe (United Kingdom, Germany, Spain, Netherlands, France, and Israel), the United States, Australia, and Canada. One family was from Mexico. All families were Caucasian except the Mexican family that was of mixed European–Amerindian descent. Only families in which at least three women were diagnosed with breast cancer under age 60 years were eligible for the study. We excluded families in which cases of either ovarian cancer or male breast cancer were observed, because these phenotypes are strong predictors of *BRCA1* or *BRCA2* mutations (1). Within these 128 families a total of 650 women were affected with breast cancer (median 5 per family); 56% of these cases were diagnosed under age 50. Samples from 409 affected individuals and 293 unaffected relatives were available for genotyping. Table 1 shows the characteristics of the families in more detail.

Exclusion of BRCA1 and BRCA2. At least one breast cancer case from each family was screened for mutations in *BRCA1* and *BRCA2*, including all coding exons and splice junctions; in general, the sampled case with the youngest age at diagnosis was screened. This screening was performed using a variety of methods, including heteroduplex analysis (HDA), conformation sensitive gel electrophoresis (CSGE), and direct sequencing. Families from The Netherlands were also screened for the large genomic rearrangements that are known Dutch founder mutations, as these would not be detected by standard PCR-based screening methods. Other families were also tested for population-specific mutations, where appropriate. Overall, we estimate that, taken together, these methods have an average sensitivity of 0.70 (1, 11).

Genotyping. Genotyping was carried out at the International Agency for Research on Cancer (62 families), Institute of Cancer Research (49 families), and University of Leiden (17 families). Genotypes were generated for 16 microsatellite markers within a 32-cM region of chromosome 13q21 spanning both BRCA2 and the putative "BRCA3" locus (see Table 2). Not all centers genotyped all markers; Table 2 gives details on which loci were genotyped at each center. Microsatellite repeats were amplified from peripheral blood lymphocyte genomic DNA by standard methods using published primer sequences (The Genome Database, http://gdbwww.gdb.org/). PCR conditions were specific to each genotyping center, as was fragment analysis. Internal consistency of allele sizing was achieved at each center by incorporating samples with known allele sizes on each gel. A common DNA sample (CEPH-1347-02) was typed to ensure consistency of allele sizing between centers. Allele frequencies were calculated separately for each center from the pedigree genotypes by using DOWNFREQ software, Version 1.1 (available through http://linkage.rockefeller.edu/soft/).

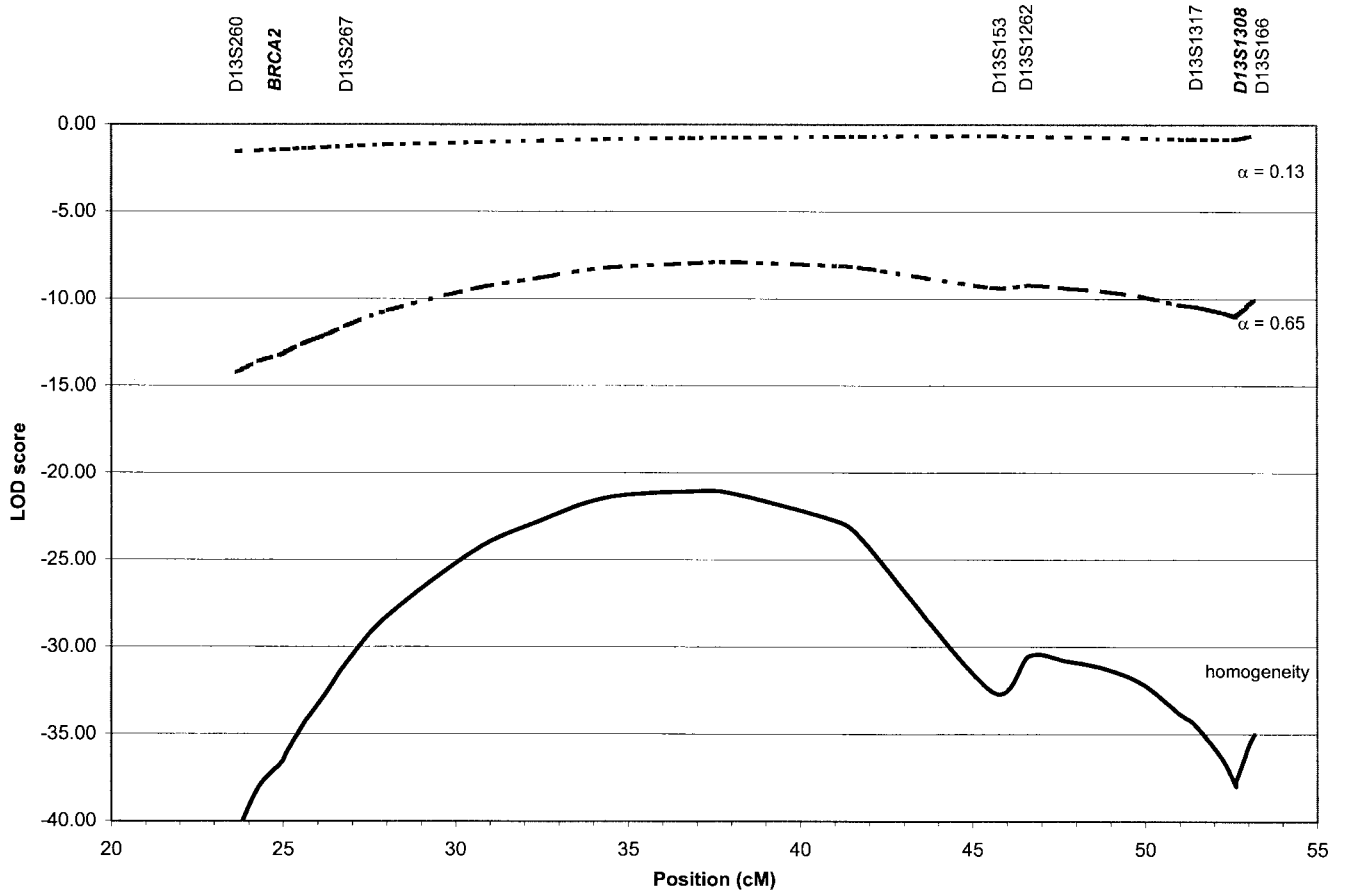

Fig. 1. Multipoint LOD scores for the 128 families analyzed are shown graphically. The solid line represents scores obtained under the assumption of homogeneity; the dashed line assumes the proportion of linked families (α) to be 65%, as estimated by Kainu *et al.* (10); and the dotted line represents the 95% upper confidence interval ($\alpha = 0.13$).

Statistical Analysis. We performed standard parametric linkage analyses, essentially identical to our previous analyses of linkage in breast cancer families (e.g., refs. 1, 5, and 8) and to the analysis conducted by Kainu *et al.* (10). These analyses assume the model of susceptibility to breast cancer based on the segregation analysis of Claus *et al.* (9). Under this model, susceptibility to breast cancer is conferred by a dominant allele with population frequency of 0.003. The risk of breast cancer by age 80 is assumed to be 0.80 in carriers and 0.08 in noncarriers. Risks are modeled in seven age categories (<30, 30–39, 40–49, 50–59, 60–69, 70–79, and 80+) as described in Easton *et al.* (12).

Multipoint linkage analyses were carried out using the programs GENEHUNTER (V. 2.0-B; ref. 13), VITESSE (14), and FASTLINK (15). GENEHUNTER was used where possible because it can analyze large numbers of polymorphic loci simultaneously and hence all of the markers we used could be incorporated into a single analysis. However, 33 families were too large to be accommodated by GENEHUNTER without discarding informative individuals. For these families we computed multipoint LOD scores by using either VITESSE (29 families) or FASTLINK (four families with multiple founders). The analyses assumed the intermarker distances as shown in Table 2.

We used the multipoint LOD scores for each family to compute heterogeneity LOD scores, using the standard admixture model, and hence estimated the proportion of families (α) linked to the putative "BRCA3" locus by maximizing the heterogeneity LOD score. A 95% confidence interval for α was derived by computing the values of the heterogeneity LOD score that were within 0.83 (corresponding to a Z value of 1.96) of its maximum value. Ninety-nine percent confidence intervals were also computed.

Because the putative "BRCA3" locus on 13q21 is linked to BRCA2, we performed a further analysis to allow for the possibility that preferential selection for families unlinked to BRCA2 may have biased the results against linkage at "BRCA3." In this analysis, we computed multipoint heterogeneity LOD scores at the candidate "BRCA3" locus, conditional on the LOD scores at BRCA1 and BRCA2, according to the formula:

$$\mathrm{LOD}(\theta_3) = \log_{10}\left[\frac{\alpha_1(1-\mu_1)10^{\mathrm{LOD}_1(\theta_1)} + \alpha_2(1-\mu_2)10^{\mathrm{LOD}_{2;3}(\theta_2)} + \alpha_3 10^{\mathrm{LOD}_{2;3}(\theta_3)} + 1 - \alpha_1 - \alpha_2 - \alpha_3}{\alpha_1(1-\mu_1)10^{\mathrm{LOD}_1(\theta_1)} + \alpha_2(1-\mu_2)10^{\mathrm{LOD}_2(\theta_2)} + \alpha_3 10^{\mathrm{LOD}_2(\theta_3)} + 1 - \alpha_1 - \alpha_2 - \alpha_3}\right]$$

In this formula α_1, α_2, and α_3 are the proportions of families meeting the eligibility criteria that are linked to BRCA1, BRCA2 and "BRCA3," respectively, and μ is the sensitivity of BRCA1/2 mutation screening. For the purposes of these analyses, α_1 and α_2 were set to 0.15 and μ to 0.7. $\mathrm{LOD}_1(\theta_1)$ and $\mathrm{LOD}_2(\theta_2)$ are the LOD scores at BRCA1 and BRCA2, respectively, whereas $\mathrm{LOD}_{2;3}(\theta_2)$ and $\mathrm{LOD}_{2;3}(\theta_3)$ are the LOD scores at BRCA2 and "BRCA3," respectively, based on markers typed at both loci; $\mathrm{LOD}_2(\theta_3)$ is the LOD score for "BRCA3" calculated using only markers at BRCA2. This calculated LOD score is the likelihood for the linkage data at "BRCA3" conditional on the existing

Table 2. Summary of markers used in the analysis

Marker	Map position, cM*	Centers typed	Multipoint LOD score: Homogeneity	Multipoint LOD score: Heterogeneity ($\alpha = 0.65$)
S1444	23.3	I	—	—
S1700	23.5	I	—	—
S260	23.7	S,L,I	−40.65	−14.25
S171	25.1	S	−36.07	−13.04
S1493	25.8	I	−33.83	−12.42
S267	26.9	S	−30.79	−11.51
S1293	26.9	I	−30.79	−11.51
S153	45.6	S	−32.54	−9.35
S788	45.6	I	−32.54	−9.35
S1317	51.0	L	−33.88	−10.35
S1262	51.0	I	−33.88	−10.35
S1308	52.6	S,L,I	**−38.00**	**−11.03**
S1296	52.6	I,L	−37.64	−10.93
S1291	53.2	L	−35.00	−10.06
S800	55.3	I	—	—
S166	55.3	S	—	—

I, IARC; S, ICR, Sutton; L, Leiden University.

*Based on published marker locations from Marshfield Medical Research Foundation (http://research.marshfieldclinic.org/genetics/). Note that BRCA2 is at position 24.8 on this map.

linkage and mutation evidence at BRCA1 and BRCA2, and hence corrects (albeit conservatively) for any bias in the "BRCA3" evidence produced by exclusion of families linked to BRCA2.

Results

Total LOD scores were strongly negative throughout the 8-cM interval between D13S153 and D13S1291 (Table 2 and Fig. 1). At the location of "BRCA3" estimated by Kainu *et al.* (10), D13S1308, the total LOD score was −38.00. Based on the admixture model, the estimated proportion of linked families (α) was 0, with an upper 95% confidence limit of 0.13. The estimated α was also zero for all possible positions in the interval D13S153-D13S1291. Of the 128 families, only four had a multipoint LOD score of greater than 0.5 at D13S1308, the highest of which was 0.67 (one additional family achieved a LOD score of 1.55 at a more distal marker, D13S800). Twelve families achieved LOD scores less than −1 at D13S1308.

We reanalyzed the data conditioning on the genotyping data at BRCA1 and BRCA2. In this analysis the total LOD score was −25.08. In the heterogeneity analysis based on these conditional LOD scores, the estimated proportion of families linked to "BRCA3" was again 0, with an upper 95% confidence limit of 0.18.

In the 95 families that could be analyzed with GENEHUNTER, we also analyzed the data by using the nonparametric method (13) to evaluate haplotype sharing among affected women. Again, no significant evidence of linkage was found (data not shown).

Discussion

Our results clearly conflict with those reported by Kainu et al. (10). Using a set of multiple case female site-specific breast cancer families analyzed for a similar set of markers within the candidate region and subjected to comparable statistical analysis, we found no evidence of linkage to 13q21. The proportion of linked families (65%) reported by Kainu *et al.* (10) is excluded with a high degree of statistical significance (the heterogeneity LOD score at $\alpha = 0.65$ was −11.03 in our dataset). This is true even after a conservative correction for possible bias due to potential exclusion of families linked at the BRCA2 locus (conditional LOD at $\alpha = 0.65$ was −7.64). In addition, under both unconditional and conditional analyses, the estimated proportion of linked families was 0, with upper 95% confidence intervals of 13% and 18%, respectively, indicating that if there is a susceptibility locus on 13q, it is likely to account for only a minority of breast cancer families. The paper of Kainu *et al.* (10) did not provide confidence limits on their estimated proportion of linked families. However, based on their LOD scores given under homogeneity and 65% heterogeneity, and assuming confidence intervals that are symmetrical about the best estimate, we have estimate a lower 95% confidence limit for α of 0.31. Thus the 95% confidence limits for the two studies do not overlap. Moreover, even when using a more stringent criteria of 99%, the upper confidence limit for our estimated proportion of linked families is 0.19 for the unconditional analysis and 0.26 for the analysis conditioning on BRCA2 markers, further indicating a minor role, if any, for this locus.

There were some differences in selection criteria between the two studies. Our study was restricted to families in which at least three cases of breast cancer were diagnosed below age 60, whereas Kainu *et al.* (10) included families with three cases diagnosed at any age. Thus, our families may be more heavily selected for genes conferring high risk. It is perhaps noteworthy that the initial hypothesis-generating family analyzed by comparative genomic hybridization (CGH) in Kainu *et al.* (10) would not have qualified for our study because only two of the five cases were diagnosed under age 60. However, in the subset of 51 families with less than three cases diagnosed under age 50 (Table 1), there is also considerable evidence against linkage to this locus (multipoint LOD = −8.06; HLOD = 0; upper 95% CI for $\alpha = 24\%$; HLOD for α of 65% = −3.57). Thus it is unlikely that difference in age criteria can explain the differences in results between the two studies.

An additional difference in selection criteria was exclusion of families with any cases of ovarian cancer in our series, given the close association of this disease with BRCA1 and BRCA2. Although no BRCA2 mutations were identified in the family set of Kainu *et al.*, the combination of detection methods applied to screening families have detection sensitivities of ≈0.70 (1, 11). Thus, although simulated linkage results allowing for up to 25% of the families in the dataset of Kainu *et al.* (10) being due to undetected BRCA2 mutations only exceeded the observed maximal lod score in 1 of 3,000 replicates, it is not known to what extent the seven families with ovarian cancer contributed to the observed overall LOD score.

The families in our study were drawn from Western Europe, or in descendent populations in North America and Australia, whereas the families studied by Kainu *et al.* (10) were from the Nordic countries. Although we have not specifically examined the ethnic origins of each family in our set, it is anticipated that the set of families from the United States and Canada ($n = 43$) are more ethnically heterogeneous, although most, if not all, are of Western European origin. Only a small minority of all of the families in our set are likely to be of Scandinavian origin, most notably the families ascertained in Minnesota, Seattle, and other parts of the Midwest, which have a high concentration of families descendent from emigrants of Sweden and Norway. One might speculate that the difference in the results observed is due to a population specific founder effect—i.e., an excess of some specific mutation in "BRCA3" in the Nordic populations.

We believe this to be unlikely. The different Nordic populations have different population histories and do not originate from a single small founder population. Although closely related, the Swedish, Icelandic, and (to a lesser extent) Finnish populations are also genetically similar to English and Dutch populations (17). If the observed linkage were due to a susceptibility allele that had reached a high frequency in the Swedish

and Finnish populations, this allele would also be expected to occur at a detectable frequency in the British and Dutch families. On the other hand, if the linkage is the result of several different mutations in the candidate "BRCA3" gene, the expectation would be that (as in the case of BRCA1 and BRCA2) mutations would also occur in the British, Dutch, and other populations, albeit the set of mutations might be different. Under either model, we would have expected to observe similar evidence of linkage in our families. Indeed, even when the prevalence of a population specific founder mutation has led to a specific susceptibility gene accounting for the majority of families of a hereditary cancer syndrome [e.g., BRCA2 in the Icelandic population accounting for 61.4% of breast cancer families (18); >50% of hereditary non-polyposis colon cancer (HNPCC) families in the Finnish population attributable to two specific MLH1 mutations (19)], these same genes account for a substantial fraction of families with the same cancer syndrome in other populations [breast cancer reviewed in (20); HNPCC (19)].

We conclude therefore that any contribution of a locus at chromosome 13q21 to familial breast cancer is likely to be small in breast cancer families of European origin. Further linkage studies in large series of multiple case families, or targeted association studies in large series of breast cancer cases and controls, will be needed to identify remaining genes underlying familial aggregation of the disease.

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