BRCA1 and BRCA2 Mutations in Breast Cancer Families with Multiple Primary Cancers[1]

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ABSTRACT

Ninety-eight women ascertained from high-risk breast/ovarian cancer clinics with breast cancer reporting at least one other primary cancer in themselves or in a relative with breast cancer were compared with 99 women with breast cancer who reported a family history of breast cancer only. All DNA was screened for coding region mutations in BRCA1 and BRCA2 using heteroduplex analysis, followed by direct sequencing.

Our data indicate that 42.9% of families reporting breast and any second nonbreast type of primary cancer in the same individual had a BRCA1 or BRCA2 mutation, as compared with the 12.1% of families reporting breast cancer only ($P < 0.001$). Among the 66 women reporting breast cancer and a nonovarian second primary cancer, 15 (22.7%) had mutations in BRCA1 or BRCA2 ($P = 0.04$). Among the 32 families where ovarian cancer was the second primary cancer, 27 (84.4%) had a mutation in BRCA1 or BRCA2 ($P < 0.001$).

BRCA1 and BRCA2 mutations were twice as common in the presence of a reported second nonovarian cancer. These data suggest that the presence of multiple primary cancer of any kind may predict for an increased likelihood of finding a BRCA1 or BRCA2 mutation and supports previous studies suggesting that BRCA1 and BRCA2 mutations may be associated with an increased susceptibility to cancers other than breast and ovarian cancer.

INTRODUCTION

Germ-line mutations in BRCA1 and BRCA2 confer a markedly increased lifetime risk of developing both breast and ovarian cancer. Factors that predict for detecting a BRCA1 or BRCA2 mutation in an affected member of a family with two or more cases of breast cancer include an average age breast cancer diagnosis <50 years, Ashkenazi Jewish descent, and a family history of ovarian cancer, particularly in a woman previously diagnosed with breast cancer (1–3). Numerous studies evaluating BRCA1 and BRCA2 mutations in breast cancer families have noted an increased incidence of other types of cancers (such as prostate, colon, and pancreas), and larger studies done by the Breast Cancer Linkage Consortium have provided stronger evidence for an association with cancers other than breast and ovarian cancer (4–13). To evaluate whether the reported presence of a multiple primary cancer of any type was associated with an increased likelihood of detecting a BRCA1 or BRCA2 mutation, as well as to further evaluate the possible association between primary cancers other than breast or ovarian cancer with BRCA1 or BRCA2 mutations, we assessed the BRCA1 and BRCA2 mutation status in families reporting breast cancer and any other nonbreast malignancy in a woman with breast cancer compared with families reporting only breast cancer.

PATIENTS AND METHODS

Patient Population. All families were recruited from clinics at the University of Michigan between 1993 and 1995 or the University of Pennsylvania between 1995 and 1998. Patients were either self- or physician referred. Subjects were ascertained from a clinic population who perceived themselves to be at an elevated risk of inherited susceptibility to breast cancer. All women consented to BRCA1 and BRCA2 testing for clinical and/or research purposes. Personal and family histories of all cancers were recorded, including ages of diagnosis of all cancers. Pathology reports were obtained on all probands and on other family members when possible. The method of ascertainment of cancer history in family members was identical in families with and without multiple primary cancers, under the assumption that this approach was the most likely means of insuring that the nature and extent of any inaccuracies in reporting of family history would be evenly distributed among all families. The testing protocol was approved by duly constituted institutional review boards at both the University of Michigan and the University of Pennsylvania.

Ninety-eight families were identified as multiple primary cancer families. These are defined here as those families that reported at least one woman affected with both a primary breast and a primary nonbreast cancer. All nonbreast malignancies were considered, including nonmelanoma skin cancers. Eighty-seven (88.8%) women had two primary cancers, and 11 women (11.2%) had three or more primary cancers. Ninety-nine fami-

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lies reporting women affected with breast cancer only were similarly ascertained and studied. DNA was available from at least one woman with multiple primary cancers in 51 families. In the remaining 47 multiple primary cancer families, the multiply-affected woman was deceased ($n = 21$) or unavailable ($n = 26$). In these families, BRCA1 and BRCA2 analysis was undertaken using DNA from the closest female relative diagnosed with breast cancer.

Mutation Analysis. DNA was extracted from peripheral blood mononuclear cells and stored in 10 mM Tris (pH 8)-1 mM EDTA at 4°C. The entire 22 exon coding domain and flanking splice site regions of BRCA1 were amplified by 31 sets of PCR primers. One set of primers was used to amplify each exon and the adjacent intronic region, except for exon 11, in which 10 sets of overlapping primer sets were used to amplify of the entire exon.

Similarly, the 26 coding exons of BRCA2 were amplified using 39 sets of intron-based primers, with overlapping primer sets for exons 10 and 11. Reaction mixtures contained 60–120 μM genomic DNA in 25 μl with 1.0 unit of Taq DNA polymerase (Boehringer Mannheim), 1× buffer [1.5 mM Mg^{2+}, 10 mM Tris-HCl, and 50 mM KCl (pH 8.3)], 0.5 μM of each of the forward and reverse primers[3], and 0.2 mM each of dCTP, dATP, dGTP, and dTTP. PCR reactions were cold started with initial heating to 94°C for 1 min, followed by a touchdown of 1°C per minute beginning 10°C above annealing temperature. Annealing temperatures were optimized for each set of primers and ranged from 55–60°C. Elongation was at 72°C, and amplification was for 20 cycles.

PCR products were analyzed by conformation-sensitive gel electrophoresis, with modifications to a recently developed protocol (14). Briefly, PCR products were heated again to 98°C for 5 min to denature the DNA and then re-annealed at 68°C over 30 min to enable heteroduplex formation. Gels consisted of 0.5× Tris-Taurine-EDTA buffer [44.4 mM Tris/14.5 mM Taurine (USB)/0.1 mM EDTA (pH9.0), filter sterile], 10% polyacrylamide with 99:1 ratio of acrylamide to 1,4-bis(acryloyl)piperazine (Fluka), 15% formamide, 10% ethylene glycol, 0.1% ammonium persulfate, and 0.69% *N,N,N′,N′*,-tetramethylethylenediamine. Gels were run overnight at 10–25 W, depending on the size of the PCR product so that the DNA had run the full length of the gel. Polyacrylamide gels (12.5%) were used to improve separation of bands in some PCR samples. Gels were stained with ethidium bromide for 15–20 min and visualized by UV light.

Sequence variants selected by atypical banding patterns on conformation-sensitive gel electrophoresis analysis were reamplified from source DNA as described, then purified by QIAquick PCR Purification Kit (Qiagen) following the manufacturer's instructions. PCR products were subsequently sequenced in both the forward and reverse directions using the ABI Prism 377.

Statistical Analyses. Contingency table analysis and Fisher's exact tests were used to evaluate differences in BRCA1/BRCA2 mutation frequency across groups defined by breast only *versus* multiple primary cancers. Odds ratios and 95% confidence intervals were also computed to estimate the magnitude of effect of BRCA1 and/or BRCA2 mutation on the development of second primary cancers. The Kruskal-Wallis χ^2 approximation was used to evaluate the differences among quantitative traits such as mean age of diagnosis across families defined by either breast only primary or multiple primary cancer cases.

[3] The sequences are at http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/.

Table 1 Nonbreast primary cancers in multiple primary cancer families

	No. of cancers[a]	BRCA1 mutation (%)	BRCA2 mutation (%)
Ovarian	32	25 (78.1)	3 (9.4)
Nonmelanoma skin	16	3 (18.8)	1 (6.2)
Colorectal	15	0 (0.0)	3 (20.0)
Cervical	8	1 (12.5)	0 (0.0)
Endometrial	8	1 (12.5)	0 (0.0)
Thyroid	7	1 (14.3)	0 (0.0)
Leukemia	5	1 (20.0)	1 (20.0)
Lymphoma	4	0 (0.0)	0 (0.0)
Others[b]	15	4 (26.7)	4 (26.7)

[a] Eleven patients had two or more nonbreast cancers; thus, the numbers of cancers does not equal the number of patients.

[b] Other primary cancers include: melanoma, brain, bladder, lung, fallopian, esophageal, pituitary, head/neck, vocal cord, bone, and Wilms' tumor.

RESULTS

Patient Population. Table 1 lists the cancers reported in the individuals with multiple primary cancers. In 32 of the 98 multiple primary families (32.7%), ovarian cancer was the second primary cancer. Nonmelanoma skin cancers (basal cell and squamous cell carcinoma) were reported in 16 of the multiple primary families (16.3%). Only one family screened reported melanoma as the second primary cancer. Fifteen families reported colorectal cancer as the second primary, endometrial and cervical cancers were noted as the second primaries in eight families each, and thyroid cancers were reported in seven families. Other cancers were reported in 5% or less of the families.

The families reporting both the presence and absence of multiple primary cancer cases were compared with respect to average age of breast cancer diagnosis and number of breast cancer cases per family. The average age of breast cancer diagnosis was 47.4 years and 48.5 years, respectively ($P = 0.36$). Bilateral cancer was diagnosed in 34 of 98 (34.7%) breast only cancer families as compared with 45 of 99 (45.5%) of multiple primary cancer families ($P = 0.12$). The average proportion of breast cancers per family (adjusted for the number of women ≥20 years) was 0.29 for multiple primary cancer families and 0.31 for families with breast cancer only ($P = 0.538$).

Mutation Status. Considering only unequivocal disease-associated mutations, 15 of 66 nonovarian multiple primary families had either a BRCA1 or a BRCA2 mutation, as compared with 12 of 99 families (12.1%) with breast cancer only ($P = 0.057$; Table 2*A*). Seven families had a BRCA1 mutation, and eight families had a BRCA2 mutation. Of the 99 families

Table 2A Unequivocal disease-associated mutations

	Breast-Ovarian Ca[a] ($n = 32$)	Breast-Non-Ov Ca ($n = 66$)	Any Multiple Ca ($n = 98$)	Breast Ca Only ($n = 99$)
BRCA1 (%)	25 (78.1)	7 (10.6)	32 (32.7)	6 (6.1)
	$P < 0.001$		$P < 0.001$	
BRCA2 (%)	3 (9.4)	8 (12.1)	11 (11.2)	6 (6.1)
BRCA1 and 2 (%)	27 (84.4)[b]	15 (22.7)	42 (42.9)[b]	12 (12.1)
	$P < 0.001$	$P = 0.057$	$P < 0.001$	

[a] Ca, cancer; Non-Ov, nonovarian.
[b] Includes one patient with both a BRCA1 and BRCA2 mutation.

Table 2B Mutations including unclassified variants of potential significance

BRCA1 (%)	25 (78.1)	8 (12.1)	33 (33.7)	6 (6.1)
	$P < 0.001$		$P < 0.001$	
BRCA2 (%)	4 (12.5)	9 (13.6)	13 (13.3)	8 (8.1)
BRCA1 & 2 (%)	28 (84.5)[a]	17 (25.8)	45 (45.9)[a]	14 (14.1)
	$P < 0.001$	$P = 0.049$	$P < 0.001$	

[a] Includes one patient with both a BRCA1 and BRCA2 mutation.

reporting breast cancer only, six families had mutations in BRCA1 and six families had BRCA2 mutations. The odds ratio associated with carrying either a BRCA1 or a BRCA2 mutation in the nonovarian multiple cancer cases as compared with the families reporting only breast cancer was 2.13 (95% confidence interval, 0.93–4.90).

Currently, there are a number of missense mutations that cannot be classified as unequivocal disease-associated mutations because of the absence of a functional assay for either BRCA1 or BRCA2. It is likely that at least some of these sequence variants are susceptibility alleles, particularly in BRCA2, where no missense mutations have been classified unequivocally. When adding these variants to the comparison of mutation frequency in the breast/nonovarian multiple primary families as compared with the breast only families, the results do not change appreciably (Table 2*B*) with $P = 0.049$ when including unclassified variants and $P = 0.057$ when considering only unequivocal mutations. All other analyses were performed using only unequivocal disease-associated mutations.

Four of 16 of the reported nonmelanoma skin cancer cases (25%) were mutation positive, three with mutations in BRCA1 and one with a mutation in BRCA2 (Table 1). Three of the 15 women (20%) with colorectal cancer reported as their second primary cancer had BRCA2 mutations, and none had a mutation in BRCA1. All other women with BRCA1 or BRCA2 mutations were single cases of cancers with no obvious association with any one type or group of cancers. Twenty-five of the 32 families (78.1%) with breast-ovarian multiple primary subjects had a germ line BRCA1 mutation ($P < 0.001$); however, only three BRCA2 mutations were found in this group (9.4%).

Overall, 42 of the 98 families (42.9%) reporting multiple primary cancers had either a BRCA1 or a BRCA2 mutation ($P < 0.001$). Thirty-two families had BRCA1 mutations, and 11 families had BRCA2 mutations. One family with a case of breast/ovary multiple primary cancer that was of Ashkenazi Jewish ancestry had both a BRCA1 (3889delAG) and a BRCA2 (6174delT) mutation. There were equal numbers of BRCA1 and BRCA2 mutations in the families with nonovarian multiple primaries, but BRCA1 mutations were eight times more frequent than BRCA2 mutations when ovarian cancer was the second primary cancer.

Of the Ashkenazi Jewish families screened, an Ashkenazi founder mutation (185delAG or 5382 insC in BRCA1, 6174delT in BRCA2) was detected in 4 of 9 multiple primary families (44.4%) and 6 of 22 families reporting breast cancer only (27.3%). Mutations within the multiple primary families included three 185delAG mutations and one 6174delT mutation. Six founder mutations (five 185delAG and one 5382 insC) were detected in the breast cancer only families. As in the complete cohort, approximately twice as many mutations were observed in the multiple primary families as compared with the families reporting only breast cancer.

Of the 55 unequivocal disease-associated mutations identified in this cohort, BRCA1 mutations included 26 frameshift mutations and 8 nonsense mutations. Two splice site mutations and two previously reported disease-associated missense mutations (C64G and C64Y)[4] also were detected in BRCA1. Fourteen frameshift mutations and three nonsense mutations were identified in BRCA2.

Seventeen of the 19 missense mutations identified in this study set either have not been previously reported or remain of uncertain significance (Table 3, *A* and *B*; Refs. 15–17). On the basis of the frequency with which they were detected within this cohort and in an analysis of samples from the United Kingdom National Case Control Study Group (18) and whether they coexisted with another mutation of known significance within the same individual, the maximum number of potentially disease-associated missense mutations was estimated. Any missense mutation with >1% prevalence was designated as a polymorphism, despite detection in a high-risk population. Missense

[4] NHGRI Breast Cancer Information Core (BIC), http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/edition, National Human Genome Research Institute, 1999.

Table 3A Missense mutations detected in families with multiple primary cancers

	No. families	Classification	Comments
BRCA1			
C64G	1	M[a]	RING finger domain mutation
C64Y	1	M	RING finger domain mutation
S1542C	1	UV	Detected in patient with bilateral breast cancer dx'd at ages 59 and 73; and colon, squamous cell carcinoma; not in BIC; outside of transactivation domain (16, 17)
M1652I	1	PM	Detected in patient with breast cancer dx'd at age 43 in a breast/ovarian cancer family; in transactivation domain (16, 17) not tested in transactivation assay, 9 cases in BIC with 1% frequency
BRCA2			
S326R	1	PM	Does not track with disease in large breast/ovarian cancer family
N991D	4	PM	2% frequency of families in this study
D1420Y	2	PM	Found in an individual with mutation of known significance
T1915M	2	PM	Found in an individual with mutation of known significance
I2285V	1	UV	Detected in patient with br ca dx'd at age 37 in a breast/ovarian cancer family; 12 cases reported in BIC
V2728I	2	PM	Individual with mutation of known significance
T3013I	1	UV	Detected in patient with breast cancer dx'd at age 59 and ovarian cancer at age 59; 3 cases reported in BIC

[a] M, functionally significant mutation; UV, unclassified variant; PM, polymorphism; BIC, Breast Cancer Information Core: http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/BIC/; dx'd, diagnosed.

Table 3B Missense mutations detected in families with breast cancer only

BRCA2	Families	Classification	Comments
Y42C	1	PM[a]	Reported as a variant that disrupts the transactivation domain but found in equal numbers in cases and controls in the United Kingdom National Case Control Study Group (15)
K314T	1	UV	Breast cancer diagnosed at age 39; amino acid charge loss; not in BIC

[a] PM, polymorphism; UV, unclassified variant; BIC, Breast Cancer Information Core: http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/.

mutations found in individuals having another mutation with known disease-association also were assumed to be polymorphisms. By these criteria, five missense mutations were considered variants of uncertain significance—three in the multiple primary cancer group and two in the group with only breast cancer. An analysis considering these five sequence variants as disease-associated mutations was performed to estimate an upper limit of mutation prevalence (Table 3, *A* and *B*). As noted above, this analysis produced results that were very similar to the analysis performed including only unequivocal disease-associated mutations.

DISCUSSION

The occurrence of multiple cancers in a single individual has long been attributed anecdotally to "bad genes." Supporting this hypothesis, there is an increased incidence of multiple primary cancers in members of families with germ-line mutations in p53 (Li-Fraumeni syndrome; Ref. 19). We now provide evidence that BRCA1 and BRCA2 germ-line mutations may be associated with multiple primary cancers (where multiple primary cancer families are defined as those that reported at least one woman affected with both a primary breast and a primary nonbreast cancer). Consistent with the published literature, this association is strongest when the second primary cancer is ovarian cancer; nonetheless, in this clinic-based series, BRCA1 and BRCA2 mutations were twice as common in women from families reporting breast cancer and a second nonovarian cancer as in women from families reporting only breast cancer.

As noted previously, a variety of cancers in carriers of BRCA1 and BRCA2 mutations have been reported in several series (4–13). Our study supports the hypothesis that although breast and ovarian cancer are the primary component tumors of these cancer susceptibility syndromes, a generalized increased risk for developing a broad spectrum of malignancies may exist in association with BRCA1 and BRCA2 mutations. In this study, in women with BRCA1 mutations, reported an array of gynecological cancers other than ovarian cancer, including cervical, endometrial, and fallopian tube cancers. Nonmelanoma skin cancer, leukemia, and thyroid cancer also were seen in this group. These cancers have been previously noted in BRCA1 mutation carriers (20) but have not been confirmed as associated tumors in a rigorous analysis of a large group of BRCA1-linked kindreds (4). BRCA2 also has been associated with a range of other cancer types (6).

In addition to the small numbers of any specific type of cancer, the association between BRCA1 and BRCA2 and other cancers may have be overlooked because the most frequently cited cancers are those that also have a high incidence in the general population, thus expected within some families with mutations. Whereas larger studies, performed in multiple primary cancer patients selected without regard to breast cancer family history, are required to search for clearer association of

BRCA1 and BRCA2 mutations with specific cancer types, the data presented herein provide evidence for a generalized cancer susceptibility in mutation carriers. This susceptibility is likely to affect both men and women because only a small proportion of these cancers occur only in women.

It is interesting that 4 of 16 women reporting a personal or family history of nonmelanoma skin cancer in association with breast cancer had either a BRCA1 or BRCA2 mutation (25%). Because a number of recent studies have provided evidence for involvement of BRCA1 and BRCA2 in the cellular response to DNA damage, this finding may reflect an inefficient response to DNA damage caused by the UV radiation in sun exposure following loss of the wild-type allele of either gene.

Our data also provide some support for an association between BRCA1 and BRCA2 mutations and leukemia. Leukemia is one of the component cancers of Li-Fraumeni syndrome and is seen in ataxia telangiectasia. In both syndromes, the inability to efficiently respond to DNA damage underlies the development of malignancy. Other sources of DNA damage also are known to predispose to leukemia, including chemotherapeutic regimens that contain alkylating agents and nuclear radiation exposure. Thus, it is plausible that BRCA1 and BRCA2 mutations also may confer an increased risk of treatment-associated leukemia. If confirmed, this finding has clinical implications because the alkylating agent cyclophosphamide is widely used as adjuvant therapy for early-stage breast cancer.

Results from the breast-ovarian cancer subset of the multiple primary cancer families reflect and support previous studies that show a strong association between ovarian cancer and BRCA1 mutations. When considering all possible variants, 85% of the breast-ovarian cancer families carry either a BRCA1 or BRCA2 mutation. Given that noncoding region mutations in BRCA1 are not detected by the methods used in this study and that these mutations may represent as many as 25% of mutations in some populations (21), it may, indeed, be that all breast-ovarian multiple primary cases are due to BRCA1 or BRCA2 mutations.

It is possible that the multiple primary cancer cases in this study that are not associated with BRCA1 or BRCA2 mutation are a manifestation of other cancer susceptibility syndromes. Possible syndromes include Li-Fraumeni syndrome (p53), Cowden syndrome (PTEN), Muir-Torre syndrome (MSH2), and Peutz-Jeghers (STK11). These genes were not studied in the present analysis, but mutations in these genes occur only rarely and explain a very small proportion of hereditary susceptibility to breast cancer. Thus, we believe that it is unlikely that a significant number of mutations in these genes will be found in this series. On the basis of the very low prevalence of mutations in most of these genes, it is more likely that undiscovered genes account for many of the multiple primary cancer cases without BRCA1 or BRCA2 mutations. This suggests that these families may be useful for linkage analysis in the isolation of novel cancer susceptibility genes.

Clearly, this study has limitations related to ascertainment. First, as in all studies of genetic susceptibility to cancer, disease-associated mortality limits access to affected family members. In addition, breast cancers associated with BRCA1 mutations are known to be of high nuclear grade and often lack estrogen receptors, both poor prognostic indicators. Thus, it is possible that using only living affected individuals might bias against selection of women with BRCA1 mutations. Furthermore, multiple primary cancer cases have an intrinsically higher mortality rate due to the additive mortality rate of each primary cancer. In this study, when DNA was unavailable from some individuals with multiple primary cancers, DNA from a closely related relative with breast cancer was analyzed with the assumption that a BRCA1 or BRCA2 mutation in the proband would also be found in the closest affected relative. This strategy should partially compensate for potential bias introduced when excluding deceased probands from analysis. Thus, our selection of testing samples was a means of increasing sample size and partially compensating for potential survivor bias without risking overrepresentation of mutations. A similar strategy was used in families with only breast cancer in designating an affected family member as the proband based on DNA sample availability. However, this strategy introduces a level of uncertainly as to the diagnoses of deceased or distant relatives. Some cancers, particularly nonmelanoma skin cancers, may be under-reported. It is likely that this occurs with the same frequency in both groups of families, but we cannot be absolutely certain this is the case. Additionally, family members may not be aware of a second cancer diagnosis and make the assumption that a death was due to a previously diagnosed breast cancer. This would result in misclassification of multiple primary families as site-specific breast cancer families, however, this would result in falsely narrowing, rather than increasing, any differences in mutation prevalence observed between these two groups of families. Despite these limitations, these data are representative of what is available to clinicians evaluating families for cancer risk, thus are likely to result in conclusions that are appropriate for discussions with patients and for the generation of hypotheses that may be tested in a rigorously designed prospective case control study.

In summary, our results suggest that as many as half of women from families with evidence for hereditary susceptibility to breast cancer who report a personal or family history of at least one multiple primary cancer may have a BRCA1 or BRCA2 germ-line mutation. In particular, these data suggest the association of BRCA1 and BRCA2 mutations with multiple primary cancer cases is not limited to breast and ovarian cancer, supporting the role of these two genes with general cancer susceptibility regardless of cancer type. Whereas a larger sample set will be required before the role of BRCA1 or BRCA2 in each of these specific malignancies can be fully defined, it may be reasonable to consider multiple primary cancers when estimating the likelihood of finding a BRCA1 or BRCA2 mutation in a clinical setting. Finally, while weighing the potential psychological distress associated with invoking a generalized cancer susceptibility, not just breast and ovarian cancer risk, these data suggest that, in general, it may be prudent to have a heightened level of suspicion in the clinical management of BRCA1 and BRCA2 mutation carriers.

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