Detection of Eight *BRCA1* Mutations in 10 Breast/Ovarian Cancer Families, Including 1 Family with Male Breast Cancer

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Summary

Genetic epidemiological evidence suggests that mutations in *BRCA1* may be responsible for approximately one half of early onset familial breast cancer and the majority of familial breast/ovarian cancer. The recent cloning of *BRCA1* allows for the direct detection of mutations, but the feasibility of presymptomatic screening for cancer susceptibility is unknown. We analyzed genomic DNA from one affected individual from each of 24 families with at least three cases of ovarian or breast cancer, using SSCP assays. Variant SSCP bands were subcloned and sequenced. Allele-specific oligonucleotide hybridization was used to verify sequence changes and to screen DNA from control individuals. Six frameshift and two missense mutations were detected in 10 different families. A frameshift mutation was detected in a male proband affected with both breast and prostate cancer. A 40-bp deletion was detected in a patient who developed intra-abdominal carcinomatosis 1 year after prophylactic oophorectomy. Mutations were detected throughout the gene, and only one was detected in more than a single family. These results provide further evidence that inherited breast and ovarian cancer can occur as a consequence of a wide array of *BRCA1* mutations. These results suggests that development of a screening test for *BRCA1* mutations will be technically challenging. The finding of a mutation in a family with male breast cancer, not previously thought to be related to *BRCA1,* also illustrates the potential difficulties of genetic counseling for individuals known to carry mutations.

Introduction

The underlying etiologic mechanism of breast cancer is poorly understood, and the annual incidence of this disease is increasing (Kelsey and Horn-Ross 1993). It has been estimated that 5%–10% of cases may be due to inherited predisposition, but the exact number and distribution of predisposing genes is unknown (Rowell et al. 1994). Genetic epidemiological studies have provided evidence for at least two genes conferring inherited susceptibility to breast and ovarian cancer. Germ-line mutations in *BRCA1* are estimated to account for 40%–50% of early onset female breast cancer families and most early onset familial breast/ovarian cancer (Hall et al. 1990; Narod et al. 1991; Easton et al. 1993). Furthermore, *BRCA1* mutation carriers face a fourfold increased risk of colon cancer, and male carriers face a threefold increased risk of prostate cancer (Ford et al. 1994). Mutations in *BRCA2* appear to account for an additional 40%–50% of familial breast cancer but for a lower proportion of familial breast/ovarian cancer (Wooster et al. 1994). In addition, *BRCA2* has been implicated in families that also contain at least one case of male breast cancer (Wooster et al. 1994), whereas no families with affected males have previously shown linkage to *BRCA1* (Stratton et al. 1994).

The recent cloning of *BRCA1* (Futreal et al. 1994; Miki et al. 1994), verified by several groups (Castilla et al. 1994; Friedman et al. 1994; Simard et al. 1994; Shattuck-Eidens et al. 1995), has implications for both clinical and basic research (Weber 1994). The most immediate impact will likely be for families with a high incidence of breast and ovarian cancer, which have the highest likelihood of harboring *BRCA1* mutations. This study describes the analysis of 24 such families. These families have been studied for several decades by the National Cancer Institute's (NCI) Genetic Epidemiology Branch because of the high incidence of ovarian cancer (Li et al. 1970; Fraumeni et al. 1975; Tobacman et al. 1982). We report the detection of eight *BRCA1* mutations in 10 of 24 families studied.

Methods

Study Population

Eligible families were selected from a registry of self-referred and physician-referred cancer-prone families of

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the NCI's Genetic Epidemiology Branch. Lymphocyte DNA was available from at least one affected individual from each of 24 families with at least one case of ovarian cancer and at least three total cases of breast or ovarian cancer verified by review of pathology material, pathology reports, or death certificate information. All subjects gave written informed consent for participation in the study. Chromosome 17 markers D17S250, D17S579, and *nm23* were typed in six families with multiple living affected individuals, and chromosome 13 marker D13S267 was typed in Family 3335, using standard methods (Smith et al. 1994). Linkage analysis was performed using the MLINK program of the LINKAGE package (Lathrop et al. 1985). Analysis parameters were those of the Breast Cancer Linkage Consortium (Easton et al. 1993).

SSCP

SSCP analysis was used to screen for mutations in *BRCA1*. Forty-one PCR assays were used to amplify the entire coding region and splice junctions of *BRCA1* from genomic DNA of the 24 probands. PCR was performed in a 10-μl reaction in a Perkin Elmer 9600 thermal cycler. Primer pairs and cycling conditions were those reported elsewhere (Castilla et al. 1994). For exons 6 and 7, a nonradioactive reaction, amplifying both exons in a single fragment, was performed first using published primer sequences (Miki et al. 1994), and 0.2 μl of this was used as template in a labeling PCR reaction using exon-specific primers (Castilla et al. 1994). Nondenaturing gels were prepared with 0.5 × MDE (mutation detection enhancement) (AT Biochem) and 5% glycerol. Samples were run at a constant 8 W for 15–20 h without cooling. Samples were run on nonglycerol gels, if there was a question of a variant band on the glycerol gel. Variant bands were cut from the gels after alignment with the autoradiograph and the DNA eluted in 100 μl of 0.25 × Tris-EDTA (pH 7.4) at 37°C overnight. For cloning and sequencing of the variant bands, 5 μl of the eluted DNA was used as template for secondary, unlabeled 50-μl reactions, using PCR conditions as above.

DNA Sequencing

PCR products were ligated with the pCRII (Invitrogen) or pT7Blue (Novagen) cloning vector and were transformed into competent DH5alpha cells (Life Technologies). DNA minipreps of 5-ml overnight cultures were prepared on Qiawell-8 strips (Qiagen) and were sequenced using both forward and reverse *Taq* dye-terminator sequencing kits on an ABI 373A DNA sequencer (Applied Biosystems). Both strands were sequenced for each PCR product from at least two independent clones in order to reduce the chance that any observed sequence changes were a consequence of polymerase errors.

Allele-Specific Oligonucleotide (ASO) Hybridization

All potential mutations were verified by ASO hybridization for the proband and all other living affected members from their family, according to methods described by Shuber et al. (1993) as modified in Hussussian et al. (1994). At least 90 parents/grandparents from the CEPH reference families (Weissenbach et al. 1992) were also screened to determine the frequency of the sequence changes in a control population. Pairs of oligonucleotides (17 nt) containing the wild-type and mutant sequences were designed for each mutation to include the variant nucleotide at the center, when possible. In brief, target DNA was amplified in independent 25-μl PCR reactions according to procedures described above, except that 40 ng of genomic DNA was used as template. Twenty microliters of PCR product were diluted with denaturing solution and dot-blotted onto nylon membranes. The oligonucleotides were end-labeled with [γ-^{32}P]ATP and hybridized in the presence of 3.0 M of tetramethylammonium chloride.

Haplotype Analysis

Individuals from families with previously published haplotype information (Simard et al. 1994) were genotyped for polymorphic markers clearly linked to *BRCA1*. Exact haplotypes could not be generated, because insufficient numbers of individuals were available for analysis. However, affected members from unrelated families were scored for allele sharing. Shared common alleles would be consistent with the mutation having been descended from a common chromosome. Markers chosen for this analysis were D17S855, D17S1322, D17S1323, and D17S1327, and estimated allele sizes were compared with those reported previously (Simard et al. 1994).

Results

The selection criteria for this study were weighted toward families with ovarian cancer. The 24 families had, on average, nearly as many cases of ovarian cancer (mean 2.9 cases; range 1–11) as breast cancer (mean 3.0 cases; range 0–8). Linkage analysis for chromosome 17q markers was completed on only six of the families, because of the unavailability of living affected individuals from the remainder. The maximum two-point lod scores for these families for markers tightly linked to *BRCA1* ranged from 0.1 to 2.1.

Eight SSCP variants occurred in three or fewer individuals and were considered potential disease-related mutations. Sequence analysis of these variants revealed five frameshift deletions, one frameshift insertion, and

two single nucleotide substitutions (table 1). One mutation, 187delAG, occurred in three families, and three additional mutations, C61G, 1294del40, and 5385insC, have been reported elsewhere (Castilla et al. 1994; Friedman et al. 1994; Miki et al. 1994; Simard et al. 1994). Two different mutations occurred in the same codon (V1713A and 5256delG), and one occurred in the penultimate cysteine of the zinc-finger-like region near the 5′ end of the gene (C61G). These eight sequence variants were confirmed by ASO hybridization using patient DNA from a different PCR reaction than the one used for SSCP analysis.

BRCA1 sequence variants were considered potential pathological mutations if they segregated with the disease in the family, and/or they were not detected in $\geq$180 control chromosomes from CEPH reference family ancestors. Lymphocyte DNA from two to six additional affected individuals per family was available for four families with potential mutations. Using ASO, all affected family members had the sequence change, and none of the CEPH reference chromosomes had the mutant sequence. An example of ASO results for one family is shown in figure 1.

Clinical characteristics of families from this study with potential mutations are also shown in table 1. Families with probable deleterious mutations had, on average, 3.3 cases of breast cancer and 3.0 cases of ovarian cancer, while families without mutations had, on average, 2.6 and 2.0 cases of breast and ovarian cancer, respectively. The mean ages at diagnosis for breast and ovarian cancer in families with mutations were 43.9 and 47.2, respectively, while the mean ages at diagnosis among families without mutations were 47.4 and 47.3, respectively. Maximum lod scores for families with mutations ranged from 0.33 to 2.1, while for the two families without mutations, for which linkage analysis was possible, the lod scores were both 0.1. The 5256delG mutation was detected in a male proband with synchronous breast and prostate cancer and in all of his living affected female relatives. This family shows linkage to chromosome 17q markers, and is unlinked to *BRCA2* marker D13S267 (lod score = -2.89; $\theta = .001$). The 1294del40 mutation was detected in a patient who developed intra-abdominal carcinomatosis one year after prophylactic oophorectomy was performed because of her perceived risk of ovarian cancer (Tobacman et al. 1982). No evidence of malignancy was noted at the time of the prophylactic surgery.

The three families from this study with the 187delAG are not known to be related to each other but are all of Ashkenazi Jewish descent. In addition, although haplotypes could not be determined, they all shared one allele for each of the four markers. The estimated sizes of these alleles were the same as in the four Canadian families with the 187delAG mutation studied by Simard et al. (1994). This suggests that the families may be related through a common ancestral chromosome carrying this mutation. Each of the seven published families with this mutation had at least two cases of ovarian cancer, and cancers of other sites occurred commonly in family NCI 62, although the mutation status of these other cases is unknown. The C61G mutation has been detected in a total of three published families, two of which contain only breast cancer. The large deletion mutation 1294del40 had been detected previously in two families (Castilla et al. 1994; Simard et al. 1994) The 5385insC mutation has been detected in five other published families predominantly with breast cancer, and those analyzed appear to share an allele (Miki et al. 1994; Simard et al. 1994).

Discussion

This study provides further evidence that inherited breast and ovarian cancer can commonly occur as a consequence of *BRCA1* mutations. Eight different mutations were detected, and only one occurred in more than a single family. While their pathological nature has yet to be confirmed, six were frameshift mutations and would be expected to result in a truncated or absent protein. These results are in general agreement with previous studies that analyzed the entire gene in a defined set of patients: of 32 mutations, only 7 have been detected in more than a single family, and they affect 27 different codons scattered throughout the gene (Castilla et al. 1994; Friedman et al. 1994; Simard et al. 1994). Despite this heterogeneity, most (24 of 32) are frameshift, nonsense, or loss-of-transcript mutations and would be expected to result in a nonfunctioning protein. This is consistent with the hypothesis that *BRCA1* functions as a tumor-suppressor gene, as originally predicted by loss of heterozygosity studies (Smith et al. 1992; Kelsell et al. 1993).

The cloning of *BRCA1* (Miki et al. 1994) will impact both clinical and basic research (Weber 1994; Hoskins et al. 1995). Breast cancer is a very common disease, with an estimated 183,400 new cases each year in the United States (Wingo et al. 1995). Sporadic breast and ovarian cancers, which account for the majority of cases, do not appear frequently to contain somatic mutations in *BRCA1* (Futreal et al. 1994), but understanding the function of *BRCA1* may still point to a common pathway for breast and ovarian cancer development. The potential value of identifying individuals at risk for breast and ovarian cancer because of predisposing germline genetic mutations is considerable. If mutations in *BRCA1* account for one half of the 5%–10% of cases due to inherited predisposing mutations, this means that ~1/300 individuals (or 400,000 women) in this country may carry germ-line *BRCA1* mutations. The estimated

Table 1

Germ-Line *BRCA1* Mutations in Breast/Ovarian Cancer Families

Designation[a]	Exon	Nucleotide Change	Codon	Effect	Family	Lod Score[b]	No. of Breast Cancers (mean age at diagnosis)[c]	No. of Ovarian Cancers (mean age at diagnosis)[c]
187delAG	2	A GAG TGT CCC→A GTG TCC	23	Stop 39	62[d]	na	6 (48)	2 (49)
					167[e]	na	8 (45)	2 (42)
					3279	na	0	3 (39)
C61G	5	TGT→GGT	61	Cys→Gly	3325	na	1 (43)	2 (40)
1294del40	11	CTG T...TCA AAT→CAA T	392-405	Stop 397	1138[f]	na	1 (48)	3 (44)
1323delG	11	GAG TCT→AGT CT	401	Stop 409	3295[g]	.67	4 (32)	1 (53)
3600del11	11	GAA GAT ACT AGT TTT→TTT T	1161-116	Stop 1163	3265	.33	3 (40)	3 (42)
5256delG	18	TGG GTA→TGG TAG	1713	Stop 1713	3335[h]	2.1	6 (44)	2 (41)
V1713A	18	GTA→GCA	1713	Val→Ala	80[i]	.35	4 (51)	11 (55)
5385insC	20	TCC CAG→TCC CCA	1756	Stop 1829	3365	na	3 (48)	1 (46)

[a] Designation of mutation according to Beaudet and Tsui (1993), conserving as much normal sequence as possible when exact starting nucleotide is ambiguous.

[b] Maximum lod score for linkage to chromosome 17 markers; na = not analyzed.

[c] Number of verified cases of breast cancer and ovarian cancer in the family. A subject with both breast and ovarian cancer was counted in each total. Fallopian tube and peritoneal carcinomatosis cases were excluded from totals.

[d] Other verified cases of cancer (and age at diagnosis): fallopian tube (43); colon (40, 60, 64); pancreas (53); uterine (61); salivary gland (43); and leukemia (5). Mutational status unknown for all cases.

[e] Other verified cases of cancer (and age at diagnosis): CNS (71); and stomach (76). Mutational status unknown for all cases.

[f] Post-oophorectomy carcinomatosis at age 43—mutation carrier.

[g] African-American family.

[h] Other verified cases of cancer (and age at diagnosis): male breast (59)/prostate (59)—mutation carrier.

[i] Other verified cases of cancer (and age at diagnosis): fallopian tube (40); colon (53, 59); kidney (64); and lung (61)—mutational status unknown.

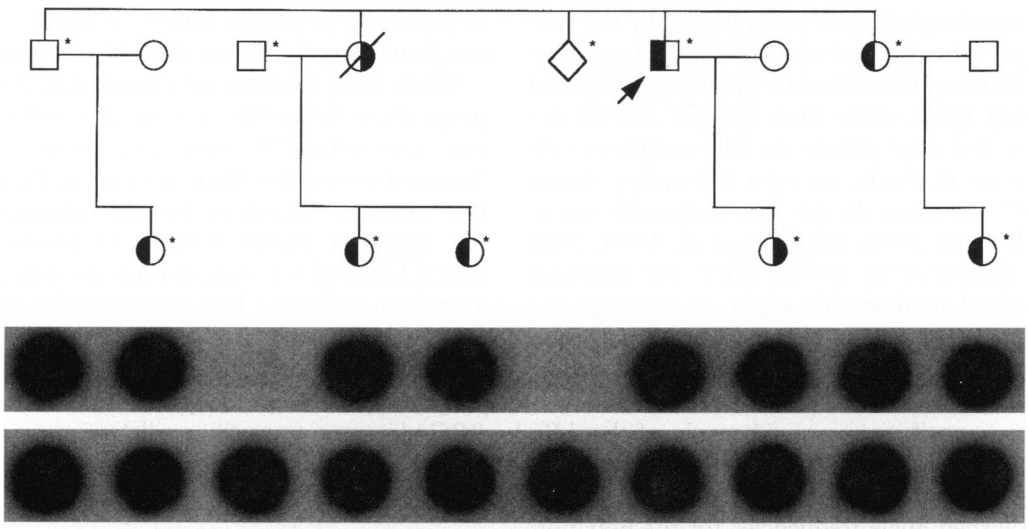

Figure 1 ASO hybridization analysis of the 5256delG *BRCA1* mutation. The pedigree is abbreviated to protect confidentiality; circles represent females, squares represent males, and the diamond represents an individual with concealed gender. Open symbols represent unaffected individuals, left-half shading represents breast cancer, and right-half shading represents ovarian cancer. Sampled individuals for whom the ASO was performed are marked with an asterisk (*). Arrow points to male proband with synchronous breast and prostate cancer at age 59 years. *Bottom row,* Hybridization of normal oligonucleotide. *Top row,* Hybridization of mutant oligonucleotide.

penetrance, or the probability of developing female breast cancer if one has a mutation, is nearly 85% in high-risk families studied thus far. However, population screening for *BRCA1* mutations is premature at this time. There are technical difficulties in identifying mutations in *BRCA1* and more profound problems in interpreting the results of testing, even in high-risk families.

The size of the *BRCA1* gene, the wide array of mutations already described, and the presence of multiple polymorphisms suggests that the development of DNA-based screening tests will be technically challenging. Since most of the mutations described thus far are frameshift or nonsense mutations, protein truncation assays may be an effective screening test. This mutation-detection method has been successfully applied to the *APC* gene in familial adenomatous polyposis (Powell et al. 1993). A functional assay would also obviate the need for DNA analysis, but, until more is known about the protein product of *BRCA1,* the feasibility of such an assay is unknown. More robust DNA-based assays, capable of detecting all possible sequence changes, may be possible in the future. An example of such a system would use machine-vision analysis of differences in hybridization of short oligonucleotides that encode for all possible nucleotide substitutions throughout the *BRCA1* gene affixed to a silicon grid or "chip" (Lamture et al. 1994; Pease et al. 1994).

In addition to technical problems, there are also difficulties in interpreting the mutation information. Given the heterogeneity in breast/ovarian cancer families, correlation of a phenotype such as a higher risk of ovarian cancer with a given mutation would be very helpful in counseling carrier individuals. While few such correlations between *BRCA1* genotype and phenotype are so far obvious, there are two mutations that have been detected in six or more families each, and they do have a somewhat different phenotype. The families with the 187delAG mutation have relatively more cases of ovarian cancer (45% of the total number of breast and ovarian cancer cases) compared with the 5385insC mutation (21% of cases). This comparison is made with caution, however, given that the limited number of families studied thus far have been selected based on varied eligibility criteria.

The finding of a putative pathological mutation in a family with male breast cancer also illustrates the current lack of solid phenotype/genotype information, which complicates genetic counseling. Epidemiological evidence has suggested that most families with one or more cases of male breast cancer are related not to mutations in *BRCA1,* but instead to *BRCA2.* (Stratton et al. 1994; Wooster et al. 1994). The deletion detected in family 3335 segregates with the disease and suggests that some families with male breast cancer may be related to mutations in *BRCA1* after all. The alternative, namely that the case of male breast cancer may have occurred by chance, unrelated to a mutation in *BRCA1,* cannot be formally ruled out. Many more families must be studied, ideally without such strong selection bias, in order to define the full spectrum of phenotypes associated with *BRCA1* mutations and to correlate this with the genotype.

Genetic counseling is further complicated by the possibility of both false-negative and false-positive test results. Some families studied thus far are probably related to predisposing genes other than *BRCA1* and so are true negatives. But there clearly are false negatives with present detection methods, as, even in families clearly linked to *BRCA1,* many do not have detectable mutations (Castilla et al. 1994; Friedman et al. 1994; Miki et al. 1994; Simard et al. 1994). SSCP, the mutation detection method used in this study, is certainly not 100% sensitive and suggests that a negative test result will be meaningful only if a relative is known to carry a pathological mutation.

False-positive results may occur because of the difficulty in distinguishing between benign polymorphisms and pathological mutations. Until epidemiological studies have established allele frequencies for the polymorphisms in the general population, interpreting sequence changes will not be straightforward. While false-positive results are less of a concern for frameshift and nonsense mutations, since they severely damage gene function, judging that a missense mutation is truly pathological will be more challenging. For example, one mutation, S1040N, segregated with disease in a large family and was not detected in 60 control individuals in one study (Friedman et al. 1994). In another study, S1040N did not segregate with disease and was present in 3 of 166 control individuals tested (Castilla et al. 1994). The V1713A missense mutation detected in this study occurred in a proband with both breast and ovarian cancer but not in 180 control chromosomes. No other affected relatives were living, so it is not known whether this mutation segregates with the disease in this family, and determination that this change is truly pathological will have to await functional studies.

After interpretation of the sequence data is resolved, the additional problem of counseling about preventive measures remains. The roles of screening mammography, transvaginal ultrasonography, or tumor markers in this population are unknown and need to be systematically evaluated (Hoskins et al. 1995). The proper role of prophylactic surgery in mutation carriers is unknown, but preliminary evidence suggests that prophylactic oophorectomy reduces the risk of ovarian/peritoneal cancer (Struewing et al., in press). Despite the surgery, there remains the finite risk of carcinomatosis, as in the patient reported here with the 1294del40 mutation (Tobacman et al. 1982). Whether increased surveillance for prostate and colon cancer in *BRCA1* mutation carriers is warranted will depend on more precise determination of the risk of these cancers for given mutations. Questions of the potential psychological and social implications of mutation testing are also serious considerations for family members (Biesecker et al. 1993; Lerman and Croyle 1994). These and other important questions will only be answered by careful follow-up studies of individuals and families with known *BRCA1* mutations.

When these findings are considered, it is not yet appropriate to move *BRCA1* mutation testing into the primary care setting. We agree with positions taken by the National Center for Human Genome Research (1994), the American Society of Human Genetics (1994), and the National Breast Cancer Coalition (1994) that *BRCA1* mutation testing should currently be considered a research endeavor. Laboratory studies of normal gene and protein function, rigorous population-based epidemiological studies of allele frequencies, and correlation of genotype and phenotype will be necessary before *BRCA1* mutation testing will be interpretable outside the context of high-risk families.

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