Germline BRCA1 Mutations and Loss of the Wild-Type Allele in Tumors from Families with Early Onset Breast and Ovarian Cancer

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ABSTRACT

The BRCA1 gene on human chromosome 17q21 is responsible for an autosomal dominant syndrome of inherited early onset breast/ovarian cancer. It is estimated that women harboring a germline BRCA1 mutation incur an 85% lifetime risk of breast cancer and a greatly elevated risk of ovarian cancer. The BRCA1 gene has recently been isolated and mutations have been found in the germline of affected individuals in linked families. Previous studies of loss of heterozygosity (LOH) in breast tumors have been carried out on sporadic tumors derived from individuals without known linkage to BRCA1 and on tumors from linked families. Loss of large regions of chromosome 17 has been observed, but these LOH events could not be unequivocally ascribed to BRCA1.

We have studied 28 breast and 6 ovarian tumors from families with strong evidence for linkage between breast cancer and genetic markers flanking BRCA1. These tumors were examined for LOH using genetic markers flanking and within BRCA1, including THRA1, D17S856, EDH17B1, EDH17B2, and D17S183. Forty-six percent (16/34) of tumors exhibit LOH which includes BRCA1. In 8 of 16 tumors the parental origin of the deleted allele could be determined by evaluation of haplotypes of associated family members; in 100% of these cases, the wild-type allele was lost. In some of these families germline mutations in BRCA1 have been determined; analyses of tumors with LOH at BRCA1 have revealed that only the disease-related allele of BRCA1 was present. These data strongly support the hypothesis that BRCA1 is a tumor suppressor gene.

INTRODUCTION

Familial early onset breast/ovarian cancer is an autosomal dominant disorder manifested as an increased susceptibility to breast cancer starting in the third decade, with an 85% lifetime risk. Breast adenocarcinomas and ovarian cystadenocarcinomas are the predominant histological types. Mucinous ovarian cancers seem to be less common in hereditary breast/ovarian families (1), but in general the familial tumors are phenotypically indistinguishable from histologically matched sporadic tumors. Atypical hyperplasia, a premalignant lesion, has been found in one study to be more prevalent in families at increased risk for hereditary breast cancer (2). The natural history of the tumors, including detectability by radiographic and clinical means, responsiveness to therapy, and overall prognosis for the patients based on stage at presentation appear to be indistinguishable from age-matched controls (1).

Since 1990, it has been known that a single locus on chromosome 17q21, termed BRCA1, is responsible for most cases of hereditary early onset breast/ovarian cancer (3, 4). Approximately 67% of families with breast cancer diagnosed before age 45 years and 95% of families with breast/ovarian cancer appear to be linked to BRCA1. Easton et al. (5) have recently reported that the penetrance, by age 70 years, is 87% (95% CI: 72–95) for breast cancer and 44% (95% CI: 28–56) for ovarian cancer. The ovarian cancer risk may be an overestimate, however, because of a bias favoring ascertainment of families with both breast and ovarian cancer. In addition, Ford et al. (6) have reported a significant increase in the relative risks for colon (4.11, 95% CI: 2.36–7.15) and prostate cancer (3.33, 95% CI: 1.78–6.20) in BRCA1 mutation carriers. The BRCA1 gene has recently been reported and disease-associated mutations have been found in breast and breast/ovarian kindreds (7–11). The study of tumors in linked families may help elucidate how BRCA1 mutations lead to disease.

Tumor analyses have proven useful not only in narrowing the candidate region, thereby expediting the search for BRCA1, but have also provided data supporting the hypothesis that BRCA1 is a tumor suppressor gene (12, 13). Work from several laboratories (12–16) has revealed that sporadic and familial breast and ovarian tumors may exhibit the phenomenon of LOH.

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3 The abbreviations used are: BRCA1, breast cancer gene; LOH, loss of heterozygosity; CI, confidence interval; SSCP, single-strand conformation polymorphism; LOD score, base 10 logarithm of the odds favoring linkage.
in large and diverse chromosomal regions, including 17q21, which supports the hypothesis that BRCA1 is likely to be a tumor suppressor gene. In other words, functional loss of both BRCA1 alleles in the affected tissue(s) may be necessary for malignant transformation to occur. In this model, affected individuals are presumed to be born with a germline defect in the BRCA1 gene, which constitutes the ‘‘first hit’’ in Knudson’s model for tumorigenesis by a tumor suppressor gene. The second defect is acquired somatically in the wild-type allele in the breast or ovarian tissue of these patients, thereby contributing to the development of cancer. One mechanism for disruption of the second allele can be an interstitial chromosomal deletion, detected through loss of alleles at flanking polymorphic markers. Thus, LOH is widely held to be an indicator of the presence of a tumor suppressor gene adjacent to the deleted markers. The observations of LOH presented here support a tumor suppressor mechanism for BRCA1.

MATERIALS AND METHODS

Specimen Selection. Thirty-four paraffin-embedded breast tumors from families likely to harbor BRCA1 germline mutations were obtained from the institutions where the patients had undergone surgical treatment, in accordance with guidelines from the University of Michigan Human Use Committee for the procurement of archival materials for research. Living family members provided informed consent to proceed with the analyses of their tumors; the results were not shared with the patients. All specimens underwent histological examination to confirm the diagnosis and to determine whether both neoplastic and normal components were present.

DNA Extraction. With the assistance of a surgical pathologist (T. S. F.), each paraffin block was cut in 4-μm sections and mounted on glass slides. Normal and neoplastic tissue fractions were dissected with a single-use disposable razor blade under a dissecting microscope. This procedure minimized mixing of normal and tumor subpopulations, and yielded tumor samples which were estimated to be at least 90% free from contamination with normal cells. A typical sample contained DNA from 100-1000 cells. The samples were deparaffinized with 100 μl xylene. An equal volume of 100% ethanol was added and the samples were pelleted for 10 min at 15,000 × g, vacuum dried, and digested overnight with 200 ng/μl proteinase K in 100 μl 50 mM Tris (pH 8.3). The samples were boiled for 8 min, iced, and centrifuged again to remove proteinaceous and other debris. Samples were extracted in groups of 8-12, each extraction containing a tube without template as a negative control. This method avoids the use of ionic detergents, sonication, or phenol/chloroform separation, all of which yield samples which may be suboptimal for PCR experiments.

All tumors were dissected and extracted at least twice in separate batches for verification of results. Each tumor/normal extraction yielded two 100-μl samples with a total DNA mass of the order of a femtogram each. Approximately 8–10 reactions could be performed with template from each extraction. Whenever further extractions were needed for a given block, these were carried out on sections immediately adjacent to the original slide. This procedure resulted in the reextraction of tumor/normal DNA from regions equivalent to those of the original extraction. It was then possible to apply uniform criteria for scoring LOH at all markers, even if more than one extraction was necessary.

PCR Amplification of Polymorphic Markers. PCR reactions were performed on each tumor/normal pair, a normal human DNA control, and two or more samples from CEPH parents known to be informative for the marker being analyzed. DNA extraction reagents and water served as negative control template. The reaction volume was 35 μl. The reaction mixture consisted of 8 μl DNA template, 3.5 μl 10X Taq polymerase buffer, 4.25 μl 1.25 mM deoxyribonucleotide triphosphate mixture, 0.2 μl Taq polymerase, a variable amount of forward and reverse primer ranging from 0.8–1.5 μl of a 10 ng/μl solution of each, and the remainder in double-distilled water. One of the primers was end labeled with [32P]dATP using T4 kinase. Annealing temperatures ranged between 53°C and 65°C, depending on the primers, with an extension temperature of 72°C. The annealing time was 1 min whereas the extension time varied from 1 min to 40 s + 5 s/cycle for 35 cycles. Optimization of conditions proceeded as follows: if amplification was not detected with an annealing temperature of 53°C, the amount of template was increased to 12 μl. Nonspecific signals were suppressed by increasing the annealing temperature and/or decreasing the primer concentration. The PCR products were analyzed in 0.4-mm thick 8% denaturing polyacrylamide gels, which were subsequently dried and autoradiographed by standard techniques.

Although it is widely acknowledged that the scoring of LOH is a qualitative assessment, we have used the following guidelines to ensure consistency of scoring. In experiments where the band intensity of the alleles varied between the tumor and normal samples, but the wild-type allele was still visible, the samples were reextracted from immediately adjacent sections at least two times and independent experiments were performed on the tumor/normal pairs. A decrease in intensity of a band of over 75% was required for scoring LOH. The criteria for scoring LOH were uniform for all markers analyzed on samples derived from a given extraction, i.e., if the wild-type allele was entirely lost in one marker, to score LOH on another marker using template from the same extraction, a qualitatively similar decrease in the wild-type allele intensity was required. The signal intensity was evaluated visually by at least three independent observers.

SSCP. PCR was carried out as outlined above in 10-μl reactions. Direct incorporation of [α-32P]dCTP was used instead of end labeling of primers. The PCR products were denatured and analyzed on MDE SSCP gels (AT Biochem) run at 6 W for 16 h at room temperature. The gels were dried and autoradiographed by standard methods. The sequences for exon 5 primers are (7): forward, 5′-CTCTTAAAGGCGATCGAG and reverse, 5′-TCTCTACTGGTGTTGCTTCC; their annealing temperature is 60°C.

RESULTS

Twenty-eight breast and 6 ovarian tumors from 14 families were studied. Six families had evidence for linkage between
The tumors were examined for LOH using polymorphic markers detectable LOH using the genetic markers described. In a related BRCA1 limited number of affected individuals available for sampling. For six families the LOD scores are not available due to the 50-75%.

Fig. 1 Clinical, pathological, genetic (LOD score), LOH, and BRCA1 mutation data on 34 tumors from breast/ovarian families analyzed with markers within and flanking the BRCA1 candidate region. Mutations are reported following the convention of Beaudet and Tsui (16). For patients affected by more than one tumor, the histological type of the tumor analyzed is listed: ⽔, LOH; ⚫, LOH, wild-type allele is lost; □, indeterminate. LOH cannot be scored; ○, retention of heterozygosity; n, not informative; blank, untested markers; B, breast; O, ovary; E, endometrium; G, stomach; AxLN, axillary lymph node; ID, infiltrating ductal carcinoma; IL, infiltrating lobular carcinoma; ILD, infiltrating lobular and ductal carcinoma; ME, medullary carcinoma; PS, papillary serous carcinoma; EN, endometrioid carcinoma; MD, metastatic ductal carcinoma; A/Am, moderately differentiated adenocarcinoma; B-Rg, Bloom-Richardson grade of the ductal carcinoma; NA, not available; +, <10%; +,+ , 10–25%; ++, 25–50%; +++, 50–75%.

breast/ovarian cancer and genetic markers flanking BRCA1 (LOD score range, 1.18–2.91), one family has a LOD score of 0.41, one family was probably unlinked (LOD score, −1.3), and for six families the LOD scores are not available due to the limited number of affected individuals available for sampling. The tumors were examined for LOH using polymorphic markers flanking BRCA1 including THRA1, D17S856, EDH17B1, EDH17B2, and D17S183. Sixteen (47%) of 34 tumors exhibited detectable LOH using the genetic markers described. In a related independent study, BRCA1 mutations were identified in some of the kindreds from which these tumors derive (8).

Fig. 1 summarizes the results of the LOH experiments for a subset of representative markers in the BRCA1 region, provides clinical data on the patients, histological data on the tumors, and genetic and mutation data on the families. Several distinct patterns of LOH are seen in these families. In family 15, four tumors (tumors 209, 191, 157, and 224) have LOH encompassing all scorable markers, whereas tumors 768 and 763 showed no LOH for any of the markers tested. In family 27, tumors 422 and 532 exhibit LOH in a region containing the BRCA1 gene. Tumor 484 belongs to a family found to be unlinked to BRCA1; we did not observe LOH for the markers.
tested. The minimum region of overlap of LOH among all of the
tumors tested lies between D17S856 (OF2) and D17S183
(SCG43), which includes BRCA1. All of the samples which
showed LOH had less than 50% normal stromal infiltration on
the slides used for DNA extraction. No relationship between the
presence of LOH and tissue of origin, histological type, or grade
was observed.

Fig. 2 displays data from experiments on a single tumor/
normal pair (sample 422) using primers which flank D17S856 (a
tetranucleotide repeat), EDH17B2 (a 12-base pair insertion-
deletion polymorphism in 17β-hydroxysteroid dehydrogenase;
Ref. 17), D17S1185 (a tetranucleotide repeat), and D17S183 (a
dinucleotide repeat). The EDH17B complex consists of two
loci, one of which is a pseudogene, arranged in tandem on
17q21 (18). The 5′ region of EDH17B2 contains a 12-base pair
insertion-deletion polymorphism (17), around which we have
designed PCR primers that yield a product of 267 and/or 255
base pairs suitable for amplification from paraffin-derived DNA
templates; PCR products less than 300 base pairs in length are
more consistently amplified from archival material than larger
segments, perhaps due to the DNA being nicked or alkylated.
We encountered rare blocks which would not yield amplifiable
DNA for any markers, possibly due to chemical modification of
the DNA during fixation.

In Fig. 2, A and D show retention of heterozygosity for
D17S856 and D17S183, respectively; B shows consistent
LOH at EDH17B2 for two different DNA extractions of
adjacent microtomed slides; and C shows LOH at D17S1185.
The assays in A, the N1/T1 normal/tumor pair in B, and C
were carried out on the same DNA sample; the assays for the
N2/T2 normal/tumor pair of B and those in D were performed
on template extracted from a slide immediately adjacent to the
previous one.

Haplotypes of additional family members for several mark-
ers flanking and within the BRCA1 region confirmed that the
allele lost in the tumor was the wild-type allele, as shown by the
pedigree in (Fig. 3). The individual marked with an arrow in
Fig. 3 is 422, whose tumor assays are depicted in Fig. 2. For
clarity, we do not present the full haplotype here, as it has been
reported previously (19) and supports linkage to BRCA1 on
17q21. We note however that the mother of 422 is a nonpen-
entrant carrier. Fig. 3 summarizes the BRCA1 haplotype data for
the constitutional and tumor DNA of sample 422 derived from
Fig. 4. SSCP of exon 5 of BRCA1 of normal control (non-BRCA1
mutation carrier) DNA (B), germline (G), and tumor (T) DNA for
sample 422. All samples were run on the same gel and autoradiographed
simultaneously. Arrow, variant band arising from the Cys64Gly muta-
tion.
The observation of LOH in 17q21 in sporadic breast tumors thereby excluding the minimum region of overlap by LOH base region of minimum overlap between D17S846 and excluded from the BRCA1 such as NME1 and pnohibitin. Both genes were subsequently encompassed by these two markers is greater than 10 cM and age. More recently, Kelsell BRCA1 has led to the hypothesis that radia carcinogenesis (15).

The phenomenon of LOH has proven to be a useful indicator of the involvement of tumor suppressor genes in the development of breast and other tumors. Following the experiments of Fearon and Vogelstein (20) and Vogelstein et al. (21) in colon cancer, Sato et al. (14) demonstrated that at least four distinct regions on chromosomes 13q (D13S52), 16q22-23, and 17p13.1–13.3 exhibit LOH in sporadic breast tumors, suggesting that as many as four different tumor suppressor genes may be implicated in sporadic carcinogenesis. Chen (15) selected probes on chromosomes 22q, 18p, 17p, 11p, 13q, and 1q, all of which are candidate regions for tumor suppressor genes, to ascertain whether LOH at these locations is present above a background level. They showed that, with the exception of 11p15, all locations tested exhibited LOH at well above background levels. Smith et al. (13) analyzed 13 (2 breast and 11 ovarian) tumors in families thought to be linked to BRCA1; they found LOH for two markers flanking BRCA1 (D17S250 and D17S855) in 9 tumors, all of which had lost the wild-type allele. The region encompassed by these two markers is greater than 10 cM and includes other genes potentially involved in breast cancer such as NME1 and prohibitin. Both genes were subsequently excluded from the BRCA1 candidate region by genetic linkage. More recently, Kelsell et al. (12) have reported LOH at marker D17S855 (248yg9) within BRCA1 in five familial breast tumors and one sporadic ovarian tumor. In all cases, the wild-type allele was shown to be lost. The marker closest to the present limits of the BRCA1 region which retained heterozygosity was located near TP53 on 17p and therefore did not contribute to narrowing the BRCA1 candidate region. The observation of LOH in 17q21 in sporadic breast tumors has led to the hypothesis that BRCA1 is implicated in sporadic carcinogenesis (15).

Recently, Cropp et al. (22) studied 130 sporadic breast tumors and found LOH in a relatively small 120–150-kilobase region of minimum overlap between D17S846 and D17S746. However, BRCA1 is located distal to D17S746, thereby excluding the minimum region of overlap by LOH observed by Cropp et al. (22). The presence of at least three tumor suppressor genes other than BRCA1 on chromosome 17 makes interpretation of LOH data derived from widely spaced markers difficult; these tumor suppressor genes may also be involved in the progression of the malignant phenotype. In this regard, carrying out LOH studies on samples obtained from families with documented BRCA1 linkage provides additional critical information: if LOH affects BRCA1, the wild-type allele should be the one lost.

The development of techniques for the extraction of relatively pure normal and tumor DNA fractions from paraffin-embedded tissue has allowed us to study 34 tumors from linked families. The success of the extraction and, ultimately, of the experiments performed with the samples hinges on the careful manual separation of tumor from normal cells under the dissecting microscope using surgical blades. This was accomplished in our study by a skilled surgical pathologist (T. S. F.). The experiments were repeated as many as five times before a definitive score was assigned. In spite of great care being taken in separating tumor from surrounding normal tissue when dissecting specimens, some samples could not be definitively scored as exhibiting LOH, although diminished intensity of the wild-type allele in the tumor sample suggested LOH.

A heterogeneous pattern of LOH was observed in these tumors. Some tumors exhibited no LOH for any of the markers tested; others showed loss of the wild-type allele flanking BRCA1. LOH might have been present and not detected in our experiments if the tumor were heavily infiltrated with stromal elements, and thus not separable. Also, if the interstitial deletion spanned an interval smaller than the distance between the markers we used, LOH would not be observed. The minimum region of overlap for the LOH seen in our familial tumors contains the now known BRCA1 gene and excludes the region between D17S846 and D17S746 described by Cropp et al. (22), which may contain another tumor suppressor gene.

Germline mutations in BRCA1 were found in four of the families shown in Fig. 1, designated by numbers 15, 27, and 51 (8). Affected members in families 15 and 27 had tumors which showed LOH. The individuals from family 27 whose tumors were analyzed and listed in Fig. 1 have been confirmed to carry the Cys64Gly germine mutation, except 532 who is diseased. Those tumors from family 27 which did not show LOH were also found to have abundant normal stromal infiltration within the tumor sections. This is a potential reason why we failed to detect LOH in these cases. When BRCA1 mutations were analyzed in a subset of familial tumors which exhibit LOH such as the one depicted in Fig. 4, the disease-related allele was the most prominent in the tumor fraction (8), providing further confirmation of the LOH results by a different method. Our data thus confirm that LOH does, in some tumors, occur specifically within the BRCA1 region and virtually always deletes the wild-type allele, thereby strongly supporting the hypothesis that BRCA1 is a tumor suppressor gene.

The role that BRCA1 plays in sporadic carcinogenesis is, in contrast, not clear at present. The two-hit tumor suppressor mechanism predicts that somatic mutations of BRCA1 would be present in the genomic DNA of sporadic tumors. However,
the first reported analyses of 35 breast and 12 ovarian sporadic tumors by Futreal et al. (11) failed to demonstrate such somatic BRCA1 mutations in the tumor samples; the four mutations described were present in both the tumor and the germline. From this experience it might be predicted that thorough searches of the BRCA1 coding and regulatory regions in many samples of breast, ovarian, and prostrate tumors may be necessary to identify a role for BRCA1 in sporadic carcinogenesis.

In some familial and sporadic tumors which do not exhibit LOH or point mutations, loss of BRCA1 function may be caused by derangement of its transcription or translation mechanisms due to alterations in regulatory elements or in other genes. One such phenomenon is postulated by Brown et al. (23) in their study of the genomic structure of the S' end of BRCA1. They showed that exon 1 of BRCA1 is centromeric to the 1A1–3B gene and that the two genes lie only 295 base pairs apart. The 1A1–3B gene had been isolated from a cDNA ovarian library with CA125 antisera. The intervening sequence between BRCA1 and 1A1–3B contains putative “CAT” boxes which may coregulate the cis-coordinate transcription of these genes. These data suggest that future studies should investigate disruptions of this regulatory mechanism in sporadic and familial tumors without LOH, especially those from breast/ovarian families for whom germ-line BRCA1 mutations have not been identified in the currently known coding region of BRCA1.

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Germline BRCA1 mutations and loss of the wild-type allele in tumors from families with early onset breast and ovarian cancer.

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