Common Nonsense Mutations in RAD52

Daphne W. Bell, Doke C. R. Wahrer, Deborah H. Kang, Melissa S. MacMahon, Michael G. FitzGerald, Chikashi Ishioka, Kurt J. Isselbacher, Michael Krainer, and Daniel A. Haber

Center for Cancer Risk Analysis, Massachusetts General Hospital Cancer Center and Harvard Medical School, Charlestown, Massachusetts 02129 [D. W. B., D. C. R. W., D. H. K., M. S. M., M. G. F., K. J. I., M. K., D. A. H.]; and Tohoku University, Sendai 980, Japan [C. I.]

Abstract

RAD51, RAD52, and RAD54 encode proteins that are critical to the repair of double-strand DNA breaks by homologous recombination. The physical interactions among the products of RAD51, BRCA1, and BRCA2 have suggested that the BRCA1 and BRCA2 breast cancer susceptibility genes may function, at least in part, in this DNA damage repair pathway. Given the observation that different genes within a common functional pathway may be targeted by mutations in human cancers, we analyzed RAD51, RAD52, and RAD54 for the presence of germ-line mutations in 100 cases with early-onset breast cancer and for somatic mutations in 15 human breast cancer cell lines. Two premature stop codons, Ser346ter and Tyr415ter, were identified in germ-line RAD52 alleles from 5% of early-onset breast cancer cases. Together, these two heterozygous mutations were also found in 8% of a healthy control population, indicating that they do not confer an increased risk for breast cancer. A rare germ-line missense mutation was identified in RAD54, whereas no sequence variants were found in RAD51. None of the three RAD genes demonstrated somatic mutations in breast cancer cell lines. We conclude that, despite their potential functional association with the BRCA gene products, RAD51, RAD52, and RAD54 are not themselves targeted by mutations in human breast cancer. The presence of common nonsense mutations in RAD52 within the population may have significance for other conditions associated with potential alterations in DNA damage repair pathways.

Introduction

RAD51, RAD52, and RAD54 encode the human homologues of yeast genes that are required for homologous recombination, an essential step in the repair of double-strand DNA breaks (reviewed in Ref. 1). This process involves the pairing of sister chromosomes and nucleolytic processing of double-strand breaks to produce single strands, which then pair with the complementary strand from the sister chromosome, leading to accurate repair and recombination. In Saccharomyces cerevisiae, Rad54 facilitates the pairing of chromosomes (2), whereas Rad52 stimulates Rad51-mediated pairing of complementary single-stranded DNA and strand exchange (3–5). Yeast strains lacking any of these genes, defined as belonging to the Rad52 epistasis group, demonstrate profound defects in recombination and increased sensitivity to DNA damage. The vertebrate homologues of the yeast RAD51, RAD52, and RAD54 genes demonstrate similar functional properties. Homozygous deletion of RAD51 and RAD54 in the mouse leads to an early embryonic lethal phenotype, and cultured embryonic stem cells display increased sensitivity to ionizing radiation, a reduction in homologous recombination, and progressive chromosome loss and genomic instability (6–8). In contrast, RAD52-null mice are viable (9), and inactivation of RAD52 in chicken B cells leads to a reduction in targeted chromosomal integration, without a detectable increase in radiation sensitivity (10). The attenuated phenotype of RAD52 deletion in vertebrates is in striking contrast to its essential role in S. cerevisiae but consistent with that of its Schizosaccharomyces pombe homologue, RAD22, pointing to potentially divergent functional pathways (11). In vertebrates, failure to repair double-strand breaks by RAD52-mediated homologous recombination may be compensated in part by Ku-dependent nonhomologous recombination (12).

The discovery that RAD51 associates directly with the product of the breast cancer predisposition gene BRCA2 (7) and indirectly with the BRCA1 product (13, 14) has implicated these tumor suppressor genes in this recombinational pathway for the repair of DNA damage. In addition to their physical association and colocalization with RAD51 within nuclear dots, the products of the BRCA genes appear to play a role in the repair of DNA damage induced by ionizing radiation. BRCA1- and BRCA2-null embryos are arrested in early embryonic development, and the latter display hypersensitivity to γ-irradiation (6, 7). Ionizing radiation leads to the hyperphosphorylation and physical relocalization of BRCA1, and mouse embryo fibroblasts expressing attenuated alleles of either BRCA1 or BRCA2 accumulate chromosomal breaks, characteristic of deficiencies in recombination-mediated repair (14–16). Taken together, these observations raise the possibility that the BRCA genes are components of a functional pathway defined by RAD51, RAD52, and RAD54 and that mutations in these genes may themselves contribute to the development of breast cancer. Of note, RAD52 maps to chromosome locus 12p12.2–p13, a frequent site for allelic losses in breast and ovarian cancer (17–19).

Germ-line mutations in either BRCA1 or BRCA2 account for 60–80% of breast cancer kindreds, suggesting the existence of additional breast cancer predisposition genes (20, 21). Population-based cohorts with an early age of onset of breast cancer have been particularly helpful in screening for genetic variations that may confer an increased risk of developing breast cancer, including highly penetrant mutations that are represented in cancer pedigrees and sequence variations in the population that may be associated with a more moderate increase in breast cancer risk. Epidemiological models have predicted that 36% of early-onset breast cancer cases (diagnosed under the age of 30 years) result from a single-gene defect (22), whereas BRCA1 and BRCA2 mutations are observed in only ~15% of these cases (23–25). Given the observation that BRCA1 and BRCA2 mutations are present in the germ-line of women with genetic predisposition to breast cancer but not in sporadic breast tumors, analysis of candidate genes for their potential role in the development of breast cancer requires analysis of both tumor and germ-line specimens. We, therefore, analyzed the transcripts for RAD51, RAD52, and RAD54 in EBV-immortalized lymphoblasts from 100 women who had been identified from Boston area hospitals as having developed breast...
cancer under the age of 40 years and from an equal number of healthy controls from the same population. Nine of the 100 early-onset cases also had a strong family history of breast cancer but did not have a BRCA1 or BRCA2 mutation and, therefore, may carry a germ-line mutation in another breast cancer susceptibility gene. To search for somatic mutations in thesegenes, we analyzed 15 breast cancer cell lines.

Materials and Methods

Study Population. EBV-immortalized lymphocytes from 100 women who were diagnosed with breast cancer at or before the age of 40 years were screened for mutations in the RAD51, RAD52, and RAD54 genes. These samples were part of a larger cohort of 408 women with early-onset breast cancer that was identified by survey of Boston area hospitals, and they represent a subset of the general population at high risk for genetic predisposition (23). Nine of the 100 early-onset cases studied also have a strong family history of breast cancer, defined as more than three affected family members, in either paternal or maternal lineage, over at least two generations. Control EBV cell lines were derived from healthy blood donors at these hospitals. Fifteen human breast cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA): MDA-MB-435, MDA-MB-468, MDA-MB-415, MDA-MB-231, MDA-MB-453, MDA-MB-175-VI, MDA-MB-436, MDA-MB-157, MCF-7, MCF-7/ADR, UACC-893, T-47D, BT-549, BT-483, and HS578.

nt4 Sequencing Analysis. Nested PCR was performed on a RAD51 primary PCR product using five sets of primers to generate overlapping PCR products for sequence analysis. To facilitate sequencing, we added the M13-21 primer sequence (GTGAAACAGCCGAGCATT) to the 5′ end of the forward primer, and the M13-28 primer sequence (AGAAACAGCATGATG) was added to the 3′ end of the reverse primer. Primers pairs used were: R51-ET-1F (ATCTGATGCAGATGC); R51-ET-1R (CAATGCTCGAGAGCCCAGAGGACC); R51-ET-2F (CTTGGCTCATGGGTACTTGACG) and R51-ET-2Ra (CACTCTTGGGTTGTGCGCAGAAGGC); R51-ET-2R (TTCCCGGAAGTCTTCTGTGGCCTG); R51-ET-3F (ATCCCTGGCAGCCATGGCTGCATC) and R51-ET-3R (GACTCCAACATGAAGGCGGAAGG); R51-ET-4F (CTGGCTGGGTC); with primers RAD52F (GATCTGCCCATTCTGGGAGCG) and RAD51R (TTCCCGGAAGTTCTTCTGTGGCCTG); R51-ET-5Ra (AAAAATGCAGACTATCAAGGTT) and R51-ET-5R (CAATCTGGTTGAGTTCATG); and R51-ET-5Fa (AGGTGTAATCACTAATCAGGG) and R51-ET-5F (CTTTCCCATTCTGGGAGCG). The coding region of RAD52 was amplified using the following primer sets: R52-ET-1F (ATCTGCCCATCTTGTACTCC) and R52-ET-1R (GATAATTACCCGGATCGGATG); R52-ET-2F (ATCCAGAAGGGCTCCGGAGCG) and R52-ET-2Ra (TGATATGGAACACCTCCTCCTGAC); R52-ET-3F (CCATCTTGGGTTGTGCGCAGAAGGC); R52-ET-3R (TTCTGAGTTCTGCCTGAG); R52-ET-4F (CTTTATCCTGGC) using 35 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 3 min; with primers RAD52F (GATCTGCCCATTCTGGGAGCG) and RAD51R (TTCCCGGAAGTTCTTCTGTGGCCTG); R52-ET-5F (GACCGAGGCCAGCCGGAGCG) and R52-ET-5R (CAGITTTCTGCTGTTGAGCTG); and R52-ET-5Fa (AGGTGTAATCACTAATCAGGG) and R52-ET-5F (CTTTCCCATTCTGGGAGCG). The region of RAD54 was amplified using the following primer sets: R54-ET-1F (CTTTGGCTCATGGGTACTTGACG) and R54-ET-1R (CAATGCTCGAGAGCCCAGAGGACC); R54-ET-2F (TCAACAGGGCCCTGTGGAGCAAGG) and R54-ET-2Ra (CTTTCCATTGGAACTAATTTAGCTGCC); R54-ET-2R (TGCCTATCCATCTGCGAAGG) and R54-ET-3F (AGGACTCCAACATGAAGGCGGAAGG); R54-ET-3R (GACTCCAACATGAAGGCGGAAGG); R54-ET-4F (CTGCAATGCAGATGC) and RAD51AR (AGCAGCCGTTCTGGCGCTAAG); and R54-ET-4R (GCCCTCCCATTCTGGGAGCG) and R54-ET-5R (CAATCGTGGTTGAGTTCATG); and R54-ET-5Fa (AGGTGTAATCACTAATCAGGG) and R54-ET-5F (CTTTCCCATTCTGGGAGCG). The coding region of RAD54 was amplified using the following primer sets: R54-ET-1F (CTTTGGCTCATGGGTACTTGACG) and R54-ET-1R (CAATGCTCGAGAGCCCAGAGGACC); R54-ET-2F (TCAACAGGGCCCTGTGGAGCAAGG) and R54-ET-2Ra (CTTTCCATTGGAACTAATTTAGCTGCC); R54-ET-2R (TGCCTATCCATCTGCGAAGG) and R54-ET-3F (AGGACTCCAACATGAAGGCGGAAGG); R54-ET-3R (GACTCCAACATGAAGGCGGAAGG); R54-ET-4F (CTGCAATGCAGATGC) and RAD51AR (AGCAGCCGTTCTGGCGCTAAG); and R54-ET-4R (GCCCTCCCATTCTGGGAGCG) and R54-ET-5R (CAATCGTGGTTGAGTTCATG); and R54-ET-5Fa (AGGTGTAATCACTAATCAGGG) and R54-ET-5F (CTTTCCCATTCTGGGAGCG). The region of RAD54 was amplified using the following primer sets: R54-ET-1F (CTTTGGCTCATGGGTACTTGACG) and R54-ET-1R (CAATGCTCGAGAGCCCAGAGGACC); R54-ET-2F (TCAACAGGGCCCTGTGGAGCAAGG) and R54-ET-2Ra (CTTTCCATTGGAACTAATTTAGCTGCC); R54-ET-2R (TGCCTATCCATCTGCGAAGG) and R54-ET-3F (AGGACTCCAACATGAAGGCGGAAGG); R54-ET-3R (GACTCCAACATGAAGGCGGAAGG); R54-ET-4F (CTGCAATGCAGATGC) and RAD51AR (AGCAGCCGTTCTGGCGCTAAG); and R54-ET-4R (GCCCTCCCATTCTGGGAGCG) and R54-ET-5R (CAATCGTGGTTGAGTTCATG); and R54-ET-5Fa (AGGTGTAATCACTAATCAGGG) and R54-ET-5F (CTTTCCCATTCTGGGAGCG).
and 72°C for 1 min 30s with primers RAD54AF, CTAGGCCCAGGATGAGGAGGAGCTTG; RAD54AR, GGTGTCAGCCTACAACAAACGACCTGC; RAD54BF, TAGGAGAGGAGCGGCTGCGGGAGC; and RAD54BR, CAGCGGAGGCCCGGCTGTTCCTC.

Primers used to amplify a RAD51 fragment encoding a frameshift were: RAD51AFmut, TTCCTGACTATGCGGGCTATCCCTATGACGTCCCGACTATGCAGAGTAATGGCAATGCAGATGC; and RAD51AR. Wild-type (RAD51AF/RAD51AR products) and mutant (RAD51AF-mut/RAD51AR products) were mixed together in variable ratios and transformed into YPH499.

Nested PCR products (10 μl) and BamHI-digested yeast expression vector, pCI-HA(ura3)-2(-leu) (10 ng), were cotransformed into competent yeast (strain YPH499; Stratagene, La Jolla, CA). Vector alone was transformed into yeast as a negative control. Transformants were selected by plating on SC medium lacking leucine. For each sample, 30 transformants were replica-streaked onto SC plates lacking either leucine or leucine and uracil. Transformants expressing a chimeric RAD51 (ORF)-URA3 fusion gene exhibit uracil-independent growth, whereas those containing a stop codon in RAD51 are unable to grow in the absence of uracil. As expected, virtually 100% of transformants derived from specimens with wild-type inserts display the ura3− phenotype, whereas ∼50% of transformants from specimens with a heterozygous truncating mutation are ura3− (see “Results”). To sequence the inserts of ura32 yeast transformants, the cDNA inserts were amplified from yeast with the ura32 phenotype by PCR amplification using primers directed against the flanking pCI-HA(ura3)-2(-leu) sequence: forward primer, CCC ATA CGA TGC TCC TGA CTA TGC G; and reverse primer, TTG GCA ACA GGA CTA GGA TGA G. PCR products were subjected to cycle sequencing using an automated fluorescence-based cycle sequencer (model 373; PE Applied Biosystems) and Taq dye terminator chemistry. Internal primers used in sequencing RAD52 clones were R52B-S2F (AGACCTCTGACACATTAGCCTTG) and R52B-S1R (TTTGCT TGTGTTTCTGTGG). The sense and antisense strands of the sequenced products were aligned with that of the wild-type sequence using the Sequence Navigator program (PE Applied Biosystems).

Results and Discussion

Analysis of RAD51. To screen for the presence of definitive RAD51 mutations in the germ-line of women at high risk for genetic predisposition to breast cancer, we first analyzed EBV-immortalized lymphocytes from a cohort of women with early-onset breast cancer (diagnosed before the age of 40 years), using a high-throughput, yeast-based protein truncation assay outlined in Fig. 1A (26, 27). To validate this truncation assay for analysis of RAD51 mutations, we mixed cDNAs with synthetic stop codons at various ratios. As predicted, specimens with reconstituted heterozygous mutations yielded

Fig. 1. Yeast based truncation assay of RAD genes. A, diagrammatic representation of yeast-based protein truncation assay for RAD51, RAD52, and RAD54. RT-PCR amplification of the transcript is performed using primers that contain a 45-nt tail, allowing for homologous recombination into the yeast URA3 gene that is interrupted by a synthetic BamHI site within plasmid pCI-HA(URA3)-2. Gap repair leads to recircularization of the plasmid and allows for selection of transformants for growth in the absence of leucine. Transformants that have an ORF within the chimeric RAD51-URA3 gene will also grow in the absence of uracil (ura3−), whereas those with a stop codon will be ura3−. In addition to rapid scoring of specimens for presence of a heterozygous truncating mutation, the separation of PCR products derived from wild-type and mutant alleles facilitates sequencing analysis. B, yeast truncation assay of reconstituted RAD51 mutants. The two agar plates represent replica plates of yeast transformed with the linearized plasmid pCI-HA(URA3)-2 and PCR-generated RAD51 cDNA. Left, plates lacking leucine, allowing for growth of all yeast transformants that have recircularized the plasmid by gap repair and homologous recombination; right, plates lacking both leucine and uracil, allowing growth of transformants with an ORF within the RAD51-URA3 chimera. Top, a homozygous wild-type specimen yields 93% ura3− colonies. Middle, a reconstituted heterozygous mutant yields 40% ura3− colonies. Bottom, a reconstituted homozygous RAD51 truncation mutant yields a frequency of leu+ transformants with the ura3+ phenotype of only 3%.

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50% ura3+ and 50% ura3− colonies, whereas those with wild-type 
RAD51 sequence produced virtually 100% ura3+ colonies (Fig. 1B).

Analysis of germ-line specimens from 120 women with early-onset breast cancer using this protein truncation assay did not identify any premature stop codons in RAD51 (Table 1). Whereas such truncating mutations might have provided definitive evidence for inactivation of RAD51 (see below), our analysis could not exclude the presence of missense mutations that might be associated with an increased risk for breast cancer. We, therefore, screened the highest-risk subset of the early-onset breast cancer cohort (27 women diagnosed before the age of 30 years) by direct automated nt sequencing of uncloned RT-PCR products, combined with analysis using software for detection of heterozygous mutations (see “Materials and Methods”). No sequence variations in RAD51 were detected in any of these cases, leading us to conclude that this gene is unlikely to contribute to genetic predisposition to breast cancer.

To extend our analysis to the potential contribution of RAD51 mutations to sporadic breast cancer, we screened breast cancer cell lines for the presence of truncating mutations. RAD51 has been localized to chromosome band 15q15.1, a region that exhibits frequent loss of heterozygosity in sporadic breast tumors (28). Loss of heterozygosity was primarily observed in specimens of metastatic breast cancer (7 of 10 cases), with less frequent allelic losses in primary breast tumors (2 of 19 cases). Analysis of 15 breast cancer cell lines derived from sporadic tumors using the yeast truncation assay identified neither heterozygous nor homozygous mutations in any cases (Table 1). Thus, definitive, truncating mutations in RAD51 are unlikely to contribute to the development of sporadic breast cancer. It is noteworthy that we detected an apparent alternative splicing variant within the 3’ end of the RAD51 transcript. This variant mRNA was detected by RT-PCR analysis in the majority of germ-line specimens and tumor cell lines. Sequencing analysis demonstrated a deletion of 122 nt (inclusive of positions 1007–1128), resulting in a frameshift and generation of a premature stop codon at nt 1195. Whereas this transcript removes the COOH-terminal region of RAD51 implicated in its interaction with the BRCT motifs of BRCA2 (29), its physiological significance is uncertain because it represents a very small proportion of the total cellular RAD51 mRNA (estimated at <1% by RT-PCR analysis, in both germ-line and cancer cell lines). However, this truncated transcript is preferentially amplified following nested RT-PCR analysis, and it should not be misconstrued as a genuine mutation.

Analysis of RAD52. In contrast to RAD51, analysis of 100 early-onset breast cancer cases, using the yeast-based protein truncation assay, revealed two recurrent nonsense mutations in RAD52: Ser346ter (TGG → TAG) and Tyr415ter (TAT → TAG; Table 1). These heterozygous germ-line mutations were confirmed by nt sequencing of uncloned PCR products derived from both cDNA and genomic DNA (Fig. 2A). The Ser346ter mutation was present in 3 of 100 cases with early onset of breast cancer, whereas the Tyr415ter mutation was detected in 2 of 100 cases. To determine whether these mutations were present within the general population, we analyzed 100 specimens from healthy blood donors. These controls are well matched with our cohort of early-onset breast cancer cases, as demonstrated by the equal prevalence of known p53 polymorphisms (data not shown). Direct sequencing analysis revealed the Ser346ter mutation in 5 of 102 controls and the Tyr415ter mutation in 3 of 102 controls (comparison with cases of early-onset breast cancer, P was not significant; Table 2). Hence, these two nonsense mutations do not appear to confer genetic susceptibility to early-onset breast cancer. Remarkably, however, these truncating mutations together are present in 8% of a diverse population. They were not restricted to a specific ethnic group, and no mutations were found in a cohort of 40 women of Ashkenazi Jewish descent, a population with an increased frequency of founder mutations in BRCA1 and BRCA2. No missense mutations in RAD52 were detected by direct nt sequencing of 27 cases with early-onset breast cancer.

RAD52 encodes a protein of 421 amino acids; the Ser346ter mutation results in loss of 75 COOH-terminal residues, whereas the Tyr415ter mutation only removes the terminal 7 amino acids. The functional properties of RAD52 remain to be defined, but known domains, including the sites of RAD51 association (30) and RAD52 self-association (31), and domains that bind DNA and interact with RPA (32) are NH2-terminal to Tyr415. To determine whether carriers of this heterozygous mutation might be more susceptible to DNA damage induced by ionizing radiation, we treated EBV-immortalized lymphoblasts from carriers with X-ray (1 Gy) and determined cell viability by vital dye staining. No significant increase in radiosensitivity was observed in the RAD52 Ser346ter/+ cells compared with RAD52 +/+ cells (data not shown). Within the limitations of these assays, carriers of these common truncating mutations in RAD52, therefore, do not appear to have increased sensitivity to ionizing radiation, although we cannot exclude more subtle abnormalities in homologous recombination and the repair of DNA damage.

Although the chromosome 12p12–13 locus encompassing the RAD52 gene is commonly targeted by allelic losses in breast cancer (18), analysis of 15 breast cancer cell lines did not demonstrate truncating mutations within RAD52. Our observations, therefore, do not indicate that inactivating mutations in RAD52 are commonly associated with sporadic breast cancer. Of note, we observed frequent aberrant RAD52 pre-mRNA splicing products, following RT-PCR amplification of the transcript from either breast cancer cell lines or germ-line of both patients and controls (Fig. 2B). However, RT-PCR of RAD52 from human thymus, a tissue in which the transcript is expressed at high levels physiologically, showed no evidence of these splicing products (Fig. 2C). As for RAD52, the aberrant RT-PCR products involved insertions of intronic sequences at nt positions 617, 736, 932, and 1236, leading to premature stop codons (Fig. 2B).

### Table 1 Mutational analyses of RAD51, RAD52, and RAD54

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutation detection assay</th>
<th>Cohort</th>
<th>n</th>
<th>Mutation detected (no. of cases)</th>
<th>Effect on protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAD51</td>
<td>Direct sequencing of RT-PCR product</td>
<td>Early-onset breast cancer (&lt;30 years)</td>
<td>27</td>
<td>None</td>
<td></td>
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<tr>
<td></td>
<td>Yeast-based protein truncation assay</td>
<td>Early-onset breast cancer (&lt;40 years)</td>
<td>93</td>
<td>No truncating mutations</td>
<td></td>
</tr>
<tr>
<td>RAD52</td>
<td>Direct sequencing of RT-PCR product</td>
<td>Early-onset breast cancer (&lt;30 years)</td>
<td>27</td>
<td>C→A at position 1306 (3 cases)</td>
<td>Truncation at codon 346</td>
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<tr>
<td></td>
<td>Early-onset breast cancer (&lt;40 years)</td>
<td>T→G at position 1514 (1 case)</td>
<td>73</td>
<td>Truncation at codon 415</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Early-onset breast cancer (&lt;40 years)</td>
<td>Ashkenazi Jewish</td>
<td>40</td>
<td>No truncating mutations</td>
<td></td>
</tr>
<tr>
<td>Yeast-based protein truncation assay</td>
<td>Early-onset breast cancer (&lt;30 years)</td>
<td>Breast cancer cell lines</td>
<td>15</td>
<td>No truncating mutations</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Early-onset breast cancer (&lt;40 years)</td>
<td>Ashkenazi Jewish</td>
<td>15</td>
<td>No truncating mutations</td>
<td></td>
</tr>
<tr>
<td>RAD54</td>
<td>Direct sequencing of RT-PCR product</td>
<td>Early-onset breast cancer (&lt;30 years)</td>
<td>27</td>
<td>C→G at position 2310 (1 case)†</td>
<td>Cys→Ser at codon 657</td>
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<tr>
<td>Yeast-based protein truncation assay</td>
<td>Early-onset breast cancer (&lt;40 years)</td>
<td>Breast cancer cell lines</td>
<td>73</td>
<td>No truncating mutations</td>
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</tr>
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</table>

† Analysis of this sequence revealed this specific mutation in 1 of 100 early-onset breast cancer cases and 0 of 100 controls.
Although they were present in a minority of the products amplified from either cases or controls, nested PCR amplification led to increased abundance of these aberrantly processed transcripts, leading to their potential misinterpretation as resulting from genuine mutations. In addition to preventing amplification of these aberrant PCR products, optimization of PCR conditions and the judicious placement of PCR primers was also required to avoid amplification of a conserved known \textit{RAD52} pseudogene (see “Materials and Methods”).

Analysis of \textit{RAD54}. Analysis of the \textit{RAD54} transcript using the yeast-based protein truncation assay did not reveal any premature stop codons in 100 cases of early-onset breast cancer. Direct nt sequencing of the 27 cases diagnosed before the age of 30 years identified a single missense mutation, Cys657Ser (Table 1). However, screening 70 additional cases of early-onset breast cancer for this specific mutation did not identify another case, and the mutation was not present in 100 controls. We, therefore, cannot distinguish a disease-associated missense mutation from a rare polymorphism. No truncating mutations in \textit{RAD54} were detected in 15 breast cancer cases.

Concluding Remarks. In summary, analysis of \textit{RAD51}, \textit{RAD52}, and \textit{RAD54} in both breast cancer cells and in the germ-line of women at high risk for genetic predisposition to breast cancer did not reveal disease-associated mutations. Our germ-line analysis was aimed at a population-based cohort of 100 early-onset cases, but it included 9 women who also have a strong family history of breast cancer and do not have a germ-line mutation in either \textit{BRCA1} or \textit{BRCA2} and, hence, are likely to harbor a mutation in another breast cancer predisposition gene. Despite their implication in a functional pathway shared by \textit{BRCA1} and \textit{BRCA2}, these \textit{RAD} genes, involved in homologous recombination and the repair of double-strand breaks in DNA, therefore, do not appear to be targeted by mutations in the development of breast cancer. A striking observation from these mutational studies is that; 8% of the United States population carries either one of two alleles encoding premature stop codons in \textit{RAD52}. Truncating mutations have typically been taken as evidence of definitive gene inactivation, and to our knowledge, a similar mutation in a gene associated with human disease has only been reported in \textit{BRCA2}, where an extreme COOH-terminal truncating mutation, Lys3326ter, is present in 2.2% of the British population (33). Like the \textit{RAD52} mutations described here, the prevalence of this nonsense mutation at codon 3326 of \textit{BRCA2} is not increased among women with early-onset breast cancer, suggesting that it may be a functionally silent polymorphism. In our own early-onset breast cancer cohort, we also detected a rare truncating mutation at codon 3308 of \textit{BRCA2}, upstream of Lys3326ter (26). In the absence of functional assays for \textit{BRCA2} or \textit{RAD52}, it is unclear at what point such progressive COOH-terminal truncations begin to affect protein function and whether they may be associated

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Cases</th>
<th>Controls</th>
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<tbody>
<tr>
<td>Ser346ter</td>
<td>3% (3/99)</td>
<td>5% (5/102)$^a$</td>
</tr>
<tr>
<td>Tyr415ter</td>
<td>2% (2/99)</td>
<td>3% (3/102)$^a$</td>
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$^a$ $P$ was not significant.
with attenuated phenotypes. As such, the study of common genetic variations within the population, ranging from subtle missense mutations to premature stop codons, is of considerable importance because even a modest increase in disease risk associated with a frequent genetic alteration may have significant consequences. This has been demonstrated most convincingly for the 11307K mutation in the APC gene, which is common in the Ashkenazi Jewish population and is associated with a moderately increased risk of colon cancer (34). Further work will be required to determine whether the truncated RAD52 products described here display any deficiency in mediating homologous recombination and whether carriers in the population are at increased risk for clinical syndromes resulting from inadequate repair of DNA damage.

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References

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