Mutation Analysis of the THRA1 Gene in Breast Cancer: Deletion/Fusion of the Gene to a Novel Sequence on 17q in the BT474 Cell Line

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ABSTRACT

We have previously described a common region of deletion and allele loss on chromosome 17q in sporadic breast cancers that is likely to contain a tumor suppressor gene. The region, mapped to 17q12–q21, was bordered by D17S259 and D17S579 on the centromeric and telomeric sides, respectively. This deletion region overlaps the BRCA1 locus, which predisposes to familial breast and ovarian cancer. The most frequent loss of heterozygosity was observed at the thyroid hormone receptor a (THRA1) locus. Southern analysis revealed a rearrangement of THRA1 in the BT474 breast cancer cell line. This rearrangement represented a deletion of exons 8–10 of one THRA1 allele that was also coamplified with ERBB2. Northern blots showed two mutant transcripts in BT474 cells. Analysis of the mutant transcripts revealed fusion of the THRA1 exon 7 by splicing to a novel sequence designated BTR for “BT474 transcribed rearrangement.” BTR was found to be highly conserved and mapped to 17q. The deletion in BT474 cells spans the entire BRCA1 region. To search for additional mutations of the THRA1 gene, all nine protein-encoding exons of THRA1 were examined for point mutations via single strand conformation analysis in a series of primary breast tumors, breast cancer cell lines, and lymphoblastoid cell lines derived from the youngest affected members of several German breast cancer families. No point mutations were detected, including the unarranged THRA1 allele in BT474. We have thus excluded THRA1 as a commonly mutated sporadic breast cancer tumor suppressor gene and as the BRCA1 gene.

INTRODUCTION

Breast cancer is one of the most common cancers in the United States (1). The molecular basis for the disease is the subject of intense investigation. Both cytogenetic and molecular genetic analyses point to involvement of multiple genes in the development of breast cancer (2). For example, previous studies have documented consistent overexpression and amplification of several genes in breast cancer, including the ERBB2 gene (3), while other work has focused on the involvement of tumor suppressor genes (4). To date, alterations of the RB gene and, more frequently, the TP53 gene have been described in breast cancers (5–8). With respect to the involvement of multiple genetic determinants in the genesis of breast cancer, a complex multistep neoplastic pathway similar to that described for colon cancer (9) is being elucidated. One key component of such a progression model is the BRCA1 gene on chromosome 17q (10). Mutant alleles of this gene predispose carriers to both breast and ovarian cancers (10, 11).

We recently reported a study of allele loss in primary breast carcinomas using microsatellite length polymorphisms (4). A common region of deletion was found in the 17q12–q21 region that was flanked by D17S259 and D17S579. Likewise, this portion of chromosome 17q is reduced to homozygosity in 53% of ovarian tumors (12). This region overlaps that of the BRCA1 locus, which led us to suggest that the gene predisposing to familial breast and ovarian cancer is allelic with a sporadic breast cancer tumor suppressor gene. Indeed, it has recently been shown that the BRCA1 gene acts as a genetically recessive locus, following the retinoblastoma tumor suppressor gene paradigm (13). The most frequent loss of heterozygosity in sporadic breast cancers was found at the thyroid hormone receptor alpha (THRA1) locus, a locus encoding the normal cellular homologue of the viral erb-a oncogene (14). Given its biological characteristics (reviewed in Ref. 12), THRA1 is a potential candidate gene for both the sporadic breast cancer 17q tumor suppressor gene and the BRCA1 gene. Here we describe the characterization of a large 17q deletion in the breast cancer cell line BT474 that truncates one allele of THRA1 and disrupts a novel coding sequence on 17q. In addition, we have performed a detailed mutation analysis of THRA1 in sporadic breast cancers and representative lymphoblastoid cell lines from several German breast cancer pedigrees (15).

MATERIALS AND METHODS

Cell Lines, Primary Tumors, and Lymphoblastoid Cell Lines. Breast cancer cell lines were obtained from American Type Culture Collection. Lines used in this study were BT474, BT20, HS578, T47D, MDA-MB-468, SKBR3, ZR751, MCF7, and HBL100. Normal diploid MRC5 cells were obtained from the Coriell/National Institute of General Medical Sciences cell repository. Normal human mammary epithelial cells (HMEC) were obtained as described (5). Primary tumor DNAs used have been described in a previous report (4). German breast cancer pedigrees and lymphoblastoid cell lines have been described elsewhere (15).

Southern/Northern Analysis and Probes. DNAs were isolated using standard protocols and poly A+ RNA was isolated using the Fast Track Kit (Invitrogen). DNAs and RNAs were run on 0.8% agarose or 1.2% formaldehyde gels, respectively, blotted onto GeneScreen Plus membranes in 10X SSC, and hybridized to random primed labeled probes under conditions recommended by the manufacturer. All probes were generated by PCR of either the THRA1 cDNA plasmid (peorthal:7 ATTCC) or from cDNA templates derived from mRNA of MRC5 normal human diploid fibroblasts using primers given in Table 1. Zoo blot and multiple tissue Northern filters were obtained from Clontech.

Polymerase Chain Reaction, SSCA, and RACE. PCR amplifications were performed in 20-μl volumes with 1.0 μM primers, 1.5 mm MgCl2, 1X PCR buffer (Promega), 0.5 units Taq Polymerase (Promega), 200 μM dNTPs, and 50 ng of genomic DNA. Samples were amplified for 25 cycles of 94°C for 1 min, 58°C for 2 min, and 72°C for 1 min. For SSAC, 5-μl aliquots of the above reactions were amplified for an additional 10 cycles in a duplicate 20-μl reaction, except that the dATP concentration was reduced to 1.25 μM, and the reaction was supplemented with 1.0 μCi of [α-32P]dATP (3000 Ci/mmol). Radiolabeled products were diluted 1:20 in stop solution (95% formamide-25 mM EDTA-0.05% bromphenol blue-0.05% xylene cyanol), heated to 95°C for 5 minutes, cooled on ice, and loaded onto appropriate gels. SSCAs were run under two conditions: (a) 5% nondenaturing acrylamide containing 10% glycerol, overnight at room temperature at 8 watts constant power; and (b) 5% nondenaturing acrylamide without glycerol, 4°C for 3–4 h at 40 watts constant power. Gels were dried and exposed overnight at room temperature. Using intron-based primers (Table 1) that would also detect splice site mutations, SSCA was performed for all nine protein-encoding exons of THRA1 on a series of genomic DNAs from 8 breast cancer cell line DNAs, 20 primary tumors

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2The abbreviations used are: poly A+, polyadenylated; PCR, polymerase chain reaction; cDNA, complementary DNA; SSCA, single strand conformation analysis; RACE, rapid amplification of cDNA ends.
Results

Southern Analysis. Southern analysis of BamHI-digested DNAs from breast cancer cell lines with a probe corresponding to exons 6–10 of THRA1 detected a novel fragment of 4.5 kilobases in the BT474 cell line (Fig. 1A, Lane 10). Comparison of signal intensities showed that the rearranged THRA1 allele was amplified. BT474 is approximately 10-fold amplified for ERBB2 (17), and THRA1 is frequently amplified with ERBB2 (4), indicating that BT474 is coamplified at these loci. Further probing of the same filter with PCR-generated probes for exons 8/9 and exon 10 of THRA1 failed to detect the mutant 4.5-kilobase fragment, demonstrating that the rearrangement was the result of a terminal deletion of exons 8–10 of the amplified THRA1 allele (Fig. 1B; data not shown). The rearrangement was confirmed by EcoRI and XhoI digests (data not shown). No other gross alterations were detected by Southern analysis of 8 other cell lines and 17 primary breast tumors.

Northern and RACE Analysis. Northern analysis of poly A+ RNAs from BT474 probed with the THRA1 exon 3–8 probe showed two overexpressed mutant transcripts of 2.5 and 4 kilobases as well as the normal 3.2- and 6-kilobase transcripts of THRA1 (Fig. 2, Lane 4).

Normal THRA1 expression was seen in eight other cell lines. RACE analysis (16) of cDNA templates generated from BT474 was used to determine the origin of the mutant, presumably truncated transcripts. Sequence analysis of RACE products revealed that the mutant transcripts were the result of a fusion of THRA1 from the 3' end of exon 7 to a novel sequence, designated BTR for BT474 transcribed rearrangement (Fig. 3). The presence of both mutant transcripts was apparently due to variant splicing of BTR because the breakpoint in THRA1 occurs prior to the natural alternative splice site exon 9 (18). Sequencing of multiple race products with THRA1 exon 7 primers did reveal two transcript groups (data not shown) consistent with this hypothesis. Attempts to amplify BT474 genomic DNA using exon 7 primers failed to detect an amplicon.
and BTR-specific primers failed to give any genomic fragments, suggesting that the perfect fusion between the natural 5' splice site of intron 7 and the BTR sequence was the result of splicing rather than a deletion breakpoint precisely at the end of exon 7.

**BTR Characterization.** The mutant 4.5-kilobase BamH1 fragment in BT474 cells as well as a 10-kilobase fragment on Southern blots (Fig. 1C, Lane 10) and the mutant 2.5- and 4-kilobase transcripts of BT474 cells on Northern blots (Fig. 2, Lane 8) were all detected using a specific BTR probe. Additionally, the BTR probe detected conserved sequences in mouse genomic DNA (Fig. 1C, Lanes 1-3) and all other mammalian DNAs tested on a zoo blot (Fig. 4), indicating that BTR is a highly conserved gene. BTR was localized to chromosome 17q by PCR and Southern analysis of somatic cell hybrids (data not shown). Sequence comparison using the FASTA programs of the GCG package (Genetics Computer Group, 1991) revealed no significant homology to any previously deposited sequences in Genbank. We have recently isolated a 2.1-kilobase lung cDNA clone for BTR that is currently being characterized.

**THRAl Mutation Screening.** The entire coding sequence of THRAl was screened for point mutations using single strand conformation analysis. A panel of 8 breast cancer cell lines, 20 primary tumors, and 17 lymphoblastoid cell lines derived from the youngest affected members of several German breast cancer families were examined, giving the potential of finding both somatic and germline mutations. Additionally, first strand cDNA was reverse transcribed from RNAs isolated from 6 of the breast cancer cell lines and 10 additional primary tumors. In total, 405 potentially mutant exons were screened from genomic DNA, and 144 exons were screened from cDNA templates under two experimental conditions. No putative point mutations were detected in the samples studied, including the presumed wild type allele of THRAl in BT474. As a control, each of three p53 point mutations was easily detected under the same conditions, including an exon 8 mutation in a primary tumor (see Fig. 5 for representative results.)

**DISCUSSION**

We recently described a common 17q deletion region in sporadic breast carcinoma that spanned a 6 Cm interval between the markers D17S250 and D17S579 (4). This region contains the BRCA1 locus that predisposes to breast and ovarian cancer in families segregating a mutant allele, which led us to suggest that the 17q sporadic breast cancer tumor suppressor gene is equivalent to BRCA1. A recent study by Smith et al. (13) demonstrated that in tumors from affected family members, the wild type chromosome is always subject to allelic loss, strongly suggesting that BRCA1 acts as a tumor suppressor gene. A (CA)n length polymorphism in the THRAl gene (19) showed the highest frequency of loss at 79% of informative sporadic cases (4).
Given the rate of loss at this locus and its attractiveness as a candidate tumor suppressor gene (14), we studied the gene for structural alterations. In examining a series of both breast cancer cell lines and primary tumor DNAs by Southern analysis using a THRA1 cDNA probe, a rearrangement of an amplified THRA1 allele in the BT474 cell line was observed. Detailed examination of the rearrangement with exon-specific probes demonstrated that the rearrangement was the result of a terminal deletion of exons 8–10 of one allele of the THRA1 gene. The truncation of THRA1 in BT474 cells resembled the v-erbA oncogene in that the ligand binding domain of the cellular gene had been deleted, similar to truncation of this region in v-erbA (14). This mutation is, therefore, considered to be of potential biological relevance.

Northern blotting of poly A+ RNAs from BT474 with the THRA1 3–8 probe detected both normal and altered transcripts. The mutant transcripts were overexpressed relative to the normal transcripts, suggesting that they arose from the amplified allele. The finding of overexpressed mutant THRA1 transcripts that lacked a ligand binding domain strengthens the plausibility of its being a v-erbA-like mutation. RACE experiments were carried out to characterize the mutant transcripts. BT474-specific RACE products were isolated and sequenced directly. Sequence analysis showed that the THRA1 transcript was truncated perfectly at the end of exon 7 and fused to a new sequence unrelated to THRA1. This new sequence was designated BT474 transcribed rearrangement. Only ten in-frame amino acids were added by BTR, thus effectively truncating THRA1. PCR experiments using primers for THRA1 exon 7 and BTR failed to amplify any fragments from BT474 DNA. Thus, the mutant transcripts result from splicing across a genomic region of less than 4.5 kilobases because THRA1 and BTR probes detect both the novel BamHI fragment in BT474 DNA.

When the BTR-specific segment was used to probe Northern blots, the mutant transcripts were recognized specifically. No presumably wild type transcripts were detected in RNA from breast cancer cell lines or normal mammary epithelial cells. A weak 2.5-kilobase transcript was seen in lung poly A+ RNA on a multiple tissue Northern blot after a 1-week exposure (data not shown). It was observed that the BTR probe hybridized specifically to mouse DNA (Fig. 1C, Lanes 1–3), indicating significant sequence conservation. Sequence conservation was further confirmed on a zoo blot where conservation across mammalian species was observed, strongly suggesting that BTR is a gene. PCR of somatic cell hybrid DNAs and Southern blotting were used to assign BTR to 17q (data not shown), indicating that the BT474 rearrangement was an interstitial deletion. Recently, BTR has been used to assign BTR to 17q (data not shown), indicating that the BT474 rearrangement is the result of a terminal deletion of an amplified THRA1 allele in the BT474 cell line. The finding of a mutated THRA1 gene in BT474 cells and this gene was frequently reduced to homozygosity in primary breast and ovarian tumors, we performed a detailed mutation screen using SSCA to search for point mutations in the gene. The sequence of the THRA1 gene had been recently reported including intron/exon borders (18); therefore, it was possible to design intron-based primers to amplify each exon. Additionally, large portions of the THRA1 transcript were quickly screened using the cDNA primers in Table 1 and by digesting the PCR products with the restriction enzymes indicated. This system of “restricted” SSCA gave very reproducible results and should prove useful for mutation screening of other genes where only the cDNA sequence is known. In total, 549 exons were screened under two experimental conditions. No point mutations were found in the coding region, including the unarranged allele in BT474 cells. Thus, if THRA1 represents an inactivated tumor suppressor in BT474 cells, it must act by a dominant negative rather than a two-hit mechanism. These results indicate that THRA1 mutations in breast cancer must be very rare and that THRA1 is neither a sporadic breast cancer tumor suppressor gene nor the BRCA1 gene. Our results with THRA1 are in agreement with the recent Breast Cancer Consortium report on genetic linkage analysis of 214 families with familial breast and ovarian cancer (21). The consortium report details several recombination events in families that rule out THRA1 as a candidate gene, consistent with the observed absence of mutations in our study set. Likewise, the partner in the BT474 rearrangement, BTR, falls below the distal recombination point; ruling it out as a candidate also. Given the hemizygous deletion of the relevant 17q region, BT474 cells may represent an excellent target cell line for gene transfection studies to aid in identification of the BRCA1 gene and elucidation of its function.

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