TBX2 Is Preferentially Amplified in BRCA1- and BRCA2-related Breast Tumors


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Abstract

The chromosome 17q23 region is frequently amplified in breast tumors. Gain of the region is present in 50% of BRCA1-associated breast tumors and 87% of BRCA2-associated breast tumors. The amplification frequency of the RPS6KB1 and TBX2 oncogenes from this amplicon was compared in 27 BRCA1 and BRCA2 mutant breast tumors, 15 breast tumors from high-risk patients with no BRCA1 or BRCA2 mutations, and 62 matched sporadic breast tumor controls. TBX2 was determined to be preferentially amplified and overexpressed in BRCA1 and BRCA2 mutant tumors, whereas RPS6KB1 was not, suggesting a role for TBX2 amplification in the development of BRCA1- and BRCA2-associated breast tumors.

Introduction

Breast tumors in BRCA1 and BRCA2 carriers are pathologically and genetically distinct from sporadic tumors and appear to follow unique developmental pathways. In contrast to sporadic tumors, BRCA1 mutant breast tumors are of higher grade, predominantly estrogen receptor and progesterone receptor negative, and contain mutant p53 (1, 2). BRCA2-associated tumors tend to be of lower grade, have estrogen receptor and progesterone receptor levels comparable to those of sporadic tumors, and have fewer p53 mutations (1, 2). In addition, BRCA1- and BRCA2-associated breast tumors analyzed by CGH display twice as many chromosomal gains and losses as sporadic tumors (3). Some specific genes displaying altered levels of amplification and expression in BRCA1- and BRCA2-associated breast tumors relative to sporadic tumors have been identified. The HER2/new gene is amplified and overexpressed in 20% of sporadic tumors and BRCA2 tumors but is detected at significantly lower levels in BRCA1 tumors (1, 4). In contrast, the Myb oncogene is amplified in 29% of BRCA1 breast tumors but is not amplified in BRCA2 tumors and is amplified in only 2% of sporadic tumors (5). Furthermore, cyclin D1 is amplified in 30% of sporadic breast tumors but is not amplified in tumors of BRCA1 or BRCA2 mutation carriers diagnosed before age 50 years (4, 6). Other chromosomal regions frequently amplified in BRCA1 and BRCA2 tumors have also been identified. For instance, the 17q22-q24 region is gained or amplified in 50% of BRCA1-associated breast tumors and 87% of BRCA2-associated breast tumors but in only 15% of sporadic tumors (3). The selection for gain of this region suggests that a gene or genes localized to this region are important in the progression of hereditary breast tumors.

We recently characterized the structure of a 4-Mb amplicon on chromosome 17q23 and have identified several independent peaks of amplification in breast cancer cell lines and in primary breast tumors (7). The RPS6KB1 and TBX2 genes are located in the amplicon, are frequently amplified in human breast cancers, and are thought to contribute to tumor development (8–12). RPS6KB1 is a ribosomal protein kinase that regulates protein synthesis and transition from G1 to S phase of the cell cycle in response to mitogenic stimuli (13). Amplification of the RPS6KB1 gene correlates with overexpression and poor survival in breast cancer patients (9). The TBX2 gene is a T-box transcription factor that regulates gene expression during embryological development and facilitates senescence bypass in Bmi−/− mouse embryo fibroblasts when moderately overexpressed (11). To determine whether these genes are preferentially amplified in hereditary breast tumors, we determined the copy number of RPS6KB1 and TBX2 in breast tumors from 27 carriers of BRCA1 and BRCA2 deleterious mutations, 15 patients who had a positive family history but were negative for deleterious mutations in BRCA1 and BRCA2 (NMD/UCV), and 62 sporadic controls using FISH.

Materials and Methods

Breast Tumors. Twenty-seven BRCA1 and BRCA2 mutation carriers and 15 patients with a significant family history but with no deleterious BRCA1 or BRCA2 mutations were identified through the Familial Cancer Program high risk breast cancer clinic and a study of patients undergoing prophylactic mastectomy at the Mayo Clinic (14). Because the 15 patients had NMD or had unique intronic variants or missense variants in BRCA1 or BRCA2 that are categorized as UCVs, this cohort will be referred to as NMD/UCV. Specimens from the NMD/UCV cohort were age-, stage-, and surgery date-matched by frequency with the BRCA1 and BRCA2 mutants. All mutations and sequence variants were identified either by conformation-sensitive gel electrophoresis followed by direct sequencing or by direct sequencing at Myriad Genetics Inc. (Salt Lake City, UT). Paraffin-embedded breast tumors from these patients were obtained from the Tissue Registry at the Mayo Clinic or from the hospital where the surgery was performed. Specimens from sporadic breast cancer controls were frequency-matched by age, stage, and surgery date with the BRCA1 and BRCA2 mutants and with the NMD/UCV cases. Note that perfect matching was not possible due to the limited availability of early-onset controls. A summary of all samples is provided in Table 1. Deleterious mutations and UCVs of BRCA1 and BRCA2 are listed in Table 2.

BAC Clones. Two BAC clones mapping to the 17q23 region, hRPK.332_H_18 and hRPC.1073_F_15, containing the TBX2 gene and RPS6KB1 gene, respectively, were obtained from Research Genetics, Inc. (Huntsville, AL) and BACPAC Resources Inc. (Oakland, CA). DNA was isolated from overnight cultures using a modified Qiagen Midi Kit (Qiagen, Valencia, CA) protocol. Probes were directly labeled with Texas Red dUTP and Texas Red dCTP (Amersham Pharmacia Biotech, Piscataway, NJ) by nick translation.
FISH Analysis. Five-μm sections were cut from paraffin-embedded specimens, and tumor cells were identified after H&E staining. The TBX2 and RPS6KB1 BAC clones were dual-hybridized with the CEP17 (Vysis, Downer’s Grove, IL) centromere probe to sections from each specimen as described previously (15). Sections were counterstained with 0.2 mM 4’,6-diamidino-2-phenylindole in a Vectashield antifade solution (Vector Laboratories, Burlingame, CA), and tumor cells were identified after H&E staining. The number of centromere signals. Low-level amplification is defined as a ratio between 1.5 and <2.0. Amplification is defined as a ratio ≥2.0 and includes moderate (ratio ≥2.0 and <3.0) and high level (ratio ≥3.0) amplification. By choosing a minimum ratio of 1.5, our frequencies of amplification closely match those from a previous CGH study of sporadic tumors (3). Likewise, with a ratio of ≥2.0, the frequencies of amplification match our previously reported Southern blot data from sporadic tumors (7).

Statistical Analysis. Statistical differences in the frequency of amplification between the BRCA1 and BRCA2 mutant tumors, the NMD/UCVs, and the controls were tested for statistical significance by Fisher’s exact test.

RNA in Situ Hybridization. A 427-bp fragment of the TBX2 coding sequence was amplified by PCR with primers F-GCG and R-GTGCAGGAAGCGGCTG and cloned into the pCRII-TOPO vector (Invitrogen). Sense and antisense digoxigenin-labeled RNA probes were generated from the linearized plasmid using the DIG RNA Labeling Kit (Roche Molecular Biochemicals). In situ hybridization of 5-μm sections from paraffin blocks of breast tumors was performed according to the manufacturer.

Results

TBX2 Amplification in Breast Tumors by FISH. To determine whether the TBX2 gene is preferentially amplified in BRCA1- and BRCA2-associated tumors, we performed FISH on tumors from BRCA1 and BRCA2 mutation carriers, NMD/UCVs, and sporadic controls with the TBX2 BAC clone hRPK.332_H.18. Amplification (hRPK.332_H.18:CEP17 ratio ≥1.5) was detected in 19 of 27 BRCA1 and BRCA2 tumors but in only 8 of 37 matched sporadic controls (P = 0.0001), as shown in Table 3. Similarly, amplification (ratio ≥2.0) was found in 8 of 27 BRCA1 and BRCA2 tumors but in only 3 of 37 sporadic controls (P = 0.04). In contrast, TBX2 amplification (ratio ≥1.5) was detected in only 3 of 15 NMD/UCVs and sporadic controls (P = 1.0). Thus, TBX2 is selected preferentially for amplification in tumors from BRCA1 and BRCA2 mutation carriers when compared with tumors from patients with a family history but no BRCA1 or BRCA2 mutations or with sporadic tumors. Examples of amplification of TBX2 in tumors are shown in Fig. 1, A and B.

Next we evaluated whether TBX2 was preferentially amplified in early-stage tumors. We compared the frequency of amplification (ratio ≥2.0) in stage I BRCA1 and BRCA2 tumors with that of stage II BRCA1 and BRCA2 tumors and found no significant difference. We also examined TBX2 amplification in DCIS specimens obtained from 20 individuals with IDC, regardless of mutation status. No difference in the frequency of TBX2 amplification in DCIS and IDC lesions was detected. This was also true for BRCA1 and BRCA2 mutant tumors, because seven of seven DCIS lesions from BRCA1 and BRCA2 mutation carriers who also had IDCs showed levels of TBX2 amplification similar to those detected in the IDC lesions. These data suggest that TBX2 is amplified equivalently in in situ and invasive tumors and that amplification of TBX2 may be an early genetic event in the development of BRCA1 and BRCA2 tumors.

A hallmark of BRCA1 and BRCA2 tumors is early age of onset. To determine whether TBX2 is preferentially amplified in patients with earlier age of onset, we analyzed the subsets of <42 years and ≥42 years to reflect the average age of onset for BRCA1 and BRCA2 tumors. TBX2 was amplified (ratio ≥2.0) in 2 of 12 BRCA1 and BRCA2 mutation carriers <42 years and in 6 of 15 BRCA1 and BRCA2 mutation carriers ≥42 years (P = 0.2). This suggests that TBX2 is not preferentially amplified in tumors with an earlier age of onset.
**Table 3** Amplification frequency of TBX2 and RPS6KB1 in BRCA1 and BRCA2 mutation carriers versus matched sporadic controls and NMD/UCVs versus matched sporadic controls

<table>
<thead>
<tr>
<th>Probes</th>
<th>BRCA1 and BRCA2</th>
<th>Controls</th>
<th>P</th>
<th>NMD/UCV</th>
<th>Controls</th>
<th>P</th>
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<tr>
<td>hRPC.1073_F_15</td>
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<tr>
<td>Ratio = 1.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11/27 (40%)</td>
<td>15/37 (41%)</td>
<td>1.00</td>
<td>1/15 (7%)</td>
<td>5/25 (20%)</td>
<td>0.38</td>
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<tr>
<td>Ratio = 2.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3/27 (11%)</td>
<td>4/37 (11%)</td>
<td>1.00</td>
<td>0/15 (0%)</td>
<td>3/25 (12%)</td>
<td>0.28</td>
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<tr>
<td>hRPK.332_H_18</td>
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<tr>
<td>Ratio = 1.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19/27 (70%)</td>
<td>8/37 (22%)</td>
<td>0.0001</td>
<td>3/15 (20%)</td>
<td>5/25 (20%)</td>
<td>1.00</td>
</tr>
<tr>
<td>Ratio = 2.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8/27 (30%)</td>
<td>3/37 (8%)</td>
<td>0.04</td>
<td>1/15 (7%)</td>
<td>3/25 (12%)</td>
<td>1.00</td>
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<sup>a</sup> A ratio of 1.5–2.0 corresponds to low-level amplification.

<sup>b</sup> A ratio of ≥2.0 corresponds to amplification.

**TBX2 and RPS6KB1 Amplification in BRCA1 Tumors Compared with BRCA2 Tumors.** Because TBX2 is apparently preferentially amplified in the tumors with deleterious mutations in BRCA1 and BRCA2, we evaluated whether TBX2 amplification was more frequent in BRCA1- or in BRCA2-associated tumors. TBX2 amplification (ratio ≥1.5) was seen in 8 of 13 BRCA1 tumors and 11 of 13 BRCA2 tumors (P = 0.38). Similarly, amplification (ratio ≥2.0) was seen in 6 of 13 BRCA1 tumors compared with 2 of 13 BRCA2 tumors (P = 0.20), suggesting that BRCA1 and BRCA2 tumors are equally likely to display amplification of this region, although the small sample size limits the ability to detect significant differences. Likewise, no difference in amplification frequency of RPS6KB1 in BRCA1 and BRCA2 tumors was detected.

**TBX2 and RPS6KB1 Coamplification in BRCA1 and BRCA2 Tumors and Controls.** To determine whether selection for specific regions of the amplicon occurs in sporadic BRCA1 and BRCA2 tumors with amplification, we differentiated between tumors with independent amplification and coamplification of the probes. Of the tumors with amplification (ratio ≥1.5), TBX2 was amplified independently of RPS6KB1 in 9 of the 20 BRCA1 and BRCA2 tumors and 1 of 16 sporadic controls (P = 0.02). In contrast, RPS6KB1 was amplified independently of TBX2 in only 1 of 20 BRCA1 and BRCA2 tumors with amplification, whereas 8 of 16 sporadic controls had independent amplification of RPS6KB1 (P = 0.005). Coamplification of both probes was seen in 10 of 20 BRCA1 and BRCA2 tumors and 7 of 16 matched sporadic controls (P = 0.75). These data suggest that TBX2 amplification is selected for in BRCA1 and BRCA2 tumors, whereas RPS6KB1 amplification is selected for in sporadic tumors.

**TBX2 Overexpression in Tumors with TBX2 Amplification.** To verify that TBX2 was overexpressed in tumors with TBX2 amplification, we performed RNA in situ hybridization of sections from paraffin blocks that were previously used for FISH analysis. The 427-bp TBX2 riboprobe was chosen from a region of minimal homology between T-box gene family members to ensure that RNA in situ signals represented TBX2 expression alone. BRCA1 and BRCA2 mutant tumors in which TBX2 was amplified showed high levels of TBX2 expression, as shown in Fig. 2, A and B. Specifically, epithelial tumor cells displayed significant expression, as did infiltrating T cells, whereas stromal and fat tissue showed little or no expression. Similarly, TBX2 was highly expressed in epithelial tumor cells from sporadic breast tumors in which TBX2 was amplified (Fig. 2C). However, the stroma and the normal ductal epithelial cells in this tumor section showed little expression of TBX2 (Fig. 2C). In contrast, BRCA1 and BRCA2 mutant tumors and sporadic tumors that did not have amplification of the TBX2 gene showed no significant expression of TBX2 (data not shown).

**Discussion**

We analyzed TBX2 and RPS6KB1 amplification in breast tumors from 27 BRCA1 and BRCA2 mutation carriers, 15 NMD/UCV cases,
and 62 sporadic controls to determine whether one or both of the genes is preferentially amplified in hereditary breast tumors. TBX2 was more frequently amplified (ratio > 1.5) in BRCA1- and BRCA2-associated tumors (70%) than in the matched sporadic controls (22%). Furthermore, TBX2 was amplified independently of RPS6KB1 in 45% of BRCA1 and BRCA2 tumors with amplification, but in only 6% of the sporadic controls with amplification. The frequent and specific selection of the TBX2 gene for amplification in hereditary breast tumors and the finding that tumors with amplification of TBX2 over-express TBX2 (whereas nonamplified tumors do not) strongly suggest that the TBX2 gene contributes to the initiation and/or progression of BRCA1 and BRCA2 mutant tumors. Likewise, frequent amplification of RPS6KB1 was detected in breast tumors, although the frequency of amplification (ratio > 1.5) was not significantly different between the tumors from BRCA1 and BRCA2 carriers (40%) and the controls (32%). However, RPS6KB1 was amplified independently of TBX2 in 5% of the BRCA1 and BRCA2 tumors with amplification and in 50% of sporadic controls with amplification. These results suggest that either (a) TBX2 and RPS6KB1 are both selected for amplification or (b) RPS6KB1 is amplified as a bystander to TBX2 in breast tumors from BRCA1 and BRCA2 mutation carriers. In contrast, RPS6KB1 appears to be specifically selected for amplification in sporadic tumors. Previous studies have shown that amplification of RPS6KB1 correlates well with overexpression of RPS6KB1 (8, 9, 10, 12) and that both amplification and overexpression of RPS6KB1 correlate with poor survival in breast cancer patients (9), suggesting that amplification of RPS6KB1 contributes to the development and/or progression of sporadic breast tumors.

Previous CGH analysis of breast tumors from BRCA1 and BRCA2 mutation carriers detected gain of the 17q22-q24 region in 50% of BRCA1 tumors, 87% of BRCA2 tumors, and 15% of sporadic controls (3). Here we report that the TBX2 gene is amplified in 62% of BRCA1 tumors, 85% of BRCA2 tumors, and 22% of sporadic controls, whereas RPS6KB1 is amplified in only 11% of BRCA1 and BRCA2 tumors and sporadic controls. This suggests that TBX2 may be the target gene driving amplification of the 17q22-q23 region in BRCA1- and BRCA2-associated tumors, whereas RPS6KB1 is not. However, given the complexity of the amplicon and the presence of several candidate genes in other amplification peaks, it is possible that other genes in the region may also be targets of amplification and may contribute to the development of breast tumors in BRCA1 and BRCA2 mutation carriers, NMD/UCV cases, or sporadic cases.

TBX2 is a member of a family of phylogenetically conserved DNA-binding proteins known to regulate gene expression during development. Low to moderate overexpression of the TBX2 protein represses the Cdkn2a (p19ARF) promoter, leading to bypass of senescence and immortalization of Bmi1 primary mouse embryo fibroblasts (11). Amplification of TBX2 appears to be selected for at high frequency in tumors from BRCA1 and BRCA2 mutation carriers and may assist early-stage tumor cells in bypassing senescence. This hypothesis is supported by the observation that TBX2 is amplified as frequently in DCIS specimens as in invasive tumors. Additional studies of TBX2 amplification in premalignant breast lesions may be helpful in determining whether this is an initiating event in the development of these tumors. However, TBX2 amplification and overexpression are not required for tumor development in all BRCA1 and BRCA2 mutation carriers because 30% of the BRCA1 and BRCA2 tumors studied did not have amplification. Thus, amplification and associated overexpression of TBX2 may be an early contributing event in initiation and development of a select group of BRCA1- and BRCA2-associated tumors.

In an effort to determine whether the preferential amplification of TBX2 is associated with a family history of breast cancer or is specific for BRCA1 and BRCA2 mutation carriers, we analyzed amplification in a group of patients with significant family history but with no deleterious BRCA1 or BRCA2 mutations. These NMD/UCV samples contain unique intronic variants or missense variants categorized as UCVs of BRCA1 and BRCA2. Unlike truncating mutations, which remove downstream sequence encoding protein domains and the nuclear localization signal, these UCVs may disrupt a single domain,
leaving the rest of the protein intact, or, more likely, may have no
effect on the protein. If UCVs of BRCA1 and BRCA2 have no role in
tumor development, the presence of a strong family history of breast
cancer in these patients suggests that other predisposition genes may
be involved. In this study, we found no significant difference in the
frequency of amplification of either TBX2 or RPS6KB1 between the
NMD/UCV tumors and matched controls, but we did detect prefer-
ential amplification of TBX2 in BRCA1 and BRCA2 tumors compared
with the NMD/UCV tumors. These data suggest that amplification of
TBX2 is unique to the BRCA1- and BRCA2-related breast cancers and
that the NMD/UCV tumors follow a different developmental pathway
than the BRCA1 and BRCA2 tumors.

In conclusion, we have found that the TBX2 gene is more frequently
amplified in breast tumors from BRCA1 and BRCA2 mutation carriers
than in NMD/UCV and sporadic tumor controls. These findings
suggest that the TBX2 gene is specifically selected for amplification
and overexpression in BRCA1 and BRCA2 tumors and may therefore
be a useful predictive/prognostic marker in familial breast tumors. On
the other hand, RPS6KB1 amplification and overexpression are more
commonly found in sporadic tumors. This apparent selection in spo-
radic tumors, in combination with the recent finding that amplification
and overexpression of RPS6KB1 correlate with poor survival in breast
cancer patients (9), indicates that RPS6KB1 may be a useful and
specific predictive/prognostic marker for patients with sporadic tu-
mors. The reasons for these differences in amplification within the
17q23 amplicon are not clear but suggest the existence of very
specific tumor developmental pathways.

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