Increased Level of Exon 12 Alternatively Spliced BRCA2 Transcripts in Tumor Breast Tissue Compared with Normal Tissue

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Abstract

The breast cancer susceptibility gene BRCA2 is expressed in a wide range of tissues as an 11-kb mRNA transcript encoding a 3418-amino acid protein, which is involved in the response to DNA damage. To obtain a better molecular characterization of BRCA2 expression in breast tissue, we analyzed full-length BRCA2 mRNA by means of reverse transcriptase-PCR with a panel of primer pairs encompassing the entire cDNA sequence. We report the identification of an exon 12 alternatively spliced BRCA2 transcript (Δ12-BRCA2) in normal human breast tissue, in a wide variety of other normal human tissues, and in several mouse tissues. The deletion observed in this transcript (96 bp) preserves the open reading frame, and translation of the transcript would result in a deletion observed in this transcript (96 bp) preserves the open reading variety of other normal human tissues, and in several mouse tissues. The PCR with a panel of primer pairs encompassing the entire cDNA sequence (exons 1–12) and translation of the transcript was higher in tumor tissue than in normal breast tissue, especially in steroid receptor-negative tumors.

Materials and Methods

Patients and Samples. Thirty-eight primary breast carcinomas were obtained at Center René Huguenin (St-Cloud, France). The samples were examined histologically for the presence of tumor cells. A tumor sample was considered suitable for this study if the proportion of tumor cells was >60%. Adjacent normal breast tissue was also taken from 12 of the 38 patients. The patients included in this study met the following criteria: primary unilateral breast carcinoma for which complete clinical, histological, and biological information was available and no other primary cancers. None of the 38 patients had received radiation therapy or chemotherapy before surgery.

Seven normal breast tissue specimens obtained from women undergoing cosmetic breast surgery were used as sources of normal RNA. Total RNA from a pool of six normal human breast tissues was also purchased from Clontech (Palo Alto, CA).

RNA Extraction. Total RNA was extracted from samples by the acid-phenol guanidium method (13). The quality of the RNA samples was determined by electrophoresis through denaturing agarose gels and staining with ethidium bromide, and the 18S and 28S RNA bands were visualized under UV light. The yield was quantified spectrophotometrically.

cDNA Synthesis. Reverse transcription was performed in a final volume of 20 μl containing 1× RT3-PCR buffer [1 mM each dNTP, 5 mM MgCl2, 50 mM KCl, and 10 mM Tris-HCl (pH 8.3)], 20 units of RNase inhibitor, 50 units of Moloney murine leukemia virus RT (PE. Applied Biosystems, Foster City, CA), 2.5 μM random hexamers, and 1 μg of total RNA. The samples were incubated at 20°C for 10 min and 42°C for 30 min, and RT was inactivated by heating at 99°C for 5 min and cooling at 5°C for 5 min.

Primers and PCR Conditions. Single-stage PCR was carried out in a final volume of 50 μl containing 2 μl of the RT reaction mix, 400 nM each primer, 200 μM each dNTP, 1.5 mM MgCl2, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 1 unit of AmpliTaq DNA polymerase (PE. Applied Biosystems).

The positions of the primers on the BRCA2 gene are shown in Fig. 1, and their nucleotide sequences were as follows: (a) BR1C1 (113U, 5′-GGAGGAGACAGATTTGTGA-3′) and BR1CIL (1099L, 5′-GGACATTTTGCAATTGCCTTT-3′) for exons 1–10 (PCR product of 987 bp); (b) BR2C2 (6974U, 5′-CCACACATCTCCTCTTACA-3′) and BR2CIL (7939L, 5′-CCTTAACAAATAACCTCA-3′) for exons 11–16 (PCR product of 966 bp); (c) BR3C3 (7767U, 5′-AAAAACATCCACTGCTC-3′) and BR3CIL (8776L, 5′-CCTTTCCTCCTCCTCTTTACA-3′) for exons 15–20 (PCR product of 1010 bp); (d) BR4C4 (8663U, 5′-GGAGGAGAACATGTTGTGGTGTG-3′) and BR4CIL (9508L, 5′-ACAAGCTAGACAAAGGGGCA-3′) for exons 19–25 (PCR product of 846 bp); (e) BR5C5 (9453U, 5′-GGAGTTTGCTGTTTGTGTG-3′) and BR5CIL (10099L, 5′-CATCAATCTCCTTCTCCTTCCCT-3′) for exons 24–27 (PCR product of 557 bp); (f) BR6C6 (9832L, 5′-ACAGTGAACAAACGATCAAGAAGACG-3′) and BR6CIL (7252L, 5′-GAGGGCTTTAGTTGTTGGAC-3′) for exons 9–13 (PCR product of 6270 bp); (g) BR7C7 (7048U, 5′-GGAGGAGCCCTTATCTGTTG-3′) and BR7CIL (8654L, 5′-GAGGAGCCCTTATCTGTTG-3′) for exons 18–23 (PCR product of 1010 bp).

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3 The abbreviations used are: RT, reverse transcriptase; WT, wild-type.
BRC7L (7254L, 5′-TGACGTTCTTAGTTGGCGA-3′) for exons 11–13 (PCR product of 207 bp).

Primers were chosen with the aid of the Oligo 4.0 (National Biosciences, Plymouth Meeting, MN) computer program. Primers are designed by the nucleotide position (relative to BRCA2 GenBank accession no. U43746) corresponding to the 5′ position, followed by the letter U for upper (i.e., sense) strand or L for lower (i.e., antisense) strand. The PCR procedure comprised: initial denaturation at 94°C for 5 min; 32 cycles of 1 min at 94°C, 1 min at 55–65°C, and 1.5 min at 72°C; and a final extension step of 10 min at 72°C, using a Perkin-Elmer 9600 DNA thermocycler. Aliquots (2 µl) of the appropriately diluted PCR products were added to 2.5 µl of deionized formamide containing 0.3 µl of a molecular size marker (Genescan 2500 ROX; PE. Applied Biosystems). The mixtures were denatured by heating and 2.5-µl aliquots were loaded onto 6% polyacrylamide gels containing 8 M urea and run for 6 h at 1200 V on the Applied Biosystems model 373A DNA sequencing system (PE. Applied Biosystems). The resulting gel data were analyzed for fragment size and peak area by using the Genescan 672 Fragment Analysis software (PE. Applied Biosystems).

### Quantitative Δ12-BRCA2 Transcript Analysis

Coamplification of all BRCA2 transcripts (Δ12-BRCA2 and WT BRCA2 transcripts) with the same primer pair (BRCA2U/BRCACL2L) provided a quantitative competitive RT-PCR method, in which the internal control was the coamplified WT BRCA2 mRNA, and comparative expression of Δ12-BRCA2 and WT BRCA2 mRNA could be determined for each sample. According to Pannetier et al. (14), the ratios between these two BRCA2 transcripts were similar regardless of the number of cycles.

The sensitive Applied Biosystems model 373A DNA sequencing system (PE. Applied Biosystems) was used, and the different fragments were quantified with Genescan 672 Fragment Analysis software (PE. Applied Biosystems), which calculates peak size and area.

For a given sample, the final result was expressed as a proportion (PΔ12, in percentage) of Δ12-BRCA2 mRNA over all BRCA2 mRNAs, was determined as follows:

\[
P_{Δ12} = 100 \times \frac{Δ12-BRCA2 \text{ (peak area)}}{Δ12-BRCA2 + \text{WT BRCA2} \text{ (peak areas)}}
\]

The reproducibility of the quantitative measurements was evaluated by three independent replicate cDNA synthesis and PCR runs. The means of the replicated measurements and their 95% confidence intervals were calculated.

**Sequencing of PCR Products.** PCR products were resolved in a 1.5% low-melting point agarose gel (Life Technologies, Inc., Gaithersburg, MD), stained with ethidium bromide. DNA fragments were eluted from gels and purified using the QIAquick PCR purification kit (Qiagen, Santa Clara, CA). The purified fragments were sequenced using the ABI PRISM Dye Terminator Cycle Sequencing kit (PE. Applied Biosystems) on the Applied Biosystems model 373A DNA sequencing system (PE. Applied Biosystems).

**Statistical Analysis.** Biological parameters were compared using the χ² test. Differences between the two populations were judged significant at confidence levels of >95% (P < 0.05).

### Results

**Identification of a Major BRCA2 Variant in Normal Human Breast Tissue.** We analyzed BRCA2 mRNA expression in normal breast tissue samples by RT-PCR, using five primer pairs (BRCA1U/BRC1L–BRC5U/BRC5L) that encompass the entire cDNA sequence of full-length BRCA2, except the large exons 10 and 11 (Fig. 1). The samples comprised seven normal breast tissue specimens obtained from women undergoing cosmetic breast surgery and a pool of six normal human breast tissues. All primer pairs yielded a major RT-PCR fragment that was exactly the size predicted from the published BRCA2 cDNA sequence (GenBank accession no. U43746). However, besides the expected band, the BRCA2U/BRC2L primer pair yielded an additional band of smaller size (~10% of the WT BRCA2 transcript amount; Table 1). Other primer pairs (BRCA1U/BRC1L and BRCA3U/3L) yielded abnormal bands, but at very low levels (<3% of the WT BRCA2 transcript). Finally, primer pairs BRCA4U/BRC4L and BRCA5U/BRC5L did not yield additional abnormal bands.

The additional band of smaller size (870 bp) than the expected band at 966 bp obtained with the BRCA2U/BRC2L primer pair (exons 11–16) was observed in all of the normal breast samples examined. This smaller transcript had a 96-bp deletion, presumably resulting from exon 12 alternative splicing of the BRCA2 mRNA. PCR ampli-

### Table 1 Proportion of Δ12-BRCA2 transcripts in human breast tissues

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Normal tissue</th>
<th>Tumor tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>13.2 ± 1.2</td>
<td>35.3 ± 0.9</td>
</tr>
<tr>
<td>P2</td>
<td>10.5 ± 0.8</td>
<td>7.1 ± 1.2</td>
</tr>
<tr>
<td>P3</td>
<td>14.1 ± 0.9</td>
<td>27.2 ± 1.3</td>
</tr>
<tr>
<td>P4</td>
<td>8.9 ± 1.1</td>
<td>23.9 ± 0.6</td>
</tr>
<tr>
<td>P5</td>
<td>8.6 ± 0.6</td>
<td>11.1 ± 1.2</td>
</tr>
<tr>
<td>P6</td>
<td>10.1 ± 1.5</td>
<td>11.1 ± 1.9</td>
</tr>
<tr>
<td>P7</td>
<td>8.6 ± 0.9</td>
<td>21.4 ± 0.8</td>
</tr>
<tr>
<td>P8</td>
<td>9.1 ± 0.6</td>
<td>11.1 ± 1.2</td>
</tr>
<tr>
<td>P9</td>
<td>7.2 ± 1.1</td>
<td>35.3 ± 0.9</td>
</tr>
<tr>
<td>P10</td>
<td>6.6 ± 0.9</td>
<td>7.1 ± 1.2</td>
</tr>
<tr>
<td>P11</td>
<td>11.8 ± 0.6</td>
<td>27.2 ± 1.3</td>
</tr>
<tr>
<td>P12</td>
<td>11.1 ± 0.8</td>
<td>23.9 ± 0.6</td>
</tr>
<tr>
<td>P13</td>
<td>9.8 ± 1.8</td>
<td>11.1 ± 1.2</td>
</tr>
<tr>
<td>P14</td>
<td>3.7 ± 1.4</td>
<td>21.4 ± 0.8</td>
</tr>
<tr>
<td>P15</td>
<td>11.4 ± 0.7</td>
<td>11.6 ± 0.9</td>
</tr>
<tr>
<td>P16</td>
<td>14.7 ± 1.1</td>
<td>12.9 ± 0.9</td>
</tr>
<tr>
<td>P17</td>
<td>13.2 ± 0.4</td>
<td>15.0 ± 0.6</td>
</tr>
<tr>
<td>P18</td>
<td>8.1 ± 0.7</td>
<td>7.9 ± 1.5</td>
</tr>
<tr>
<td>P19</td>
<td>7.7 ± 1.1</td>
<td>8.2 ± 0.8</td>
</tr>
<tr>
<td>P20</td>
<td>6.0 ± 0.9</td>
<td>6.8 ± 1.1</td>
</tr>
</tbody>
</table>

*For each sample, the mean proportion (%) of BRCA2 transcripts was calculated as the mean of the proportions found for each measurement. Triplicate cDNA and PCRs were performed for each sample. Data represent 95% confidence intervals of the means.
fication with primer pair BRC7U/BRC7L, which encompasses only exon 12 (Fig. 1), and sequence analysis (Fig. 2) confirmed the existence of a splice variant lacking exon 12.

Putative alternative splicing involving large exons such as exons 10 (1116-bp) and 11 (4932-bp) was studied by Northern blot analysis. This analysis showed a single 11-kb BRCA2 transcript in all samples tested (data not shown), confirming a previous study (15). However, because BRCA2 is remarkably similar to BRCA1, both having a large exon 11 (3426 bp for BRCA1 and 4932 bp for BRCA2), and because several authors have shown the existence of an mRNA variant of BRCA1 with deletion of exon 11 (10–12), we confirmed our Northern blot results by RT-PCR analysis using a primer pair (BRC6U/BRC6L) encompassing exons 10 and 11 of BRCA2 (Fig. 1). This primer pair not only did not yield the expected 6270-bp fragment because of the inefficiency of RT-PCR over such a length of sequence, but it also did not yield the putative 222- and 1338-bp fragments when exon 11 (with and without exon 10, respectively) had been spliced out.

**Detection of the Δ12-BRCA2 Variant in Other Normal Human Tissues.** Expression of the Δ12-BRCA2 mRNA variant in a variety of normal human tissues, including peripheral blood leukocytes, kidney, smooth muscle, stomach, colon, skin, liver, bone marrow, ovary, placenta, and prostate, was examined.

The Δ12-BRCA2 variant was observed in all tested tissues, with a proportion of ~10%, compared with the WT BRCA2 transcript.

**Detection of the Δ12-BRCA2 Variant in Mouse Tissues.** To verify the presence of the Δ12-BRCA2 alternative splicing variant in mouse tissues, we carried out RNA RT-PCR with primers encompassing exon 12. One transcript isoform of the expected size, comparable to the human profile (96 bp smaller than the WT BRCA2 PCR product), was amplified from all mouse tissues tested (smooth muscle, placenta, and liver). The mouse isoform showed a deletion of exactly the same 96 nucleotides of exon 12 as in the human counterpart.

**Increased Level of the Δ12-BRCA2 Variant in Tumor Breast Tissue Compared with Normal Tissue.** Because BRCA2 is responsible for the development of a subset of familial breast tumors, the possible role of Δ12-BRCA2 alternative splicing in sporadic breast tumorigenesis warrants investigation. We examined the expression level of the Δ12-BRCA2 variant in tumor breast tissue compared with normal tissue. We analyzed matched normal and primary tumor breast tissues from 12 patients (patients P8–P19; Table 1). For each sample, three different cDNA and PCRs were performed, and the mean value was determined.

To check the reliability of our molecular analysis, we used several controls: (a) triplicate PCRs were carried out, one for 30 cycles, another for 32 cycles, and the last for 35 cycles, to check whether 32 PCR cycles corresponded to the exponential phase of the reaction; (b) PCR was carried out with the two primer pairs BRC2U/BRC2L and BRC7U/BRC7L, which both reveal the Δ12-BRCA2 variant (Fig. 1); (c) PCR was carried out using three DNA polymerase conditions: AmpliTaq DNA polymerase (PE. Applied Biosystems), AmpliTaq DNA polymerase (PE. Applied Biosystems) plus TaqStart Antibody (Clontech), and AmpliTaq Gold DNA polymerase (PE. Applied Biosystems). These three controls were used to check whether the two PCR fragments were equally amplified. Indeed, in certain PCR conditions, smaller fragments could be more efficiently amplified than larger fragments. For each sample, the qualitative and quantitative measurements in different PCR conditions were not significantly different from each other, showing that the smaller fragments were not more efficiently amplified, therefore confirming the accuracy of the molecular analysis.

Because the levels of Δ12-BRCA2 variant in the 12 matched normal samples, together with the 8 previously tested normal samples (7 normal breast tissue specimens obtained from women undergoing cosmetic breast surgery and a pool of 6 normal human breast tissues), consistently fell between 6 and 15 PA12 value, values of 20 or more were considered to represent overproduction of the Δ12-BRCA2 variant in individual breast cancer tissue. Among the 12 patients in whom both primary breast tumors and normal breast tissue were investigated, 4 (P8, P10, P11, and P13; 33.3%) clearly showed a higher proportion of the Δ12-BRCA2 variant in tumor than in normal tissue (Table 1).

**Overproduction of the Δ12-BRCA2 Variant Is Associated with Steroid Receptor Status.** BRCA2 expression is regulated by estrogen in human breast cancer cell lines (16), so it was important to determine whether a high level of Δ12-BRCA2 variant is associated with the steroid receptor status of the tumors. We analyzed Δ12-BRCA2 mRNA expression in 26 additional breast tumors selected on the basis of steroid receptor status: half (n = 13) were both estrogen receptor and progesterone receptor negative and other half were both estrogen receptor and progesterone receptor positive. The level of the Δ12-BRCA2 variant ranged from 5.9 to 41.9% in this additional series. Fig. 3 represents tumors in which the proportion of Δ12-BRCA2 variant was 5.9% (T444), 22.6% (T182), and 41.9% (T327). Overproduction of the Δ12-BRCA2 variant was found in 9 (34.6%) of these 26 tumors and was significantly associated with steroid receptor negativity (P = 0.0005). None of the 13 steroid receptor-positive tumors showed overproduction of the Δ12-BRCA2 variant, compared to 9 of the 13 (69%) that were steroid receptor negative.

**Discussion**

Alternative mRNA splicing is a common mechanism for regulating gene expression in higher eukaryotes, and there are many examples of development-, tissue-, and tumor-specific differences in splicing events. To search systematically for the existence of alternative BRCA2 variants, we carried out RT-PCR with primer pairs covering the whole sequence. We used only a single-stage PCR strategy to avoid the overexpression of shorter PCR products frequently observed by nested PCR. It is interesting to note that Xu et al. (12), using a nested RT-PCR strategy, identified multiple variants of the BRCA1 gene. Here, we report the identification of a major alternative BRCA2 transcript with an expression level of ~10% in normal breast tissue relative to the WT BRCA2 transcript. Sequencing of this alternative transcript shows that it arises from alternative splicing of exon 12. We cannot rule out the existence of other important BRCA2 variants. Indeed, we observed several BRCA2 variant transcripts in the NH2-terminal and central domains (primer pairs BRC1U/BRC1L and BRC3U/BRC3L), but they had very low expression levels (<3%) relative to the WT BRCA2 transcript (data not shown). Thus, we
observed the Δ3-BRCA2 variant reported by Siddique et al. (17). We did not find any variants in the COOH-terminal domain (exons 19–27; primer pairs BRC4U/BRC4L and BRC5U/BRC5L).

The number of nucleotides (96 bp) missing from the Δ12-BRCA2 transcript isosform is a multiple of three, suggesting the maintenance of the open reading frame; translation of this transcript would result in a BRCA2 protein lacking 32 amino acids between codons 2280 and 2311. The function of this region of BRCA2 protein is unknown. Indeed, several different domains have been recognized in BRCA2. A coding sequence for a translational activation domain was identified in the 5’ end of the transcript (exon 3; Ref. 18) and a domain that interacts with RAD51 through its BRC repeats located at the 5’ portion of exon 11 (19). None of these functional domains is encoded by exon 12. However, several of our results suggest that the Δ12-BRCA2 transcript may give rise to a protein with an important biological function. (a) The Δ12-BRCA2 transcript was observed in a large variety of normal adult human tissues, including peripheral blood leukocytes, and it is conserved in the mouse. (b) The expression of the Δ12-BRCA2 transcript was higher in tumor tissue than in normal breast tissue, suggesting the existence of mechanisms generating the BRCA2 mRNA variant in normal breast tissue and the possibility that these may be dysregulated in breast tumor tissues. It is not clear why or by what mechanism the BRCA2 mRNA variant level is increased, but it could be caused by factors at the transcriptional or posttranscriptional level or both. (c) Overproduction of the Δ12-BRCA2 variant is associated with steroid receptor-negative tumors. Spillman and Bowcock (16) have previously shown that estrogen regulates BRCA2 mRNA expression mediated by the estrogen receptor. Taken together, these results suggest that the Δ12-BRCA2 transcript isoform must make a significant contribution to overall BRCA2 function.

In conclusion, we have identified an alternatively spliced BRCA2 transcript that is widely expressed in all normal tissues examined. This Δ12-BRCA2 transcript is overexpressed in steroid receptor-negative breast tumor tissue, suggesting that dysregulation of the Δ12-BRCA2 isoform may contribute to progression in human breast cancer. Characterization of the functional properties of the protein derivative and direct assessment of protein produced in various cell types will be necessary to determine the significance of this variant. Alternative splicing of the BRCA2 gene in lymphocytes may also have an important practical implication: in some cases, it may complicate the detection of germ-line BRCA2 mutations based on the screening of lymphocyte RNA.

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References


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