Methylation of the BRCA1 Gene in Sporadic Breast Cancer

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Abstract

Mutations of the BRCA1 gene in tumor DNA from patients with sporadic breast cancer have been reported, and BRCA1 activity is markedly decreased in invasive breast tumors. Previous reports have shown that hypermethylation of the promoter region is an alternative mechanism to mutation for the inactivation of tumor suppressor genes. We examined the BRCA1 promoter region for hypermethylation by Southern blotting. Hypermethylation was observed in two of seven sporadic breast carcinomas but not in any normal tissues. The hypermethylation was not an artifact because a control region was unmethylated in the two tumors. Although not all tumors were hypermethylated, these observations are consistent with an important role for epigenetic mechanisms in human cancer.

Introduction

Familial breast cancer and familial breast and ovarian cancer often result from germ-line mutations of the BRCA1 gene (1). However, no BRCA1 mutations in sporadic breast cancer have been reported, and BRCA1 mutations in sporadic ovarian cancer are rare (2, 3). This markedly contrasts with other tumor suppressor genes first identified through the study of familial cancer. Tumor suppressor genes such as RB, APC, and VHL are frequently mutated in the corresponding sporadic cancers (4-6). Nevertheless, BRCA1 does seem to play a role in sporadic tumors because BRCA1 expression is very much reduced in invasive breast tumors, relative to normal breast epithelium and ductal carcinoma in situ (7). Alleles of some genes are known to be silenced by hypermethylation of the promoter region associated with CpG island in breast cancer (8-12). Here, we show that the BRCA1 promoter region CpG island is hypermethylated in some sporadic breast cancers.

Materials and Methods

BRCA1 Sequence and Restriction Fragments. The numbers used refer to the sequences of Smith et al. (13) for BRCA1 and Barker et al. (14) for the IA1.3B promoter region. The position of CpG islands was determined by the CPGS program of John McCarrey (Southwest Foundation for Biomedical Research, San Antonio, TX). In the BRCA1 region, BRCA1 exon 1a is at 3344-4346, exon 1b is at 3621-3998, and exon 2 is at 4620-4718. The AvaII 1008-bp fragment is between 3008 and 4016. In the IA1.3B promoter region, the duplicated BRCA1 exon 1a is at 1675-1795, the duplicated BRCA1 exon 1b is at 1954-2385, and the duplicated BRCA1 exon 2 is at 3288-3385. The AvaII 1994-bp fragment is between 1621 and 3615. The probe used was a 217-bp fragment (nucleotides 3301-3517) amplified by the following primers: 5'-GGTTTTCCGGCAAAGGAAAACG-3' and 5'-AGTACCCCAAGCATCATTGGG-3'.

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Tumor Samples. Tumor samples from patients with infiltrating adenocarcinoma were frozen in liquid nitrogen following surgery. Samples were obtained after informed consent was obtained and the protocols were approved by the Ethics of Human Research Committee of the Queen Elizabeth Hospital. Genomic DNA was prepared by digesting 20-10 μm slices in 500 μl of 10 mm Tris-HCl, 2 mm EDTA, and 400 mm NaCl, pH 8.0, with 250 μg of proteinase K (Merck, Darmstadt, Germany) and 0.5% SDS. After successive extractions with equal volumes of phenol, phenol:isoamyl alcohol:chloroform (25:24:1), and isooamyl alcohol:chloroform (24:1), the aqueous phase was precipitated with 2 volumes of ethanol, washed with 70% ethanol and resuspended in TE (10 mm Tris-HCl and 1 mm EDTA, pH 8.0).

Southern Blot Analysis of Methylation. Ten μg of DNA were cut with 50 units of AvaII (New England Biolabs, Beverly, MA) and 100 units of either of the methylation-sensitive enzymes, HpaII (New England Biolabs) or CfoI (Boehringer Mannheim, Mannheim, Germany), in the buffer recommended for the methylation-sensitive enzyme for 16 h. AvaII cutting may itself be affected by methylation if the terminal C of its recognition sequence is methylated. However, neither of the two sites in the BRCA1 promoter region is followed by a G or NpG residue. One AvaII site at the duplicated region (nucleotide 1664) is followed by a NpG residue, but no evidence for its methylation was seen. After digestion, samples were precipitated, washed in 70% ethanol, and run on a 20-cm 1.5% agarose gel at 100 V in 0.5X TBE buffer for 5-6 h. The gel was blotted overnight without prior depurination onto Hybond N+ (Amer sham, Amersham, United Kingdom) in 400 mm NaOH. After rinsing in 2X SSC, the gel was cross-linked using a Stratagene UV cross-linker (Stratagene, La Jolla, CA). The blot was prehybridized at 42°C in 50% deionized formamide, 1 mm NaCl, 1% SDS, 10% dextran sulfate (Pharmacia, Uppsala, Sweden), and 400 μg/ml sheared, denatured herring sperm DNA. The probe (25 ng) was labeled by random priming, added to the prehybridization mix, and incubated at 42°C overnight. The membrane was rinsed and washed at 65°C in 0.5% SSC and 0.5% SDS and autoradiographed.

Results and Discussion

BRCA1 has two alternative first exons, 1a and 1b, each with its own promoter region. Exon 1a-containing transcripts are the forms predominantly expressed in mammary epithelium (15). BRCA1 is within 50 kb of the IA1.3B gene, which was identified using antisera against the CA125 tumor antigen (16). The promoter regions of these two genes have a complex organization (14, 17). A partial duplication of IA1.3B (exons 1a, 1b, and 3 and intervening sequences) is in a head-to-head orientation with BRCA1, and a partial duplication of BRCA1 (exons 1a, 1b, and 2 and intervening sequences) is in a head-to-head orientation with IA1.3B. BRCA1 exon 1a is located 295 bp from exon 1a of the IA1.3B duplication. Similarly, the duplicated BRCA1 exon 1a lies adjacent to exon 1a of IA1.3B, but the intergenic region, which is otherwise highly homologous to the 295-bp region associated with BRCA1 exon 1a, is larger due to the insertion of a 343-bp ARPP1 pseudogene (17).

Analysis of the 5' region of the BRCA1 gene, using the criteria of Gardiner-Garden and Frommer (18), shows that there is a CpG island (a G+C-rich region with reduced suppression of CpG dinucleotides) extending over more than 1200 nucleotides, which begins at exon 1a of the IA1.3B duplication and includes exons 1a and 1b of BRCA1 and their associated promoter regions [nucleotides 2962-4209, using the sequence of Smith et al. (13)].

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We used a 217-bp probe spanning BRCA1 exon 1a to assess methylation of the BRCA1 CpG island by Southern blotting. The chosen bordering enzyme AvaII gives a 1008-bp fragment (nucleotides 3008–4016) that spans most of the island and includes part of the duplicated exon 1a of 1A1.3B, the intergenic region and BRCA1 exons 1a and 1b (Fig. 1). This 1008-bp region has five HpaII sites and five CfoI sites. The probe also detects an AvaII band of 1994 bp (nucleotides 1621–3615, using the sequence of Barker et al. (14]) that includes the duplicated BRCA1 exons 1a, 1b, and 2 associated with the 1A1.3B gene. The 1008- and 1994-bp bands only remain intact after digestion with HpaII or CfoI if all of the HpaII or CfoI sites within the AvaII fragments are methylated.

Seven invasive tumors from patients with sporadic breast cancer, two biopsies of normal breast tissue, two specimens of peripheral blood mononuclear cells, and four breast cancer cell lines were examined. No evidence for methylation of either region was seen with DNA extracted from either peripheral blood or normal breast tissue because only bands consistent with complete digestion by the methylation-sensitive enzymes were present. Similarly, no evidence for methylation was seen in four tumors and the cell lines T-47D, MDA-MB453, and MDA-MB468.

Tumors from two patients with advanced breast cancer showed hypermethylation of the BRCA1 promoter region (Fig. 2a shows one of these tumors). The duplicated region was unmethylated in these patients. The duplicated region thus acted as a control for digestion and showed that the persistence of the 1008-bp band was not an artifact of incomplete digestion with the methylation-sensitive enzymes. The age of onset was quite early for both of these patients (Table 1), but neither had any family history of breast or ovarian cancer.

A third breast tumor showed evidence of methylation at the duplicated region with CfoI digestion but was unmethylated at the BRCA1 promoter region (Fig. 2b). A similar pattern was seen in the cell line MCF-7 (Fig. 2a). Here, the methylated band was slightly smaller, indicating that a terminal CfoI site was unmethylated. In both cases, methylation was not observed using HpaII digestion. This could be explained by a lack of methylation of the HpaII sites between exons 1a and 1b (Fig. 1). The significance of partial methylation in this region remains unclear, although it may indicate methylation of the 1A1.3B promoter CpG island. Because the 1994-bp AvaII band detected by our probe is more than 500 bp upstream of exon 1a of 1A1.3B, these findings require further investigation by the use of a different probe or bordering enzyme.

It is unclear whether methylation is acting on one or both alleles of the BRCA1 gene for two reasons. First, the amount of normal cell contamination is unknown, and it is impossible to obtain sufficient tumor DNA for Southern blotting by microdissection. In those patients that appear substantially methylated, loss of heterozygosity of the normal allele may have occurred.

Despite the lack of mutation of the BRCA1 gene, several lines of evidence suggest that BRCA1 has tumor suppressor activity in sporadic tumors. The activity of the gene is markedly lower in sporadic tumors once they become invasive (7). Antisense oligonucleotides to BRCA1 increase the proliferation of mammary epithelium and the MCF-7 cell line (7). Most importantly, introduction of a normal copy of the BRCA1 gene into cell lines derived from sporadic tumors results in decreased tumorogenicity (19).

There are several explanations for the lower BRCA1 activity in invasive breast tumor cells. One is that an upstream regulator of activity is involved (7). A second is that BRCA1 becomes inactivated as the tumor becomes either dedifferentiated or enters a different and abnormal developmental program. These explanations are not mutually exclusive because alterations in control of BRCA1 by upstream genes may result from an altered developmental program.

Another possibility is that an epigenetic change such as methylation has inactivated theocus. Promoter methylation of other genes is associated with loss of activity in breast cancer (8–10). However, hypermethylation of the BRCA1 promoter region may not be the sole explanation for the decreased BRCA1 transcription seen in invasive breast cancer because we did not detect altered methylation in the majority of patient specimens and the cell lines that were examined. A role for methylation in these tumors is not definitively excluded thus far because methylation at a few critical CpG sites other than...

Table 1. Summary of results with clinical characteristics of the patients

<table>
<thead>
<tr>
<th>Tumor sample</th>
<th>Patient age</th>
<th>BRCA1 promoter region</th>
<th>BRCA1 duplicated region</th>
<th>Bloom and Richardson grade</th>
<th>Estrogen receptor status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>42</td>
<td>Methylated</td>
<td>Not methylated</td>
<td>2</td>
<td>Negative</td>
</tr>
<tr>
<td>Patient 2</td>
<td>45</td>
<td>Methylated</td>
<td>Not methylated</td>
<td>3</td>
<td>Negative</td>
</tr>
<tr>
<td>Patient 3</td>
<td>56</td>
<td>Not methylated</td>
<td>Methylated</td>
<td>3</td>
<td>Positive</td>
</tr>
<tr>
<td>Patient 4</td>
<td>55</td>
<td>Not methylated</td>
<td>Not methylated</td>
<td>3</td>
<td>Positive</td>
</tr>
<tr>
<td>Patient 5</td>
<td>45</td>
<td>Not methylated</td>
<td>Not methylated</td>
<td>1</td>
<td>Positive</td>
</tr>
<tr>
<td>Patient 6</td>
<td>93</td>
<td>Not methylated</td>
<td>Not methylated</td>
<td>3</td>
<td>Positive</td>
</tr>
<tr>
<td>Patient 7</td>
<td>67</td>
<td>Not methylated</td>
<td>Not methylated</td>
<td>3</td>
<td>Positive</td>
</tr>
</tbody>
</table>

4 A. Dobrovic and T. Bianco, unpublished results.
The 546-bp band is from the duplicated BRCA1 region (Fig. 1). When the tumor DNA from patient 1 was digested with Avail and CfoI (Lane 2), the 1994-bp band was absent, but either heterozygosity for the methylation or the presence of substantial normal tissue (or both). The Avail and MspI digest and the Avail and HpaII digests (Lanes 3 and 4, respectively) confirmed by Avail and HpaII digest of the same tumor (Lane 3), in which, once again, the 1008-bp band remains, whereas the 1994-bp Avail band is absent. When MspI, the methylation-insensitive isoschizomer of HpaII, was used, bands of 351 and 299 bp, deriving from the BRCA1 region, and a 186-bp band, deriving from the duplicated BRCA1 region, are seen. The 351-bp band is weak because it only has a 40-bp overlap with the probe (Fig. 1). For tumor 2, the digest with Avail and HpaII (Lane 5) gives an identical pattern to the Avail and MspI digest, indicating that both regions are unmethylated, which is confirmed by the Avail and CfoI digest (Lane 7). Likewise, MDA-MB468 gives a totally unmethylated appearance with the Avail and CfoI digest (Lane 8). However, when MCF-7 was digested by Avail and CfoI (Lane 11), a 1928-bp band remains, indicating that, whereas the first CfoI site of the duplicated BRCA1 region is unmethylated, the more distal CfoI sites are methylated. The absence of the duplicated region’s 550-bp band supports this interpretation. B, methylation studies of the BRCA1 promoter and BRCA1 duplicated regions. DNA was obtained from the cell line MCF-7 (Lanes 1 and 2) and patient 3’s tumor (Lanes 3–5). The Avail and HpaII digest (Lane 1) is identical to the Avail and MspI digest (Lane 2) for MCF-7. The 1994-bp band is absent, indicating that HpaII sites within the duplicated BRCA1 region are unmethylated. Although this appears to be contradictory with the CfoI digests, the results can be reconciled because of the different distribution of the HpaII and the CfoI sites within the Avail fragment from the duplicated region (Fig. 1). The probe only detects the proximal part of this region, and there is a cluster of fairly closely spaced HpaII sites in this region. Thus, the more distal HpaII sites may well be methylated. Patient 3 shows a strong 1994-bp band for the Avail and CfoI digest (Lane 5), but the 550-bp band is strong as well, indicating either heterozygosity for the methylation or the presence of substantial normal tissue (or both). The Avail and MspI digest and the Avail and HpaII digests (Lanes 3 and 4, respectively) are identical, again indicating that the proximal HpaII sites within the duplicated BRCA1 region are unmethylated.

those recognized by CfoI or HpaII may be sufficient to reduce the activity of the BRCA1 gene. Genomic sequencing (20) or methylation-specific PCR (21) will be necessary to resolve this question. Methylation may also be a marker of another type of epigenetic event, such as a regional alteration in chromatin structure, rather than the primary mechanism of gene silencing. Silencing may then occur without concomitant methylation (22).

It is possible that the methylation of the BRCA1 promoter region reflects a widespread hypermethylation of Cpg islands in those patients in which it is observed. It may be significant that the two patients that showed BRCA1 promoter region hypermethylation were also estrogen receptor negative and thus may be hypermethylated at the estrogen receptor as well (8). Hypermethylation of the BRCA1 promoter region represents the first BRCA1 lesion reported in sporadic breast cancer. It is an intriguing possibility that hypermethylation might also act to inactivate the normal allele in individuals carrying a germ-line mutation in which the tumors do not show loss of heterozygosity.

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