Advances in Brief

Methylation of the *BRCA1* Gene in Sporadic Breast Cancer[1]

Alexander Dobrovic[2] and David Simpfendorfer

Department of Haematology-Oncology [A. D.] and University of Adelaide Department of Medicine [A. D., D. S.], The Queen Elizabeth Hospital, Woodville, South Australia 5011, Australia

Abstract

Mutations of the *BRCA1* gene in tumor DNA from patients with sporadic breast cancer have not yet been observed. Nevertheless, *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 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 *1A1.3B* promoter region. The position of CpG islands was determined by the CPGS program[3] of John McCarrey (Southwest Foundation for Biomedical Research, San Antonio, TX). In the *BRCA1* region, *BRCA1* exon 1a is at 3344–3464, exon 1b is at 3621–3998, and exon 2 is at 4620–4718. The *Ava*II 1008-bp fragment is between 3008 and 4016. In the *1A1.3B* 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 *Ava*II 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'-GGTTTCCGTGGCAACGGAAAAGCG-3' and 5'-AGTACCCCAGAGCAT-CACTTGGG-3'.

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 isoamyl 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 *Ava*II (New England Biolabs, Beverley, MA) and 100 units of either of the methylation-sensitive enzymes, *Hpa*II (New England Biolabs) or *Cfo*I (Boehringer Mannheim, Mannheim, Germany), in the buffer recommended for the methylation-sensitive enzyme for 16 h. *Ava*II 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 *Ava*II 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 0.5× TBE buffer for 5–6 h. The gel was blotted overnight without prior depurination onto Hybond N+ (Amersham, Amersham, United Kingdom) in 400 mM NaOH. After rinsing in 2× SSC, the gel was cross-linked using a Stratalinker UV cross-linker (Stratagene, La Jolla, CA). The blot was prehybridized at 42°C in 50% deionized formamide, 1 M 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 *1A1.3B* 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 *1A1.3B* (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 *1A1.3B*. *BRCA1* exon 1a is located 295 bp from exon 1a of the *1A1.3B* duplication. Similarly, the duplicated *BRCA1* exon 1a lies adjacent to exon 1a of *1A1.3B*, 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 *1A1.3B* 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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Fig. 1. Fragments recognized by the 217-bp BRCA1 exon 1a probe. The 1008- and 1994-bp AvaII fragments (not shown to scale) are from the BRCA1 promoter region and the duplicated BRCA1 promoter region adjacent to the 1A1.3B gene, respectively. The heavy black line indicates the region of each sequence that is homologous to the probe. CfoI sites are indicated by an upward stroke, and HpaII sites are indicated by a downward stroke. ■, BRCA1 exons; □, 1A1.3B exons. Exon numbers are indicated by large characters above the exons. The size of fragments detected by the probe, when the region is fully unmethylated, are indicated. The numerals above the line refer to CfoI fragments, and the numerals below the line refer to HpaII fragments. □, inserted sequences; that preceding the duplicated BRCA1 exon 1a is part of the ARPP1 pseudogene and that within the duplicated BRCA1 exon 1b is a previously undescribed insertion of an Alu element.[4] The ARPP1 pseudogene is inserted immediately prior to the duplicated BRCA1 exon 1a.
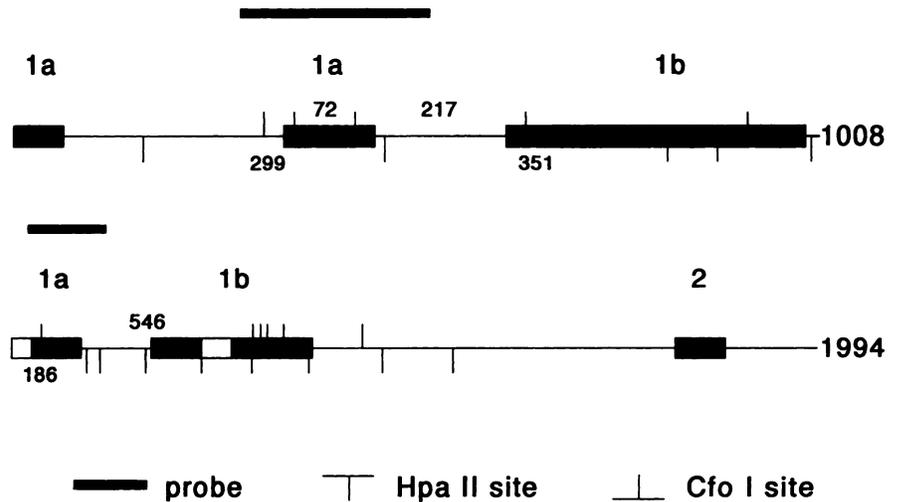
We used a 217-bp probe spanning BRCA1 exon 1a to assess methylation of the BRCA1 CpG island by Southern blotting. The chosen bordering enzyme AvaII gives a 1008-bp fragment (nucleotides 3008–4016) that spans most of the island and includes part of the duplicated exon 1a of 1A1.3B, the intergenic region 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 all of the 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 tumorigenicity (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 the locus. 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

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[4] A. Dobrovic and T. Bianco, unpublished results.

Table 1 Summary of results with clinical characteristics of the patients

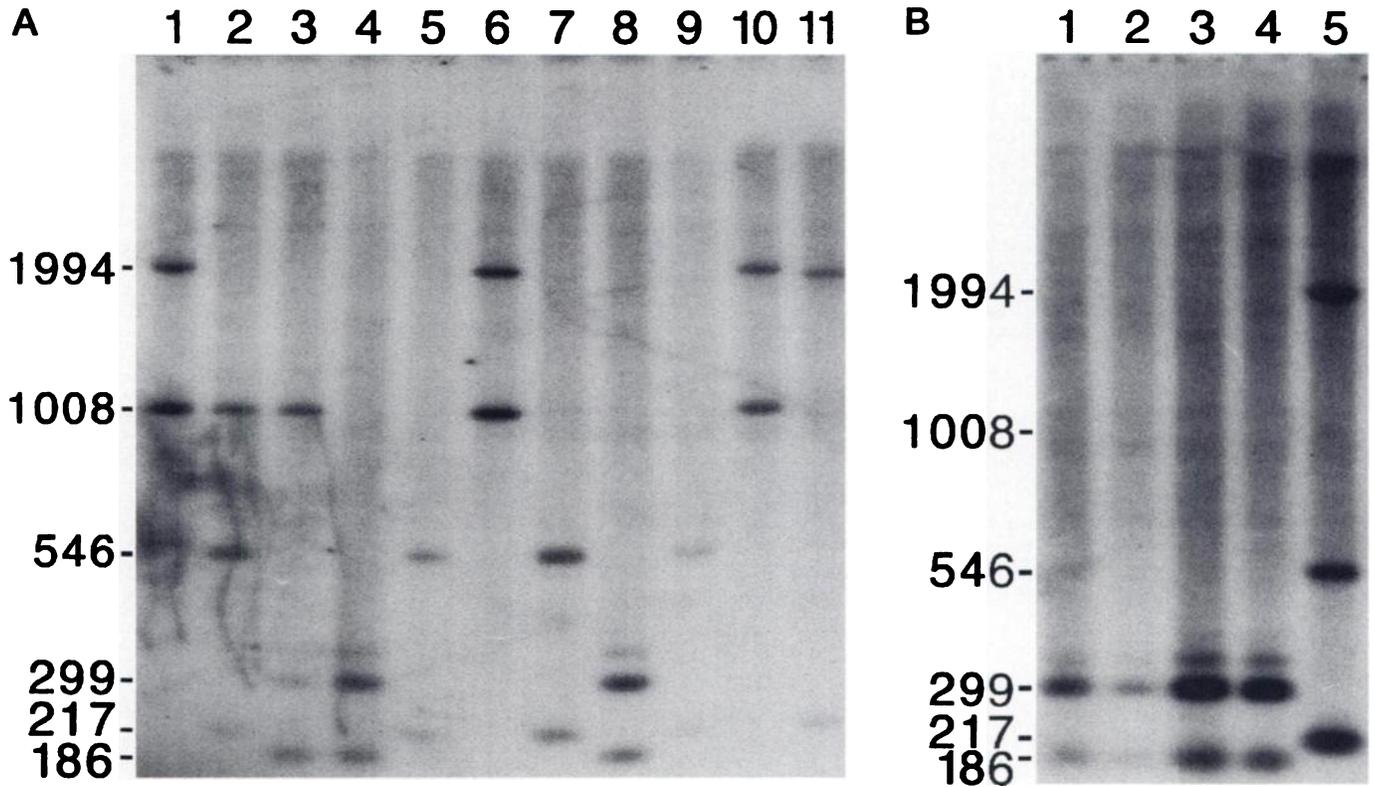| Tumor sample	Patient age	BRCA1 promoter region	BRCA1 duplicated region	Bloom and Richardson grade	Estrogen receptor status
Patient 1	42	Methylated	Not methylated	2	Negative
Patient 2	45	Methylated	Not methylated	3	Negative
Patient 3	56	Not methylated	Methylated	3	Positive
Patient 4	55	Not methylated	Not methylated	3	Positive
Patient 5	45	Not methylated	Not methylated	1	Positive
Patient 6	93	Not methylated	Not methylated	3	Positive
Patient 7	67	Not methylated	Not methylated	3	Positive

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Fig. 2. *A*, methylation studies of the *BRCA1* promoter and *BRCA1* duplicated regions. DNA was obtained from patient 1's tumor (*Lanes 1–4*), patient 1's normal breast tissue (*Lane 5*), patient 5's tumor (*Lanes 6–8*), and the cell lines MDA-MB468 (*Lane 9*) and MCF-7 (*Lanes 10* and *11*). All lanes were digested with *Ava*II. *Ava*II digests alone show two bands of 1994 and 1008 bp (*Lanes 1, 6*, and *10*). When normal breast tissue is digested with *Ava*II and *Cfo*I, these bands disappear (*Lane 5*). The 217-bp band is from the *BRCA1* region. The 546-bp band is from the duplicated *BRCA1* region (Fig. 1). When the tumor DNA from patient 1 was digested with *Ava*II and *Cfo*I (*Lane 2*), the 1994-bp band was absent, but the 1008 bp band remained, albeit at reduced intensity, indicating hypermethylation of the *BRCA1* promoter region but not of the duplicated *BRCA1* region. The hypermethylation was confirmed by *Ava*II and *Hpa*II digests of the same tumor (*Lane 3*), in which, once again, the 1008-bp band remains, whereas the 1994-bp *Ava*II band is absent. When *Msp*I, the methylation-insensitive isoschizomer of *Hpa*II, 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 *Ava*II and *Hpa*II (*Lane 8*) gives an identical pattern to the *Ava*II and *Msp*I digest (*Lane 1*) is identical to the *Ava*II and *Msp*I digest (*Lane 2*) for MCF-7. The 1994-bp band is absent, indicating that both regions are unmethylated, which is confirmed by the *Ava*II and *Cfo*I digest (*Lane 7*). Likewise, MDA MB468 gives a totally unmethylated appearance with the *Ava*II and *Cfo*I digest (*Lane 8*). However, when MCF-7 was digested by *Ava*II and *Cfo*I (*Lane 11*), a 1928-bp band remains, indicating that, whereas the first *Cfo*I site of the duplicated *BRCA1* region is unmethylated, the more distal *Cfo*I 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 *Ava*II and *Hpa*II digest (*Lane 1*) is identical to the *Ava*II and *Msp*I digest (*Lane 2*) for MCF-7. The 1994-bp band is absent, indicating that *Hpa*II sites within the duplicated *BRCA1* region are unmethylated. Although this appears to be contradictory with the *Cfo*I digests, the results can be reconciled because of the different distribution of the *Hpa*II and the *Cfo*I sites within the *Ava*II 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 *Hpa*II sites in this region. Thus, the more distal *Hpa*II sites may well be methylated. Patient 3 shows a strong 1994-bp band for the *Ava*II and *Cfo*I 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 *Ava*II and *Msp*I digest and the *Ava*II and *Hpa*II digests (*Lanes 3* and *4*, respectively) are identical, again indicating that the proximal *Hpa*II sites within the duplicated *BRCA1* region are unmethylated.

those recognized by *Cfo*I or *Hpa*II 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.

Acknowledgments

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