Down-regulation of BRCA1 in human sporadic breast cancer; analysis of DNA methylation patterns of the putative promoter region

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Germ-line alterations of *BRCA1* are responsible for about 50% of familial breast cancers. Although its biological function(s) has not yet been fully determined, it has been suggested that it may act as a tumor suppressor gene in breast and ovarian cancers. In sporadic breast cancers alterations of *BRCA1* have not been detected and *in vitro* experiments have indicated that *BRCA1* negatively regulates cellular proliferation. The present study was designed to identify and quantify, the *BRCA1* mRNA levels, in normal and neoplasic human breast tissue. *BRCA1* mRNA molecules were quantified using competitive reverse transcriptase PCR assays. DNA methylation patterns of this gene have been analysed by Southern blot experiments using methylation sensitive restriction enzymes. We found that *BRCA1* mRNA levels were significantly lower in sporadic breast cancers (37 cases analysed, 24 cases of invasive ductal carcinomas not otherwise specified (NOS), two lobular carcinomas *in situ* two medullary carcinomas, four invasive lobular carcinomas, two invasive mucinous carcinomas and three invasive ductal carcinomas with predominantly *in situ* component) compared with normal breast tissues ($P = 0.0003$). This down-regulation of *BRCA1* is observed in all histologic types analysed. In invasive ductal carcinomas NOS, this down-regulation does not correlate with any of the prognostic factors studied (tumor size, node status, histologic grade, hormone receptor status). In the samples analysed, alterations of DNA methylation patterns were not dectected in the vicinity of the major transcription start site. These data suggest the involvement of *BRCA1* in the carcinogenesis of these histologic types.

Keywords: *BRCA1*; human breast cancers, mRNA; PCR; DNA methylation; prognostic factors

Introduction

Germ-line alterations of the *BRCA1* gene have been shown to be responsible for about 50% of familial breast cancer (Friedman *et al*., 1994; Easton, 1997). Although its biological function is still unknown, it has been suggested that *BRCA1* may act as a tumor suppressor gene (Smith *et al*., 1992). Indeed, as expected for a tumor suppressor gene, loss of the wild type allele is observed in more than 90% of the tumors carrying germ-line alterations of *BRCA1* (Easton *et al*., 1997). In addition, recent findings demonstrating an association between BRCA1 and Rad 51 proteins suggest a role for this protein in DNA repair (Scully *et al*., 1997a). However, the presence of a transactivation domain in BRCA1 and its stable association with RNA Polymerase II holenzyme (Scully *et al*., 1997b) indicate that BRCA1 might also be involved in the regulation of transcription. These two hypotheses, which are not mutually exclusive, stress that *BRCA1* may have an important role in cellular differentiation and proliferation. Direct evidence for an essential role of *BRCA1* in mammalian development has been obtained from transgenic mice, since homozygous disruption of the *BRCA1* gene results in embryonic lethality (Hakem *et al*., 1996), and the progressive changes of *BRCA1* expression during mouse embryogenesis also imply a role for *BRCA1* in the differentiation process (Marquis *et al*., 1995).

Alteration of the *BRCA1* mRNA level has been observed in sporadic breast cancers, microdissections of biopsies from sporadic invasive breast tumors have shown a decrease in *BRCA1* expression in the tumoral component of the samples (Thompson *et al*., 1995). In addition, the involvement of *BRCA1* in cellular proliferation has been also addressed in human cell lines, using antisense oligonucleotides (Thompson *et al*., 1995) and in mouse cell lines, using antisense RNAs (Rao *et al*., 1996). All these experiments indicate that *BRCA1* negatively regulates cellular proliferation.

Taken together these data suggest that alterations in *BRCA1* mRNA levels might be a feature of human breast cancers. We have, therefore, investigated this hypothesis in human breast cancer biopsies. Tumors were assessed for histologic type, grade and prognostic factors and a total of 37 representative samples were analysed for their *BRCA1* mRNA content using a quantitative RT-PCR method (Ribieras *et al*., 1997).

Since somatic mutations of *BRCA1* were not detected in human breast cancers (Futreal *et al*., 1994; Merajver *et al*., 1995), alterations of DNA methylation patterns might be an alternative explanation for its down-regulation. It has been recently shown, by Southern blotting, that CpG sites around the exon 1a region were hypermethylated in some (two out seven) sporadic breast cancer cases (Dobrovic *et al*., 1997). Alterations of DNA methylation patterns of *BRCA1* gene have also been detected using the bisulfite genomic method (Mancini *et al*., 1998). In two out of six breast cancers analysed, 21% and 16% of DNA molecules exhibit abnormal methylated CpGs at a cyclic AMP regulatory element binding protein (CREB) (position -172 and -167) in the vicinity of the major transcription start site (Xu *et al*., 1995).

In order to investigate the potential relationship between *BRCA1* expression and DNA methylation,

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DNA methylation patterns in the 5′ region and within the body of the gene were investigated by Southern blot experiments, after digestion with methylation sensitive restriction enzymes. Since first reported (Frommer *et al*., 1992), the bisulfite genomic sequencing method has been widely used for the analysis of DNA methylation patterns. This method is based on specific deamination of unmethylayed cytosines. After strand-specific amplification of the deaminated DNA, the pattern of methylation is deduced from the sequence of PCR fragments. However, it has recently been shown that the quantitation of the relative amounts of methylated and unmethylated DNA at particular loci might be altered by PCR bias, depending on the sequence and/or the strand analysed (Warnecke *et al*., 1997). Although the Southern blot method is less sensitive than bisulfite genomic sequencing and does not provide information about the CpGs outside restriction sites, this method does provide an evaluation of the methylation profiles of all the DNA molecules from a given sample. Therefore, the methylation status of *Hpa*II sites, including one at a CREB site (position −167), was determined in normal and pathological breast tissues and compared with the steady-state *BRCA1* mRNA level in these samples.

Results

Low abundance of BRCA1 *mRNA in human sporadic breast cancer*

The steady state level of *BRCA1* mRNA was investigated in normal and pathological breast tissues using a competitive RT–PCR method (Ribieras *et al*., 1997). Germ-line alterations of *BRCA1* are more frequently localized in the 3′ one-third of the gene (Gayther *et al*., 1995) and only one case of alteration at the exons 6 and 8 has been reported (The Breast Cancer Information Core). Therefore, in order to amplify *BRCA1* transcripts in a large panel of samples, primers were chosen in these two different exons together with a chimeric RNA derived from the same sequence but containing an insert of 48 bp, which was used as a competitor (Ribieras *et al*., 1997). Some representative assays are shown in Figure 1. Data obtained are summarized in Figure 2 and indicate that the *BRCA1* mRNA level is significantly decreased ($P = 0.0003$) in tumoral tissue compared with normal breast tissues (37.054 ± 42.4 and $179.2 \pm 40.8 \times 10^3$ copies/μg of total RNA, respectively).

The samples analysed correspond to various histologic types (Table 1). However, this low level of

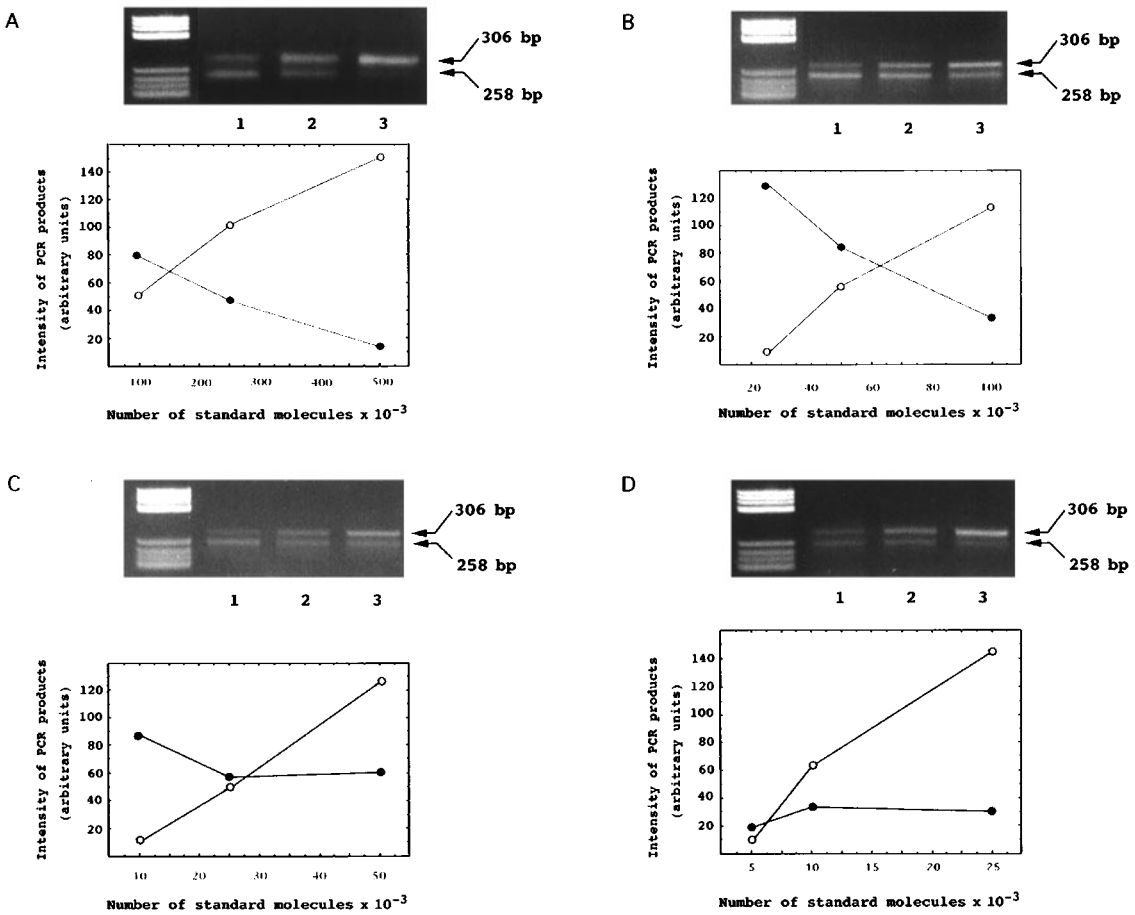

Figure 1 RT–PCR assay of *BRCA1* mRNA in breast tissues. Fifteen μl of the RT–PCR reaction mixture (total volume 100 μl) performed from serial dilutions of chimeric *BRCA1* RNA and 0.6 μg of sample total RNA were analysed on ethidium bromide 2% agarose gel. The 306 bp band corresponds to the chimeric *BRCA1* RNA. The 258 bp band corresponds to the wild type *BRCA1* mRNA. The intensity of the bands were plotted against the initial number of standard molecules added. The RT–PCR reactions were performed with 0.6 μg of total RNA from (**a**) normal breast tissue and (**b**, **c**, **d**) neoplasic breast samples

BRCA1 mRNA does not seem to be associated with a specific histologic type of cancer (Table 1). In invasive breast ductal carcinomas not otherwise specified (NOS) a 5–6-fold decrease in *BRCA1* expression is observed ($33.3 \pm 40.1 \times 10^3$ copies/μg RNA, $P = 0.0004$) compared with normal breast tissue. However, this low level of expression is also found in the other histologic types analysed, lobular carcinomas *in situ*, medullary carcinomas, invasive lobular carcinomas, invasive mucinous carcinomas and invasive ductal carcinomas with predominantly *in situ* component (Table 1).

The abundance of BRCA1 *mRNA is not associated with a specific prognostic factor in invasive breast ductal carcinomas NOS*

The biological and clinical characteristics of the invasive breast ductal carcinoma NOS samples analysed and their *BRCA1* mRNA content are reported in Table 2. From these data there is no meaningful association between *BRCA1* expression and any other prognostic factor. However, although not statistically significant, estrogen receptor rich (ER+) invasive breast carcinomas NOS exhibit a higher level of *BRCA1* mRNA than that observed in the estrogen receptor negative samples (ER−). In line with this observation, *in vitro* studies indicate a potential relationship between estrogen receptors and the control of *BRCA1* expression (Gudas *et al*., 1995; Xu *et al*., 1997).

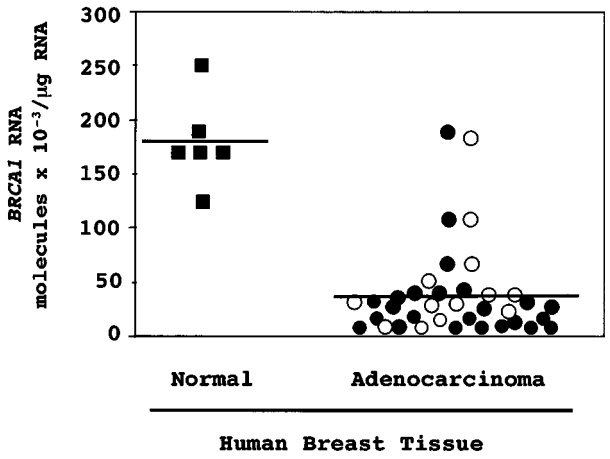

Figure 2 *BRCA1* mRNA in normal and neoplasic breast tissues. ■ normal breast tissue; ● invasive ductal carcinoma NOS; ○, other histologic types of breast carcinomas. Horizontal lines, median values. The difference between neoplasic ($37.054 \pm 42.4 \times 10^3$ copies/μg of total RNA) and normal breast tissues ($179.2 \pm 40.8 \times 10^3$) is significant (Kruskal-Wallis test, $P = 0.0003$)

Table 1 Histologic type and *BRCA1* mRNA level in 37 patients with sporadic breast carcinomas

	Patients No.	BRCA1 *mRNA*
Age		
⩽50 yr	15	38.7±56.2
⩾51 yr	22	36.6±28.1
Tumor size (cm)		
⩽1	3	38±29
1–3	23	31.5±39.3
>3	9	52.6±58.6
Histologic type		
Invasive lobular carcinoma	4	38.3±46.9
Invasive mucinous carcinoma	2	34±5.7
Invasive ductal carcinoma with predominantly *in situ* component	3	40±10.2
Lobular carcinoma *in situ*	2	38±41
Medullary carcinoma	2	103±113.1
Invasive ductal carcinoma NOS	24	33.3±40.1

BRCA1 mRNA level is expressed as the number of *BRCA1* mRNA molecules $\times 10^{-3}$ per μg of total RNA. Results are expressed as the mean±s.d.

DNA methylation patterns of BRCA1 *in normal and pathological breast tissues*

Site-directed deletion mutagenesis of the 5′ region of the *BRCA1* gene lead to the identification of a bidirectional promoter, position −258 to +43 from the major transcription initiation start site (nt+1 of exon 1a) in the human mammary gland (Xu *et al*., 1995). This region contains several putative regulatory elements, Sp1 sites are present at positions −163 and −233 from exon 1a and a CREB site at position −176 (Smith *et al*., 1996).

Consequently, we first analysed this 5′ region of *BRCA1*, using a cloned DNA fragment (probe 1, Figure 3), in 37 neoplasic breast cancers and in two normal breast tissues. Probe 1 spans the *BRCA1* gene nucleotide 3294 to 3606 and contains the region corresponding to the exon 1a (Smith *et al*., 1996) and maps *Hpa*II sites within the promoter region, at positions −167 (CREB site), +133 and +488, (Figure 3).

Since the *BRCA1* pseudogene is the result of the duplication of the *BRCA1* region containing the exons 1a, 1b and 2, probe one also matches (90% similarity) the *BRCA1* pseudogene from nucleotides 1683–1937 (Barker *et al*., 1996). After *Taq*I

Table 2 Prognostic factors and *BRCA1* mRNA level in 20 patients with invasive breast ductal carcinoma NOS

	Patients No.	BRCA1 *mRNA*	P
Tumor size (cm)			0.33
⩽1	2	38±41	
1–3	13	22±12.2	
>3	5	74±73.8	
Nodal status			0.47
0	10	25.2±18.5	
1–3	5	36.6±41.7	
>3	4	81.7±92.8	
Histological grade of Scarff, Bloom and Richardson			0.07
I	1	9	
II	15	37.7±44.5	
III	4	16.5±7.7	
Estrogen receptor status			0.16
<10 fmol/mg protein	12	33.3±27.5	
⩾10 fmol/mg protein	8	17.6±12.25	
Progesterone receptor status			0.31
<10 fmol/mg protein	11	33.8±27.6	
⩾10 fmol/mg protein	9	23.4±19	

BRCA1 mRNA level is expressed as the number of *BRCA1* mRNA molcules $\times 10^{-3}$ per μg of total RNA. Results are expressed as the mean±s.d. Significance of differences between each subgroups were assessed by the Kruskal-Wallis test

digestion, probe one reveals two bands at 2.7 and 1.5 kb corresponding to the *BRCA1* gene and to the *BRCA1* pseudogene respectively (Figure 4a, lane 1). Addition of *Msp*I generates two DNA fragments at 0.35 kb and 0.3 kb (Figure 4a, lane 2), consistent with the presence of the CCGG sites in this region (Figure 3a and 3b). The 0.3 kb band corresponds to the *BRCA1* gene and the 0.35 kb band corresponds to two unresolved bands, one at 0.351 kb from *BRCA1* (Figure 3a) and the other at 0.364 kb from the pseudogene (Figure 3b). When DNAs were cleaved by *Hpa*II, the methylation sensitive enzyme, the patterns obtained were identical to those observed with *Msp*I (Figure 4a, lanes 3–5 and 8–14), indicating that the CCGG sites at positions −167, +133 and +484 are unmethylated.

However, in two samples out the 37 neoplasic breast tissue samples analysed, a selective hypermethylation of the *BRCA1* pseudogene was observed indicated by the partial cleavage of the 1.5 kb *Taq*I-band (Figure 4a, lanes 6 and 7).

In order to map the CCGG sites within the body of the gene, filters were also hybridized with several cloned cDNA fragments and the representative patterns obtained with the probe two, encompassing cDNA region corresponding to the 3′ end of the exon 11 to the exon 24, are shown in Figure 4b. Hybridization with probe two reveals, after *Taq*I

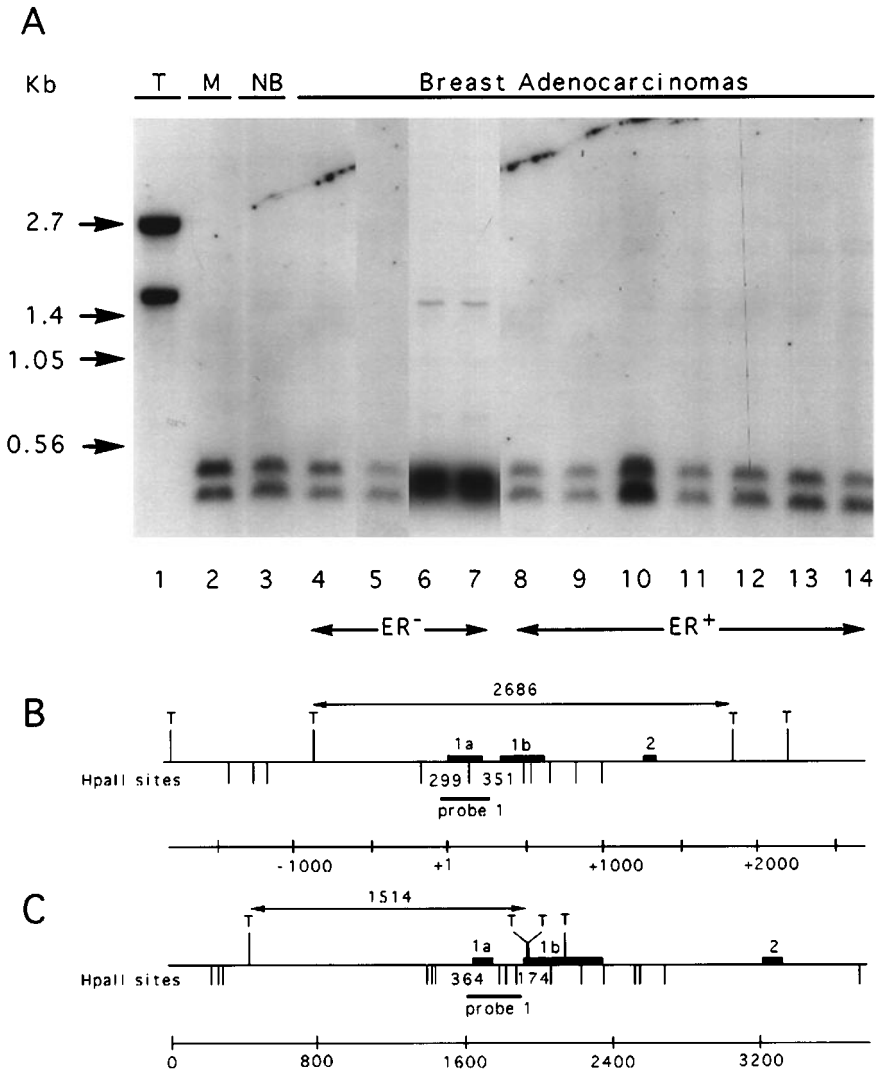

Figure 3 DNA methylation pattern of the 5′ region of the *BRCA1* gene and pseudogene. (**a**), DNA extracted from the HeLa cell line was digested with the restriction endonuclease *Taq*I (T) or by *Taq*I and *Msp*I (M). DNA extracted from normal breast (NB) or breast adenocarcinomas were cleaved by *Taq*I and the methylation sensitive enzyme *Hpa*II. Southern blot experiments were performed as described in the Materials and methods section. DNA methylation patterns shown are representative of those observed in the normal and tumoral tissues studied. Lane 1, the 2.7 kb band corresponds to the exon 1a region of the *BRCA1* gene. The probe 1 also reveals a 1.5 kb band corresponding to the *BRCA1* pseudogene. Lane 2: the probe 1 reveals two bands for the *BRCA1* gene: 299 and 351 bp and only one band for the *BRCA1* pseudogene (364 bp). The same pattern is observed (lane 3) for normal breast tissue, (lanes 4–7) for ER^- breast adenocarcinomas and (lanes 8–14) for ER^+ breast adenocarcinomas. The two ER^- samples, exhibiting an hypermethylation of the 5′ region of the *BRCA1* pseudogene, are shown (lanes 6 and 7). (**b**) Partial restriction map of the 5′ region of the *BRCA1* gene and (**c**) pseudogene. The position of the different exons (exon 1a, exon 1b and exon 2) is indicated by black boxes and *Taq*I restriction sites by T. The probe used is a 350 bp fragment corresponding to the exon 1a region of the *BRCA1* gene. The size of the fragments revealed by probe 1, after *Taq*I or *Taq*I-*Hpa*II digestion, are indicated in bp. In the Southern blot experiments the 174 bp fragment was not detected using probe 1

digestion, several bands corresponding to different regions of the gene (Figure 4b, lane 1). Since the intronic regions contain several CCGG sites (Smith *et al*., 1996), *Msp*I digestion produces several novel bands. The different regions were identified using several probes corresponding to the different exons present in this region (Figure 4b, lane 2). *Hpa*II patterns are similar to *Taq*I patterns, indicating that the CCGG sites are equally methylated in the normal and pathological samples analysed (Figure 4b, lanes 3 to 12). Taken together these data indicate that the DNA methylation pattern of *BRCA1* is not altered in the breast cancer samples analysed and, as described for *BRCA2* (Collins *et al*., 1997), the putative promoter region of *BRCA1* is unmethylated at CCGG sites.

Discussion

Using a quantitative RT–PCR method (Ribieras *et al*., 1997) we have shown that *BRCA1* transcripts can be quantitated in normal and pathological breast tissues. Our data provide evidence that the majority (33 out of 37) of the tumor samples analysed exhibit a 10–12-fold decrease in *BRCA1* mRNA, compared to that observed in normal breast tissue. In addition, the number of *BRCA1* mRNA copies/μg of total RNA, in 12 out of

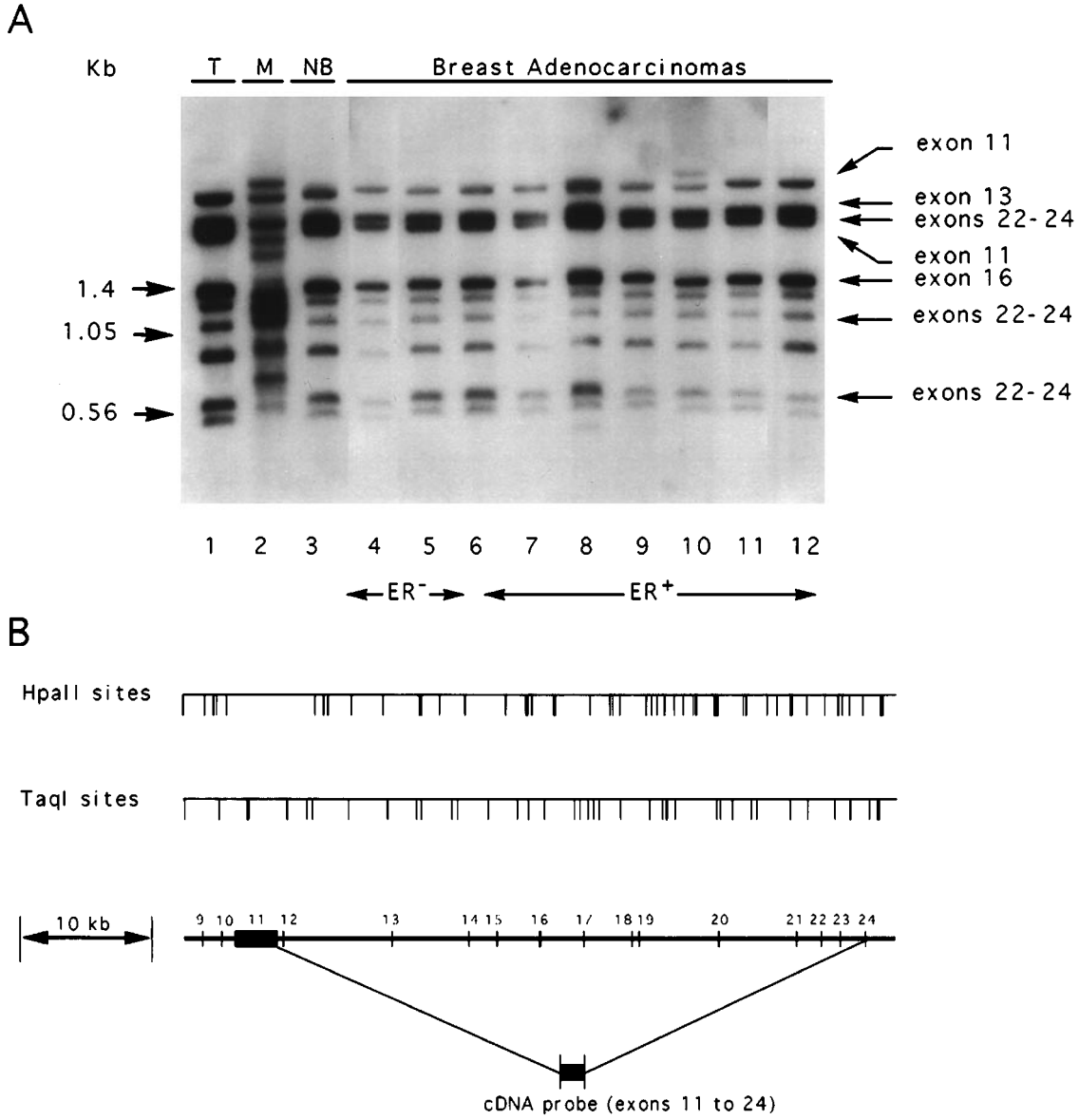

Figure 4 Representative DNA methylation patterns of the *BRCA1* 3′ region. (**a**) The filter was hybridized with probe 2, a cDNA fragment encompassing the end of exon 11 to the end of exon 24. Since the pseudogene corresponds to the duplication of the first exons of the *BRCA1* gene, this probe reveals only the *BRCA1* gene. DNAs were cleaved with *Taq*I (T-lane 1), *Taq*I and *Msp*I (M-lane 2) or *Taq*I and *Hpa*II (lanes 3–12). Lane 3, normal breast tissue; lanes 4–12, breast adenocarcinomas. The DNA digested with *Taq*I and *Hpa*II gives an identical pattern to the *Taq*I digest, indicating that all the CCGG sites are methylated. The filter was successively hybridized with probes corresponding to the different exons. The band corresponding to each exon is indicated by arrows on the right of the figure. (**b**) Partial restriction map of the 3′ region of the *BRCA1* gene. The position of the different exons (exon 9 to exon 24) is indicated by black boxes. The probe two is a 1.7 kb cDNA fragment from nt 3707 to nt 5711 of the *BRCA1* cDNA (Miki *et al*., 1994)

37 samples is 12–22-fold lower than in normal breast tissue. These results, therefore, suggest that the down-regulation of *BRCA1* is a feature of sporadic breast cancers, since alteration of the *BRCA1* mRNA level is observed in all histologic types of tumors analysed.

However, two samples (an invasive ductal carcinoma NOS and a medullary carcinoma) exhibit a *BRCA1* mRNA content similar to that found in normal tissue. These data may suggest that the down-regulation of *BRCA1* is not a necessary event in the carcinogenic process or, alternatively, that the absence of any change in the *BRCA1* mRNA level in cancer cells can be compensated for by other genic alterations, since it has been shown that breast cancer may have a multifactorial origin. In line with this hypothesis, the interaction of BRCA1 protein with Rad51 protein, the human homologue of the procaryotic RecA protein, suggests a role of BRCA1 in the double-stranded DNA repair mechanism. In addition, alterations in the *BRCA1* mRNA level are not restricted to invasive breast carcinomas, similar alterations having been observed in all the histologic types of tumors analysed, including the two samples of lobular carcinomas *in situ*. Furthermore, in the fibroadenoma samples analysed, the *BRCA1* mRNA level ($134.7 \pm 10.44 \times 10^3$ copies/μg of total RNA, $n=4$) was simililar to that found in normal breast tissue. Although the detection of BRCA1 proteins was not systematically performed, Westerns blot experiments have indicated, in cell lines, that the level of *BRCA1* mRNA is correlated with the amount of BRCA1 proteins (data not shown).

Several lines of evidence indicate that *BRCA1* negatively regulates cellular proliferation and may act as a suppressor gene in breast cancers. However, alterations in *BRCA1* were not detected in sporadic breast cancer and were only exceptionally found in ovarian cancers (Futreal *et al*., 1994; Merajver *et al*., 1995), suggesting that other mechanism(s) might be involved in its down-regulation in sporadic cancers.

Alteration of DNA methylation patterns is a very common event in cancer cells (Laird and Jaenisch; 1994; Counts and Goodman, 1995) and it has been shown that hypermethylation is associated with the down-regulation of several suppressor genes in human cancers (Graff *et al*., 1995; Huynh *et al*., 1996; Baylin *et al*., 1998). In addition, the CCGG and GCGC sites located around the exon 1a of *BRCA1* were found to be hypermethylated in some breast cancers samples (Dobrovic *et al*., 1997) and abnormal CpG methylations were detected in a small percentage (16% and 21% at CpG sites −172 and −167) of DNA molecules from two out six breast cancer samples analysed using the bisulfite genomic sequencing method (Mancini *et al*., 1998).

In our work we found, in the majority of tumor samples (33 out of 37), a 10–12-fold decrease in *BRCA1* mRNA level. Furthermore, in 12 of these samples the number of *BRCA1* mRNA molecules/μg of total RNA was 12–22-fold lower than in normal breast tissue. Since the amount of *BRCA1* mRNA is relatively low in normal breast tissue, ranging from six to 38 copies in breast cell lines (Ribieras *et al*., 1997), these data indicate that the *BRCA1* mRNA level is dramatically reduced in the majority of cancer cells within the samples analysed.

Southern blot experiments, performed with a DNA methylation sensitive enzyme from the same samples, show that the methylation pattern at the CCGG sites of the *BRCA1* gene is not altered in sporadic breast cancers. Despite the fact that the sensitivity of this method does not allow for the detection of small percentage of abnormal methylation, these results indicate that the down-regulation of *BRCA1* in sporadic breast cancer may not be associated with DNA hypermethylation or, alternatively, that DNA methylation might be involved in other regulatory regions not yet characterized.

Materials and methods

Sample preparation

For both normal and pathological samples, specimens were fixed in formalin, paraffin embedded, sectioned and stained with hemaluneosin-safran. Breast tumors were classified according to the WHO classification of breast tumors (Poulsen *et al*., 1975). After examination of the sections (Department of Pathology, Dr L Frappart), the region corresponding to the tumoral tissue was selected for further analysis. Neoplasic lesions comprised invasive ductal carcinomas NOS ($n=24$), lobular carcinomas *in situ* ($n=2$), medullary carcinomas ($n=2$), invasive lobular carcinomas ($n=4$), invasive mucinous carcinomas ($n=2$) and invasive ductal carcinomas with predominantly *in situ* component ($n=3$). Normal breast tissues were obtained from patients ($n=6$) undergoing mammoplastic surgery.

DNA/RNA extraction

DNA was extracted from frozen tissues by standard procedures (Sambrook *et al*., 1989). RNA was extracted using the RNAzol method (Bioprobe, Paris, France), a modification of the guanidium procedure (Dante *et al*., 1994). After isopropanol precipitation, the pellet was washed in 70°C ethanol and dissolved in an appropriate volume of sterile water. The integrity and quantity of RNA was then examined by gel electrophoresis. Total RNA was quantified by densitometry on agarose gel in comparison with serial dilutions of known amounts of standard RNA (Boehringer Mannheim, Meylan, France).

RT–PCR assay

RT–PCR assay was performed as previously described (Ribieras *et al*., 1997). Briefly, 0.6 μg of total RNA was coamplified with different amounts of chimeric *BRCA1* RNA as a competitor in a final volume of 100 μl containing 10 mM Tris-HCl, 3 mM $MgCl_2$, 50 mM KCl, 0.1 mg/ml Gelatin, 200 μM each of the four deoxyribonucleoside triphosphates and 0.25 μM of each of the C3 primer; 5′ TGT GCT TTT CAG CTT GAC ACA GG 3′, position 390–412; 5′ CGT CTT TTG AGG TTG TAT CCG CTG 3′, position 624–647 (Friedman *et al*., 1994). After initial denaturation at 94°C for 2 min, 17.5 units of Expand Reverse Transcriptase (Boehringer Mannheim, Meylan, France) were added to the reaction mixture and incubated for 35 min at 42°C. Reverse Transcriptase was inactivated by heating for 2 min at 94°C and then, cooling to 0°C. PCR amplification of the cDNA was accomplished by adding 0.6 units of *Taq*I DNA polymerase (Boehringer Mannheim, Meylan, France). The PCR amplification was performed after 35 cycles in a thermocycler under the following conditions: 1 min denaturation at 94°C, 2 min annealing at 65°C and 3 min extension at 72°C. In addition, control experiments for each RNA sample were performed omitting reverse transcriptase to ensure that the

signal was the result of RNA and not DNA amplification. PCR products were analysed on a 2% agarose gel containing 0.1 μg/ml ethidium bromide. The gels were photographed under ultraviolet transillumination, the photographs were scanned and the intensity of the PCR-band corresponding to wild-type *BRCA1* mRNA was plotted against the intensity of the PCR product corresponding to the competitor.

Southern blot analysis of DNA methylation patterns

Methylation patterns were determined by Southern blot experiments using the pair of isoschizomeric restriction endonucleases, *Hpa*II and *Msp*I. *Msp*I yielded fragments resulting from the cleavage of all CCGG sites, whereas *Hpa*II only cleaved CCGG with unmethylated internal cytosine. In order to map these sites, all DNAs were also cleaved with *Taq*I, a methylation insensitive endonuclease. To control the efficiency of *Hpa*II digestion, several samples were digested once or twice, with a tenfold excess of this enzyme. Southern blot experiments have shown that the pattern of *Hpa*II digested DNAs was not modified in the double digested samples.

Restriction endonuclease digests were fractionated on a 1.2% agarose electrophoresis gel and then transferred to Hybond N+ Nylon membrane as previously described (Ribieras *et al*., 1994). Membranes were hybridized for 16 h at 42°C to randomly primed ^{32}P-labeled probes (Random Primed DNA labeling kit, Boehringer Mannheim, Meylan, France) in a buffer containing 50% Formamide, 10% Dextran Sulfate, 0.7 M NaCl, 1% SDS and 300 μg/ml denatured herring sperm DNA. Membranes were washed with increasing stringency (from 2 × to 0.2 × SSPE; 1% SDS) at 65°C and exposed to Hyperfilm (Amersham, France) for 1–7 days using an intensifying screen. Nylon membranes could be used for rehybridizations after two successive treatments with SDS 1% at 80°C for 30 min. The same filters were successively hybridized to several probes.

Gene probes

The probes used for Southern blot analysis were synthesised by PCR or RT–PCR (Friedman *et al*., 1994). The PCR or RT–PCR fragments were cloned into a pGEM-T vector (Promega, Lyon, France). Following digestion with the appropriate enzymes, the inserts were purified by agarose gel electrophoresis followed by electroelution. Probes one and two fully matched the *BRCA1* gene sequence (results not shown).

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