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Epigenetic inactivation of *BRCA1* is associated with aberrant expression of *CTCF* and DNA methyltransferase (*DNMT3B*) in some sporadic breast tumours

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ABSTRACT

We assessed expression of the *BRCA1*, *CTCF* and *DNMT3b* methyltransferase genes along with *BRCA1* promoter methylation to better define the epigenetic events involved in *BRCA1* inactivation in sporadic breast cancer. These gene expression patterns were determined in 54 sporadic breast tumours by immunohistochemistry and the methylation status of the *BRCA1* promoter was evaluated using methylation-specific PCR. We observed significant *DNMT3b* expression in 80% of the tumours and that 43% of tumours exhibited novel cytoplasmic *CTCF* expression. Pairwise analyses of gene expression patterns showed that 28/32 tumours lacked *BRCA1* expression and also exhibited cytoplasmic *CTCF* staining, while 24/32 of these tumours also overexpressed *DNMT3b*. Furthermore, 86% of the *BRCA1* low-expressing tumours were methylated at the *BRCA1* promoter and a subset of these tumours displayed both cytoplasmic *CTCF* and increased *DNMT3b* expression. Thus, tumour subsets exist that display concurrent decreased *BRCA1* expression, *BRCA1* promoter methylation, cytoplasmic *CTCF* expression and with *DNMT3b* over-expression. We suggest that these altered *CTCF* and *DNMT3b* expression patterns represent (a) critical events responsible for the epigenetic inactivation of *BRCA1* and (b) a diagnostic signature for epigenetic inactivation of other tumour suppressor genes in sporadic breast tumours.

1. Introduction

Most cancers result from multiple genetic and epigenetic alterations that transform a normal cell into an invasive and/or metastatic phenotype. This process includes altered DNA methylation patterns occurring as global hypomethylation and localised hypermethylation events that lead to the inappropriate expression of tumour suppressor genes in sporadic cancers.[1] In breast cancer, in particular, hypermethylation of the *BRCA1* promoter has been reported in up to 20% of sporadic breast tumours and corresponds with a reduction in *BRCA1* transcription.[2,3] However, the mechanisms responsible for disrupting normally methylation-free promoter regions of tumour suppressor genes, leading to transcriptional repression and tumourigenesis, are unclear. Identifying these molecular events is critical if we are to exploit epigenetic changes as targets for novel clinical therapies that could re-establish proper DNA methylation and gene expression patterns, in a gene and cell-specific manner.

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DNA methylation is regulated by a complex machinery that includes DNA methyltransferases (DNMTs) and methyl binding domain proteins (MBDs).[1] *DNMT-1*, *3a* and *3b* are essential for proper development and for somatic cell function, with over-expression of the DNMTs being described in bladder, colon, kidney and pancreatic tumours.[4] An increase in *DNMT3b* mRNA has been shown in breast tumours,[5] and a novel promoter polymorphism increases *DNMT3b* expression and the risk of developing breast cancer in patients.[6]

We have previously implicated two proteins, *CTCF* and *SP1*, in maintaining a methylation-free *BRCA1* promoter in normal breast tissue.[1] We have identified *CTCF* binding sites and *in vivo SP1* binding at sequences flanking the hypomethylated promoter region of *BRCA1*.[7] *CTCF* is of particular interest, since the gene is implicated in genomic imprinting and is located at 16q22, a commonly deleted region in sporadic breast cancer.[8,9] In addition, in many breast tumours, *CTCF* is inappropriately expressed in the cytoplasm and is absent from the nucleus,[10] suggesting that the functional loss of nuclear *CTCF* could contribute to the loss of methylation boundaries in genes like *BRCA1* that possess *CTCF* binding sites.

Here, we have assessed the epigenetic regulation of *BRCA1*, *DNMT3b* and *CTCF* expression in the context of *BRCA1* promoter methylation, in sporadic breast tumours. *DNMT3b* expression was observed in most *BRCA1*-deficient tumours, and we observed that a loss of *BRCA1* correlates with the inappropriate cytoplasmic expression of *CTCF* in tumours that lack or express low levels of *BRCA1*. Furthermore, this cytoplasmic *CTCF* expression correlates with the over-expression of *DNMT3b*, and a methylated *BRCA1* promoter in these tumours. Our data suggest that alterations in *DNMT3b* and *CTCF* expression are at least partially responsible for this inappropriate methylation within the *BRCA1* promoter. As a consequence, loss of *BRCA1* expression may lead to the disregulation of numerous cell functions and chromosome instability that together predispose to the formation and progression of sporadic breast tumours.

2. Materials and methods

2.1. Archival breast tumours

Sixty anonymous tissue samples (54 tumours and 6 normal breast tissues) were obtained from the Manitoba Breast Tumour Bank for this study. The tumours were selected by the tumour bank from patients (over the age of 55) and all tumours were ductal infiltrating, lobular infiltrating or a combination of the two. This age of diagnosis was chosen based on the criteria for *BRCA1* genetic screening in Ontario[11] and minimised the inclusion of tumours possessing hereditary *BRCA1* or BRCA2 mutations. None of the patients providing tumour material had received chemotherapy, radiation therapy or hormone treatments that may have resulted in treatment-related epigenetic changes. No family history, hormone exposure, race or other clinical and demographic data were available for the cases selected. The anonymous control tissues were collected from reduction mammoplasty and the age of the patients was not indicated.

2.2. Immunohistochemistry

Serial 5 μm sections of formalin-fixed and paraffin embedded tissue were deparaffinised through 3 × 5 minute (min) washes in xylene, followed by rehydration in descending alcohols. Slides were then soaked in 1× phosphate buffered saline (1× PBS). Deparaffinised sections were treated with sodium citrate (0.1 M) to retrieve antigens by boiling at 1350 W for 7 min and at 945 W for 15 min in a microwave. The slides were allowed to cool and then rinsed in water. Endogenous peroxidase activity was blocked by incubation in 1% hydrogen peroxide followed by a wash in deionised water and 2 × 5 min rinses in 1× PBS.

The sections were immunoperoxidase stained following the manufacturer's instructions for the ABC staining system (rabbit sc-2018, goat sc-2023, or mouse sc-2017 as required by the antibody: Santa Cruz Biotechnology, Santa Cruz, CA). Briefly, tissues were incubated for one hour (h) in 1.5% blocking serum in PBS and the slides were incubated in primary antibody and 1.5% blocking serum overnight at 4 °C. Antibodies were used at the following concentrations: *BRCA1*-Ab1, 1:150 dilution (OP92; Oncogene Research Products, Cambridge, MA); *CTCF*-C20, 1:50 dilution (sc15914; Santa Cruz Biotechnology, Santa Cruz, CA); *DNMT3b*, 1:50 dilution (IMG-184; Imgenex, San Diego, CA); and *Ki67*, 1:50 dilution (H300; sc15402; Santa Cruz Biotechnology, Santa Cruz, CA). We validated this immunohistochemistry using paraffin slides of human tumour xenograft tumours generated in mouse tissue, which allowed us to identify positive signals against human antigen in the mouse background. All staining was done with batches of slides containing a slide to which only secondary antibody was added. In addition, we identified non-staining cells in stromal tissue on individual tumour slides as a control for non-specific staining.

Sections were washed 3 × 5 min in 1× PBS, incubated with 1 mg/ml biotinylated secondary antibody for 30 min and then washed 3 × 5 min in 1× PBS. Avidin and biotinylated horseradish peroxidase conjugates were added to the sections that were then incubated for 30 min and washed 3 × 5 min in 1× PBS. Peroxidase substrate containing DAB (3,3′-diaminobenzidine) chromogen was added to the sections for 6–10 min to develop the stain. Sections were then washed in deionised water for 5 min, counterstained with haemotoxylin, and dehydrated using ascending alcohols and xylene. Images of immunohistochemical staining at 200× magnification were recovered using a Olympus AX70 upright microscope fitted with a Cooke Sensicam digital camera (Romulus, MI) using Image-Pro Plus software.

2.3. Cell counting and statistical analysis

Nuclear or cytoplasmic staining was counted using the cell counter function of the Image J software[12] to differentiate four separate staining parameters on two or three fields per section. *BRCA1*, *DNMT3b* and *Ki67* expression was assessed on the basis of nuclear staining while *CTCF* expression was also assessed on the basis of cytoplasmic staining. Specific categories for *BRCA1*, *DNMT3b* or *CTCF* (percent of cells staining positive) and for *Ki67* (as a measure of proliferation status) are shown in Table 1. Staining intensity was counted as neg-

Table 1 – Scoring criteria and results of BRCA1, CTCF, DNMT3b and K167 immunohistochemistry

		Normal ($n = 6$)		Tumour	
(a)	BRCA1	($n = 6$)		($n = 54$)	
(O)	Negative: 0–10% of cells	0		19	
(+)	Weak: 10–20% positive cells	2	(6)[a]	13	(35)[a]
(++)	Positive: >20% cells	4		22	
(b)	DNMT3b	($n = 6$)		($n = 54$)	
(O)	Negative: 0–10% of positive cells	6		11	
(+)	Weak: 10–20% positive cells	0		10	(43)[b]
(++)	Positive: 20–40% positive cells	0	(0)[b]	4	
(+++)	Strongly postive: >40% positive cells	0		29	
(c)	CTCF	($n = 6$)		($n = 54$)	
(O)	Negative: 0–10% of positive nuclel	0		1	
(N)	Nuclear staining: >10% of cells	5		14	
(C)	Cytoplasmic staining: >10% of cells	0	(1)[c]	23	(39)[c]
(N/C)	Nuc+Cyto staining:>10% of cells	1		16	
(d)	K167	$n = 4$		$n = 50$	
(O)	Negative: 0–10% of positive cells	4		18	
(+)	Weak: 10–20% positive cells	0		0	
(++)	Positive: 20–40% positive cells	0		0	
(+++)	Strongly postive: >40% positive cells	0[d]		32[d]	

Percentages indicate the percent of cells with positive expression of the particular protein.
Significance (p)values were calculated for number pairs, as indicated in Section 3.
a $p = 0.08$; NS.
b $p < 0.0001$.
c $p = 0.01$.
d $p = 0.01$.

ative (0–10% of cells), weak (10–20%) or positive (greater than 20% of cells).[13,14] Where samples size permitted, the χ^2 test was used to test for differences in proportions and the Fisher's exact test was used in categories with small sample sizes.

2.4. Sodium bisulphite conversion and methylation specific PCR

To determine DNA methylation status, slides were deparaffinised and sections were removed from the glass slides using a sterile razor blade, placed into small eppendorf tubes and then digested and sodium bisulphite treated as described previously.[15] Sample lysis buffer (20–50 μl; 0.5% Tween-20 in TE) was added to each of the samples, along with proteinase K (10 mg/ml) and the samples were then incubated for 72 h at 65 °C, with the addition of proteinase K every 24 h. DNA samples were then mixed with 0.2 M NaOH at 95 °C for 5 min and then at 70 °C for 10 min to denature the DNA, and two volumes of low melt agarose (2%) were added to this mixture.[16] A 10 μl aliquot of the agarose/DNA slurry was dropped into ice-cold mineral oil (500 μl) and sodium bisulphite conversion of agarose-embedded genomic DNA was performed.[16]

Methylation-specific PCR was used to distinguish unmethylated from methylated *BRCA1* alleles. Individual samples were collected from single tissue sections to provide DNA for methylation assays that were performed in duplicate. Primer sequences for 86 bp unmethylated product were 5′-TTGGTTTTTGTGGTAATGGA AAAGTGT (sense) and 5′-CAAAAAATCTCAACAAACTCA CACCA; and for the 75 bp methylated DNA reactions: 5′-TCGTGGTAACGGAAAGCGC (sense) and 5′-AAATCTCAACG AACTCACGCC.[17] The unmethylated sense primer begins at −46 and the methylated sense primer begins at −38 from the transcriptional start site (+1). DNA from MCF-7 cells was treated with *Sss*1 methylase as a positive methylation control and untreated DNA was used as a negative control based on the constitutive unmethylated status of *BRCA1* in the MCF-7 cell line (data not shown). The bisulphite-treated DNA was PCR amplified in duplicate, from separate PCRs. The amplification conditions included an initial start at 95 °C (5 min) to melt the sample agarose bead, then 5 cycles at 95 °C (45 s), 62 °C (2 min), 72 °C (2 min) followed by 25 cycles at 95 °C (45 s), 62 °C (1 min), 72 °C (1 min) followed with a 72 °C incubation for 8 min using *Taq* polymerase (Invitrogen, La Jolla, CA). Aliquots of each PCR were loaded onto 10% polyacrylamide gels, stained with ethidium bromide and visualised under UV illumination.

3. Results

3.1. Immunohistochemical staining in normal breast and tumour samples

Protein expression patterns were evaluated by immunohistochemistry in six normal breast tissue samples (Table 1). *BRCA1* was expressed in all normal tissues (in over 10% of nuclei), a result consistent with previous reports of *BRCA1* expression in normal breast tissue.[13,18] In contrast, *DNMT3b*

expression was absent in normal breast tissue samples. *CTCF* was expressed in the nuclei in all six samples, although weak cytoplasmic staining was observed in one of these samples. *Ki67*, a marker of cell proliferation, was expressed at low levels (or was absent) from the samples we assessed. To summarise, the predominant gene expression patterns in normal breast tissue involve the nuclear expression of *BRCA1* and *CTCF* and the absence (or low levels) of *DNMT3b* and *Ki67* (Table 1a–d).

Breast tumour sections were also immunostained for *BRCA1*, *CTCF* and *DNMT3b*. Tumours could be separated into three groups on the basis of their *BRCA1* expression patterns (Table 1a). One group (19/54; 35%) expressed *BRCA1* in less than 10% of the tumour cells or cells were negative for *BRCA1* expression. A second group of tumours (13/54; 24%) expressed *BRCA1* in only 10–20% of cells. The remaining tumour group (22/54; 41%) was *BRCA1* positive in greater than 20% of the cells. These results showing varied levels of *BRCA1* expression are not surprising, since *BRCA1* is one of a number of gene targets having a causative role in sporadic breast cancer.[21] Representative images of *BRCA1* staining are shown in Fig. 1a and b.

Although none of the normal samples showed expression of *DNMT3b*, we observed statistically significant expression of *DNMT3b* in the tumour samples ($p < 0.0001$; Table 1b). Eighty percent of the breast tumours (43/54) exhibited *DNMT3b* expression in greater than 10% of the tumour cells, while only 11 tumours expressed low amounts of protein. In the *DNMT3b* expressing tumours, 67% (29/43) were strongly positive in that over 40% of the tumour cells expressed *DNMT3b*, while 10 other tumours (10/54; 23%) weakly expressed *DNMT3b*. Representative tumour expression patterns for *DNMT3b* are shown in Fig. 1e and f.

Tumours were also assessed for *CTCF* expression (Table 1c). Overall we observed a significant shift in *CTCF* expression in tumours, when compared with normal tissues ($p = 0.01$). Nuclear *CTCF* was present in all six normal samples; one of which also displayed cytoplasmic *CTCF* expression. However, 39/54 tumours expressed cytoplasmic *CTCF* expression (23/39) or nuclear/cytoplasm expression (16/39), revealing a dramatic change in subcellular localisation of *CTCF*. Representative images of *CTCF* staining are shown in Fig. 1c and d. Finally, the presence of *Ki67* expression in 32/50 breast tumours (in comparison to none of the normal samples) indicates that a

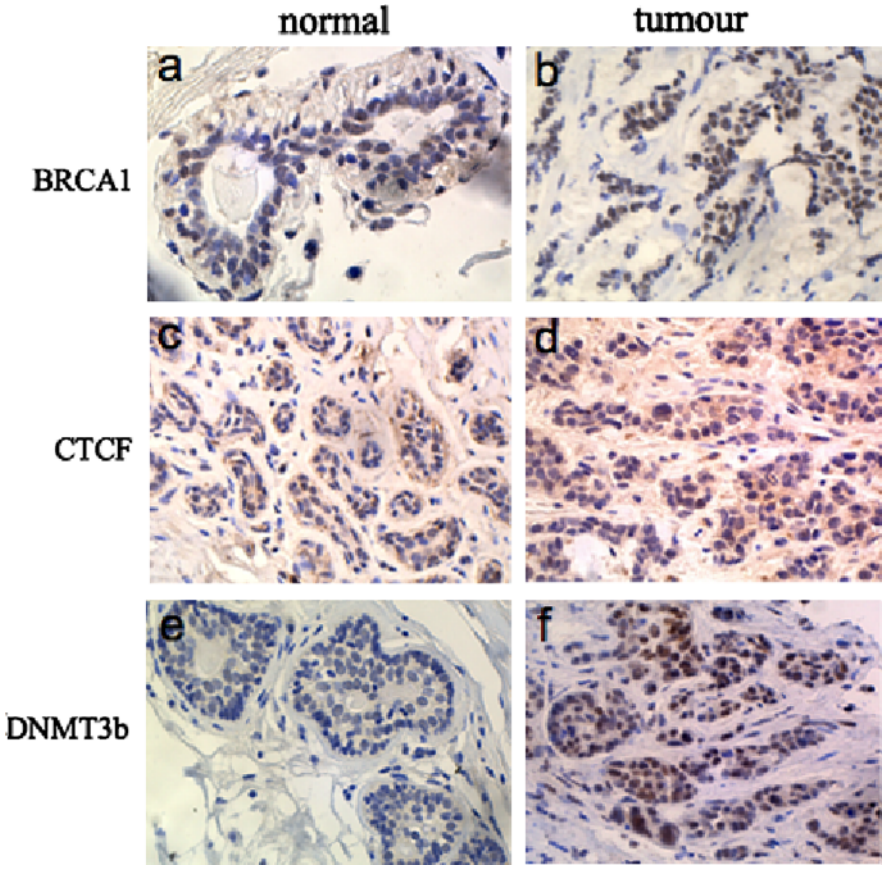

Fig. 1 – Immunohistochemical staining of normal breast and breast tumour tissues. Representative normal and tumour tissue samples stained for *BRCA1* (a,b), *CTCF* (c,d) and *DNMT3b* (e,f) are shown. In normal breast tissue, (a) *BRCA1* is expressed in the nucleus in greater than 20% of the cells, (c) nuclear expression of *CTCF* is usually observed and (e) *DNMT3b* is expressed at low levels or is absent from the nuclei. We observed breast tumours in which (b) *BRCA1* is expressed in the nucleus in greater than 20% of the cells and (d) cytoplasmic expression of *CTCF* is present. *DNMT3b* is also frequently expressed in tumour cell nuclei (f). Original magnification: 200×.

significant majority of the tumours ($p = 0.01$; Table 1d) possessed proliferating cells.[19]

3.2. *Overlapping patterns of expression in breast tumours*

We analysed the overlapping, pairwise expression patterns of these proteins. First, *BRCA1* and *DNMT3b* expression patterns were compared (Table 2a) and we found that most of the tumours (24/32) that lacked *BRCA1* (or expressed it weakly) were positive for *DNMT3b* expression. Only 9/22 *BRCA1*-expressing tumours were over-expressing *DNMT3b*, suggesting a correlation between *DNMT3b* overexpression and the decreased expression of *BRCA1* (24/32 *versus* 9/22; $p = 0.01$).

Next, *BRCA1* and *CTCF* expressions were compared (Table 2b) and we found that 28/32 tumours that lacked *BRCA1* (or expressed it weakly) either exhibited exclusive cytoplasmic staining for *CTCF* (19/32) or had *CTCF* staining in both the cytoplasm and the nucleus (9/32). In contrast, only four tumours expressed *CTCF* exclusively in the nucleus (19/32 *versus* 4/32, $p = 0.0001$). In the 22 tumours expressing *BRCA1*, only 5 tumours exhibited exclusive cytoplasmic staining for *CTCF*, whereas 10 tumours expressed *CTCF* solely in the nucleus (5/22 *versus* 10/22, $p = 0.11$). The remaining 7 tumours had *CTCF* staining in both the cytoplasm and the nucleus. These data show that the exclusive presence of *CTCF* in the cytoplasm correlates with a loss of *BRCA1* expression (19/32 *versus* 5/22; $p = 0.01$), while *CTCF* expressed solely in the nucleus is more likely to be found in cells expressing *BRCA1*, rather than in *BRCA1* non-expressing cells (10/22 *versus* 4/32; $p = 0.01$). In general, a greater proportion of tumours with low levels of *BRCA1* exhibited cytoplasmic *CTCF* than did *BRCA1* expressing tumours (28/32 *versus* 12/22; $p = 0.01$).

A comparison of *DNMT3b* and *CTCF* expression patterns (Table 2c) was also done. When *DNMT3b* was absent (or weakly expressed), *CTCF* was expressed in the nucleus (9/21), in the cytoplasm and the nucleus (6/21) or in the

Table 2 – Pairwise analyses of BRCA1, CTCF and DNMT3b expression patterns

	BRCA1			
	0–10%	10–20%	+20%	Total
(*a*) *DNMT3b*				
0–10%	2	1	8	11
10–20%	4	1	5	10
20–40%	2	0 (24)[a]	2 (9)[a]	4
+40%	11	11	7	29
Total	19 (32)	13	22	54
(*b*) *CTCF*				
nuclear only	3 (4)[b]	1	10[c,e]	14
cytoplasmic only	12 (19)[b,d]	7 (28)[f]	5[c,d] (12)[f]	24
cytoplasmic and nuclear	4[e]	5	7	16
Total	19 (32)	13	22	54

	DNMT3b				
	0–10%	10–20%	20–40%	+40%	Total
(*c*) *CTCF*					
nuclear only	8	1	1 (5)[g]	4	14
absent/cytoplasmic only	2	4	1 (18)[g]	17	24
cytoplasmic and nuclear	1	5	2	8	16
Total	11	10	4 (33)	29	54
(*d*) *Ki67*					
0–10% positive	5	5	3 (8)[i]	5	18
>40% positive	4 (9)[h]	5	1 (23)[h,i]	22	32
Total	9 (19)	10	4 (31)	27	50

Percentages indicate the percent of cells with positive expression of the particular protein. In some cases, columns have been added together and these values are noted in brackets. Superscripts indicate that significance (p) values were calculated for number pairs, as indicated in the results section.

a ($p = 0.01$).
b ($p = 0.0001$).
c ($p = 0.11$).
d ($p = 0.01$).
e ($p = 0.01$).
f ($p = 0.01$).
g ($p = 0.001$).
h ($p = 0.0005$).
i ($p = 0.06$).

cytoplasm alone (6/21). However, when *DNMT3b* was strongly expressed (in more than 20% of cells), *CTCF* was expressed exclusively in the cytoplasm of 18/33 of these tumours. Aa additional 10 tumours displayed nuclear/cytoplasmic *CTCF* expression (10/33), while only 5 tumours displayed *CTCF* expression exclusively in the nucleus (18/33 *versus* 5/33, $p = 0.001$). Thus, these pairwise analyses show that cytoplasmic expression of *CTCF* correlates with over-expression of *DNMT3b* in tumours that lack *BRCA1*. Our results suggest that when *CTCF* is absent from the nucleus in breast tumour cells, methylation boundaries may be compromised by the presence of excess *DNMT3b*, leading to promoter hypermethylation in genes such as *BRCA1*.

Finally, we observed that the majority of tumours (64%) were proliferating,[19] as determined by greater than 10% of tumour cells expressing *Ki67* (Table 2d). In addition, *Ki67* was expressed in 23/32 tumours that overexpressed *DNMT3b* (23/32 *versus* 9/32, $p = 0.0005$).[20] The frequency of expression of *DNMT3b* in *Ki67* negative tumours (8/18) was not statistically different from tumours expressing *Ki67* (23/32, $p = 0.06$). In tumours lacking *Ki67*, there was no statistical difference between tumours that expressed *DNMT3b* and those that did not. Although there was a wide range of *DNMT3b* expression (from 40% to 100% of tumour cells), high *DNMT3b* expression levels did not correlate to the expression levels of *Ki67*. It therefore appears that proliferation is related to *DNMT3b* expression but does not determine the level of *DNMT3b* expression.

3.3. *BRCA1* expression and methylation status of the *BRCA1* promoter

Lastly, we used methylation specific PCR (MSP) to evaluate *BRCA1* promoter methylation in these tumour samples. While a few *BRCA1* non-expressing tumours were not methylated at *BRCA1* (Fig. 2a), the majority of tumours that lacked or expressed low *BRCA1* levels (24/28; 86%) were also methylated at the *BRCA1* promoter (Fig. 2b). These data show that a significant proportion of *BRCA1* negative tumours also possess a methylated promoter region, consistent with methylation-associated transcriptional inactivation of *BRCA1*. To investigate a potential mechanism linking *BRCA1* inactivation to DNA methylation, we compared the expression patterns of *CTCF* and *DNMT3b* with both the expression pattern and promoter methylation status of *BRCA1*.

Nineteen tumours with limited expression of *BRCA1* (in less than 10% of tumour cells) also expressed *CTCF* in the cytoplasm. Eleven of these tumours lacked *BRCA1* expression, and of these 11 tumours, 7 displayed BRCA1 promoter methylation and were concurrently positive for *DNMT3b*. Interestingly, 5 of these 7 tumours are also ER–/PR– (ER < 3.0 fmol/mg; PR < 10 fmol/mg; data not shown), while only 7 of the

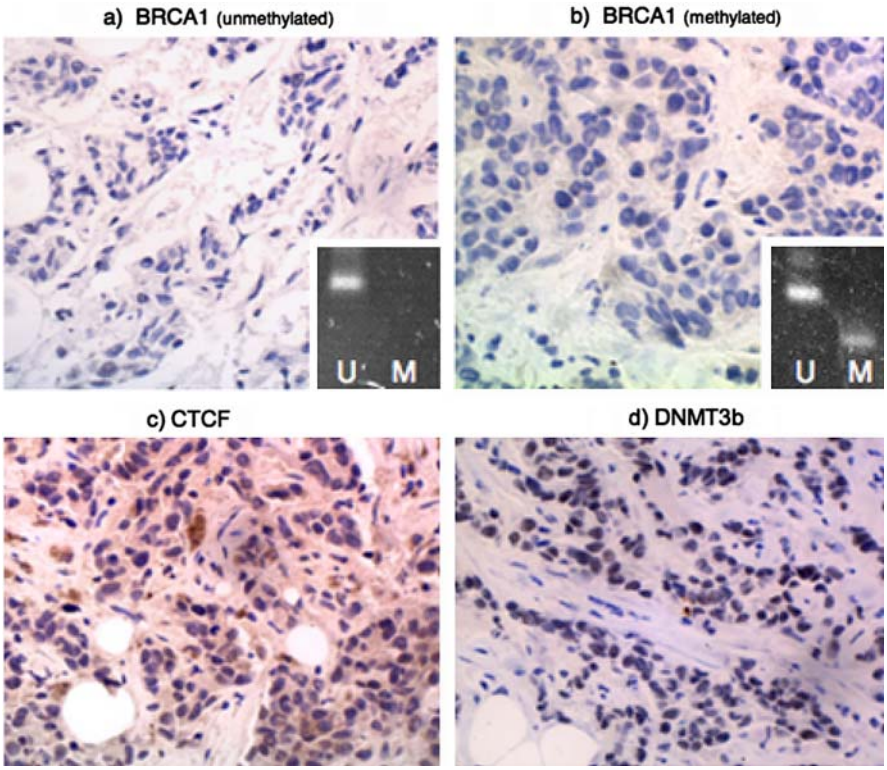

Fig. 2 – Immunohistochemical staining of *BRCA1* and the methylation status of the *BRCA1* promoter. DNA methylation of the *BRCA1* promoter was determined by methylation specific PCR (MSP), which distinguishes between unmethylated (U) and methylated (M) templates. We show here (a) a tumour lacking *BRCA1* expression and concurrently exhibiting a lack of methylation at the *BRCA1* promoter (U). In contrast, a second tumour (b) lacks *BRCA1* expression while displaying methylation within the *BRCA1* promoter (M). This same tumour also displays (c) cytoplasmic expression of *CTCF* and (d) the nuclear expression of *DNMT3b*. Original magnification: 200×.

54 tumours in the entire tumour set were ER–/PR–. The other 4 tumours in this 11 tumour subset expressed *DNMT3b* in some cells, and interestingly, three displayed methylation at the *BRCA1* promoter. In addition, 8 of the 32 tumours that express *BRCA1* at low levels (and display cytoplasmic *CTCF*) also express high levels of *DNMT3b* and have a methylated *BRCA1* promoter (Fig. 2c and d). Our results show that specific subsets of breast tumours exist that display *BRCA1* promoter methylation, inappropriate expression patterns of both *CTCF* and/or *DNMT3b* and decreased expression of *BRCA1* and perhaps other epigenetic targets including ER and PR.

4. Discussion

The downregulation of *BRCA1* results in the loss of normal cellular growth and development and predisposes cells to tumourigenesis.[21] In some sporadic breast cancers the levels of *BRCA1* mRNA are repressed epigenetically, in the absence of identifiable *BRCA1* mutations. Such hypermethylation within the *BRCA1* promoter represses *BRCA1* transcription and predisposes for tumourigenesis.[3,22] A primary research focus has been to identify how the methylation machinery is recruited to methylation-sensitive gene promoter regions and how altered methylation patterns are generated when recruitment goes awry. The ultimate goal of this approach is to target these protein complexes, to reestablish normal methylation patterns and thereby reactivate silenced genes such as *BRCA1*.

This is the first report that correlates complex patterns of *BRCA1*, *DNMT3b* and *CTCF* expression with *BRCA1* promoter methylation in a set of sporadic breast tumours. We determined that the loss of *BRCA1* correlates with the cytoplasmic expression of *CTCF* in these tumour samples. Furthermore, the likelihood of *DNMT3b* expression is significantly higher in the tumours that do not express *BRCA1*. In fact, a significant proportion of the tumours in this sample set do not express *BRCA1* protein, but overexpress *DNMT3b* and display cytoplasmic localisation of *CTCF*. In addition, the *BRCA1* promoter is methylated in these tumour cells. These data suggest that a fundamental error in DNA methylation involving at least two components of the methylation machinery, *DNMT3b* (an essential DNA methyltransferase) and *CTCF* (a methylation boundary protein), can target tumour suppressor genes such as *BRCA1*, lead to its inactivation and predispose individual cells towards a tumourigenic fate.

In normal breast tissues, the unmethylated *BRCA1* promoter[7] enables essential transcription factors to bind and initiate transcription.[23] As a result, nuclear *BRCA1* can function in DNA repair, checkpoint control, chromatin remodeling and transcriptional regulation.[21] We observed that *BRCA1* was absent or expressed at low levels in a majority of breast tumours. In addition, *BRCA1* promoter methylation was found in 86% of these *BRCA1* deficient tumours. This percentage is much higher than previously reported[3,24] and may be due to the fact that only ductal infiltrating and/or lobular infiltrating tumours were chosen for analysis, and/or because we excluded women who were diagnosed prior to age 55. Thus, women from this age group and with this tumour type may be either more prone to epigenetic inactivation of *BRCA1* by DNA methylation or epigenetic inactivation may be a 'late' event in multistage breast tumourigenesis. The expression of *DNMT3b* protein in these breast tumours is a novel observation. Normally, *DNMT3b* is expressed during human and mouse development[25] and then is expressed later in only a few adult human somatic tissues, including the pancreas, testis, thyroid and bone marrow.[26] In contrast, increased expression of *DNMT3b* in different tumour types has previously been described,[4] and in our study *DNMT3b* is expressed in 80% of the breast tumours.

We have previously reported multiple *CTCF* binding sites flanking the unmethylated *BRCA1* promoter that may function to separate methylated (condensed) from unmethylated (accessible) chromatin.[7] In this present report, we observed nuclear expression of *CTCF* in normal breast tissue, supporting its normal role as a methylation sensitive insulator that can block the spread of heterochromatin[1,27] and block enhancer function.[8,9,28] In contrast, some tumours we analysed showed concurrent *DNMT3b* expression and *CTCF* mis-localisation to the cytoplasm suggesting a fundamental disregulation of the methylation machinery.[29] Our data suggest that along with common deletion events in sporadic breast cancer involving the *CTCF* locus,[30,31] impaired nuclear transport of *CTCF* may lead to the loss of methylation boundaries, thus exposing the *BRCA1* promoter to inappropriate DNA methylation. Although no mutations within the nuclear localisation signal sequence of *CTCF* have been reported,[32,33] mutations in the importin protein karyopherin-α have been associated with aberrant cytoplasmic localisation of p53.[34] Another possibility may involve the displacement of *CTCF* from its binding sites by its homologous family member BORIS.[1] Overexpression of BORIS[35] may disrupt the methylation boundary functions of *CTCF*, permitting DNMTs access to CpG islands while still allowing the nuclear localisation of *CTCF* we observed in some tumours.

We suggest that *BRCA1* promoter methylation may not be the first epigenetic hit in the development of sporadic breast cancer. Instead, that event may be preceded by the disregulation of genes involved in maintaining and establishing genomic methylation patterns. In our model (Fig. 3) the normal, unmethylated *BRCA1* promoter is maintained by *CTCF* bound to flanking consensus sequences that permit the access of appropriate transcription factors to their binding sites.[23] The aberrant localisation of *CTCF* to the cytoplasm, and/or its displacement by BORIS would result in the loss of these methylation boundaries flanking the *BRCA1* promoter. Furthermore, the loss of *CTCF* from nuclear matrix associated regions[36] would cause global alterations in chromatin structure, facilitating the recruitment of histone deacetylases and histone methyltransferases.[23] Consequently, the overexpression and recruitment of *DNMT3b* would lead to the hypermethylation within the *BRCA1* promoter, the recruitment of methyl binding proteins[37] and the inactivation of *BRCA1* transcription. The subsequent loss of *BRCA1* activity would predispose a cell to tumourigenesis, due to defects in *BRCA1*-dependent DNA repair, cell cycle regulation and proliferation.

It is unlikely that altered *BRCA1* hypermethylation represents a single epigenetic event. A global shift in promoter methylation patterns, due to the *CTCF* localisation and the overexpression of DNA methyltransferases, could have

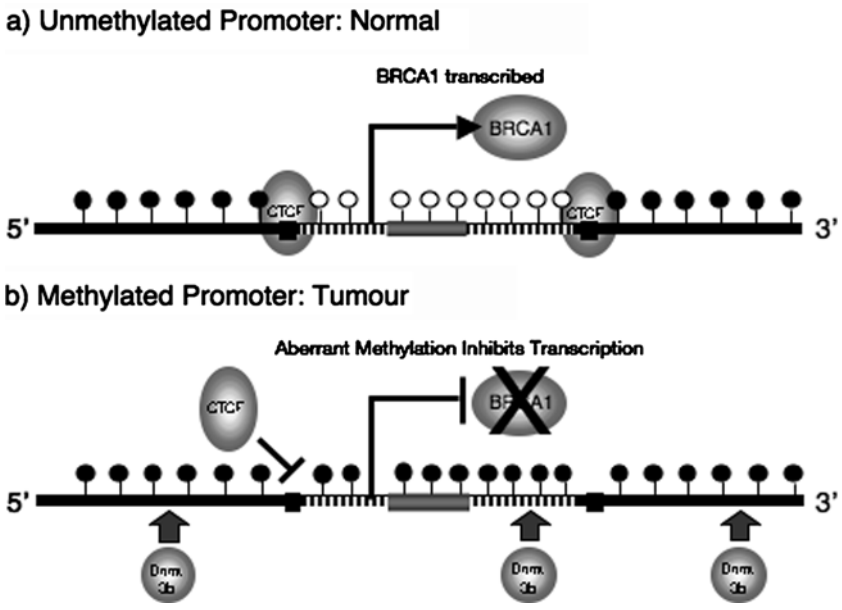

Fig. 3 – A model for *BRCA1* inactivation by DNA methylation in sporadic breast tumours. (a) In normal breast tissue the *BRCA1* promoter is not methylated, *DNMT3b* is expressed at low levels and *CTCF* is expressed in the nucleus. In this context, *BRCA1* is expressed and is able to participate in its usual pathways. (b) In a subset of sporadic breast tumours, *CTCF* is localised in the cytoplasm, and therefore is not able to act as a boundary to protect the *BRCA1* promoter, while *DNMT3b* is overexpressed and can *de novo* methylate the promoter region. The expression of *BRCA1* is inactivated due to the newly established pattern of methylation. Loss of *BRCA1* expression can predispose to the development of sporadic breast tumours. This scenario provides a potential target for demethylating agents that would reestablish appropriate *BRCA1* expression.

profound consequences on other genes normally expressed in breast tissue such as ER, PR and E-cadherin.[38–40] Thus, the accumulation of multiple epigenetic events throughout the genome would provide a selective advantage to individual cells and predispose them to tumour formation.

DNMT3b overexpression in breast tumours is potentially treatable with currently used anti-cancer therapy. Hypermethylated gene promoters have the potential to be reactivated by nucleoside analogues, such as 5-azacytidine and 5-aza-2-deoxycytidine (Decitabine), both of which have been approved for clinical usage. Azacitidine traps DNMT as a DNA adduct,[41] thereby depleting *DNMT3a* and *3b* and inhibiting DNA methylation.[42] Clinical trials for Decitabine are already in the early stages for solid tumours,[43,44] and azacitidine and decitabine are currently in use to treat myelodysplastic syndrome and acute myeloid leukemia (reviewed in [45]). Our data suggest that these specific stages of sporadic breast tumours displaying *DNMT3b* overexpression would be good candidates for targeted epigenetic therapy to sequester *DNMT3b*, decrease levels of aberrant methylation within the *BRCA1* promoter and potentially reactivate *BRCA1* expression. Clearly, the transcriptional consequences of aberrant DNA methylation are part of a complex picture in which both genetic and epigenetic alterations in gene expression contribute to breast tumour formation and progression.

Conflict of interest statement

None declared.

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