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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-